Reactivation of Vanadate-inhibited Enzymes with Desferrioxamine B, a Vanadium(V) Chelator

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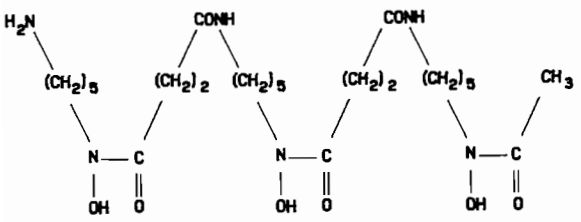
Vanadate ($H_2VO_4^-/HVO_4^{2-}$) has been used to inhibit a large number of ATPases and phosphate ester hydrolyzing enzymes [1]. The similarities of vanadate and phosphate in terms of geometry, charge, bond lengths and ability to adopt a trigonal bipyramidal coordination probably explain the efficacy of vanadate as an inhibitor of many phosphate-binding enzymes [2]. The use of vanadate as a tool to probe the mechanisms of phosphate-metabolizing enzymes would be facilitated if vanadate inhibition could be rapidly reversed, such as by complexation of vanadium(V), which must be accomplished under mild, aqueous conditions without complexing essential metal cofactors (e.g., Mg^{2+} and Ca^{2+}). Ethylenediamine tetraacetic acid (EDTA) and analogues often are not satisfactory for this reason. Hydroxamic acid-containing ligands, which coordinate V(V) strongly, have been used for quantitative determination of vanadium(V) [3, 4] and do not strongly complex Mg^{2+} or Ca^{2+}. Desferrioxamine B, the water-soluble polyhydroxamic acid siderophore, originally isolated from *Streptomyces pilosus* [5], coordinates V(V) [6, 7] and thus was investigated as a reagent to effect reactivation of vanadate-inhibited phosphate-metabolizing enzymes. Desferrioxamine offers an advantage over *N*-phenylbenzohydroxamic acid [3] and *N*-*m*-chlorophenylpalmitohydroxamic acid [4], both of which have been used as ligands for the quantitative determination of V(V), because of the entropic driving force of the chelate effect and because desferrioxamine is water soluble, whereas the other hydroxamate ligands are not.



Desferrioxamine B

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Experimental

Reagents

Desferrioxamine mesylate (*N*-[5-[3-[(5-aminopentyl)hydroxycarbamoyl]propionamide]pentyl]-3-[[5-(*N*-hydroxyacetamido)pentyl]carbamoyl]propionohydroxamic acid monomethane sulfonate or Desferal mesylate®) was a kind gift from Mr Samuel DiMaulo of Ciba-Geigy Corp. (Suffern, NY). Sodium orthovanadate was obtained from Aldrich Chemical Co. and the bicinchoninic acid assay was obtained from Pierce Chemical Co. Stock Solutions of 100 mM vanadate were prepared by dissolving Na_3VO_4 in 100 mM NaOH to avoid the formation of decavanadate. Synaptic vesicles prepared from the electric organ of *Torpedo californica* were concentrated by centrifugal pelleting, resuspended in 0.7 M glycine, 0.1 M HEPES*, 1 mM EGTA, 1 mM EDTA, pH 7.8 and stored at −70 °C as described [8]. The amount of EGTA and EDTA diluted into the assay was negligible. All other chemicals and biologicals were of the highest grade available from the usual commercial sources. The pH of all solutions was adjusted with NaOH to 7.4 and buffered by 59 mM HEPES.

Assays

Frozen synaptic vesicles were thawed, homogenized gently and the protein concentration determined by bicinchoninic acid assay to be 3.4 mg/ml. Vesicle ATPase activity was assayed using a pyruvate kinase–lactate dehydrogenase coupled spectrophotometric assay, as described [8]. The 1 ml assay volume contained 68 μg vesicle protein, 20 units/ml each of pyruvate kinase and lactate dehydrogenase, 59 mM HEPES, 3 mM MgATP, 2 mM potassium phosphoenol pyruvate, 1 mM $MgCl_2$, 0.05 mM K_2NADH and 1 mM ouabain. Four samples were assayed in triplicate. The control for total ATPase activity contained vesicles only. The vanadate control contained 0.5 mM vanadate in addition to vesicles. For reactivation, the desferrioxamine samples contained vesicles, 0.5 mM vanadate and 2 mM or 4 mM desferrioxamine added after a 10-min incubation with vanadate. The levels of vanadate and desferrioxamine used in the ATPase assay system were found to have no significant effect on assay performance. The vanadate control and 2 mM desferrioxamine samples were used for ^{51}V NMR spectroscopy.

