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Original article

^{51}V NMR investigation of cell-associated vanadate species in *Phycomyces blakesleeanus* mycelium

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Abstract

^{51}V NMR spectroscopy was used for detection and identification of cell-associated vanadate (V^{5+}) species after exposure of *Phycomyces blakesleeanus* mycelium, in exponential phase of growth, to sodium orthovanadate. Complete disappearance of monomer and dimer signals and decreased intensity of the tetramer signal were observed about 40 min after treatment. Simultaneously, a signal at −532 ppm, with increasing intensity, was detected in spectra. The time-dependent rise in this signal was