^{51}V NMR Spectroscopy

The ^{51}V NMR spectra were obtained at room temperature using a General Electric GN 300 spec-

*The abbreviations used are: HEPES = *N*-2-hydroxyethyl-*N*'-2-piperazine ethane sulfonic acid; EGTA = [ethylenebis-(oxoethylenenitrilo)]tetraacetic acid.

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trometer (frequency for ^{51}V, 79.0 MHz) equipped with a multinuclear broadband probe. Spectra were acquired unlocked, in non-spinning samples under the following conditions: 20 μs 90° pulse length, 100 ms pulse repetition rate and a 100 000 Hz spectral width. Chemical shifts were reported relative to neat $VOCl_3$ as an external reference.

Results and Discussion

The ATPase activity of highly purified synaptic vesicles isolated from the electric organ of the marine ray *Torpedo* was determined using the assay system described above. Figure 1 shows that the vesicles exhibited an ATPase activity of 200 nmol/min/mg, and 80% of this activity is inhibited by 0.5 mM vanadate, which is saturating. The inhibited ATPase is a phosphointermediate-forming enzyme [8]. The remaining 20% ATPase activity is due to the presence of a vanadate-insensitive vacuolar ATPase present in the synaptic vesicles [8]. Addition of 2 or 4 mM desferrioxamine rapidly leads to rapid recovery of up to 95% of the inhibited activity, successfully reversing the inhibition of the phosphointermediate-forming ATPase.

The ^{51}V NMR spectrum (Fig. 2(A)) shows that in the ATPase assay system containing 0.5 mM vanadate, most of the vanadium(V) is present as the monomeric $H_2VO_4^-/HVO_4^{2-}$ species at −556 ppm, although the presence of dimeric $H_2V_2O_7^{2-}$ at −571 ppm and tetrameric $V_4O_{12}^{4-}$ at −576 ppm is evident, as previously identified [9]. Upon addition of 2 mM desferrioxamine, the ^{51}V resonances shift to a new resonance at −515 ppm with a half width of 773 Hz (Fig. 2(B)). The UV–Vis absorption spectrum also

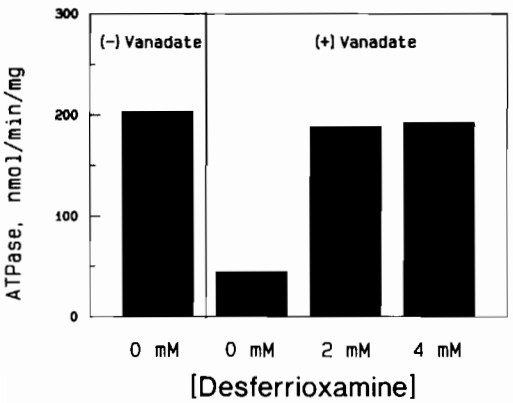

Fig. 1. Reactivation of vanadate-inhibited ATPase by desferrioxamine. The first bar shows uninhibited ATPase activity. The second bar shows inhibition of ATPase activity by 0.5 mM vanadate. The third and fourth bars show recovery of ATPase activity after chelation of vanadium(V) with desferrioxamine at 2 and 4 mM, respectively. The average standard deviation was ±1.1 nmol min^{-1} mg^{-1}.

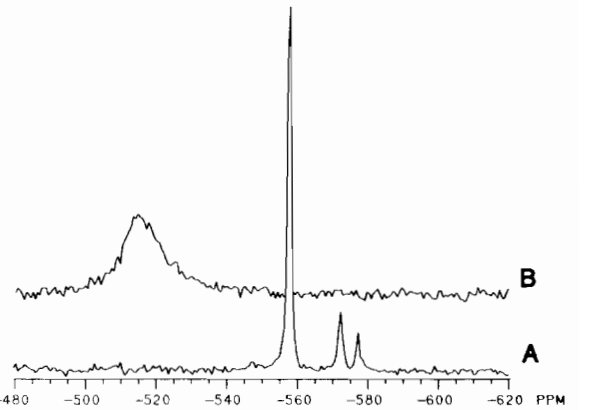

Fig. 2. ^{51}V NMR spectra of: (A) 0.5 mM vanadate and (B) 0.5 mM vanadate plus 2 mM desferrioxamine in the ATPase assay buffer, 0.1 M HEPES, pH 7.4 (see Experimental section). The samples were prepared as described in the Experimental section.

reflects rapid 1:1 complexation [7] of vanadium(V) by desferrioxamine, as shown by the appearance of a new absorption maximum at 320 nm ($\epsilon \approx 2440 \pm 100$ M^{-1} cm^{-1}). The maximum absorbance is attained in <1 min under equimolar vanadate and desferrioxamine concentrations. Thus vanadate is quantitatively and rapidly complexed by desferrioxamine. The relatively high extinction coefficient of the vanadium(V)–desferrioxamine complex at 340 nm ($\epsilon \approx 2070$ M^{-1} cm^{-1}) limits the concentration of vanadate and desferrioxamine which can be utilized in the coupled pyruvate kinase–lactate dehydrogenase spectrophotometric assay used for many ATPase studies since the λ_{max} of NADH is at 340 nm ($\epsilon \approx 6220$ M^{-1} cm^{-1}). This absorbance, however, will not affect a ^{32}P-release assay.

Vanadate polymerization is a potential complication in a vanadate pulse experiment. Polymerization is dependent on concentration and pH and can lead to the irreversible formation of decavanadate [10]. Although probably acting by a different mechanism from orthovanadate, decavanadate has been found to bind to and affect the properties of at least one ATPase [11]. Other potential complications include complexation of vanadate by common amine buffers [12], and possible reduction of vanadate under certain conditions [13]. Because the ^{51}V NMR spectrum of the vanadate-inhibited ATPase assay solution is identical to the spectrum of equimolar vanadate in water, including resonance positions and total intensity, Fig. 2 demonstrates that HEPES buffer does not complex vanadate, decavanadate was not formed and NADH or other agents did not cause reduction of vanadate.

In summary, desferrioxamine complexation of vanadium(V) should prove to be a useful tool in mechanistic studies of phosphate-metabolizing

enzymes. Desferrioxamine rapidly (<1 min) complexes V(V) over a wide pH range [7], without complexing other essential metal ions. We are continuing our investigations of the exact nature of the vanadate–desferrioxamine complex and the kinetics and mechanism of complex formation.

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References

1 S. G. O'Neal, D. B. Rhoads and E. Racker, *Biochem. Biophys. Res. Commun., 89* (1979) 845.
2 R. L. VanEtten, P. P. Waymack and D. M. Rehkop, *J. Am. Chem. Soc., 96* (1974) 6782.
3 Y. K. Agrawal and G. D. Mehd, *J. Environ. Anal. Chem., 10* (1981) 183.
4 R. M. Cassidy and D. E. Ryan, *Can. J. Chem., 46* (1968) 327.
5 H. Bickel, R. Bosshardt, E. Gaumann, P. Reusser, E. Vischer, W. Voser, A. Wettstein and H. Zahner, *Helv. Chim. Acta, 43* (1960) 2118.
6 S. Luterotti and V. Grdinic, *Analyst, 111* (1986) 1163.
7 I. Batinic, M. Birus and M. Pribanic, *Croatica Chem. Acta, 60* (1987) 279.
8 S. K. Yamagata and S. M. Parsons, *J. Neurochem.*, (1989) in press.
9 O. Borgen, M. R. Mahmoud and I. Skauvik, *Acta Chem. Scand., Ser A, 31* (1976) 329.
10 E. Heath and O. W. Howarth, *J. Chem. Soc., Dalton Trans.* (1981) 1105.
11 C. Coan, D. J. Scales and A. J. Murphy, *J. Biol. Chem., 261* (1986) 10394.
12 D. C. Crans and P. K. Shin, *Inorg. Chem., 27* (1988) 1797.
13 N. D. Chasteen, *Struct. Bonding (Berlin), 53* (1983) 107.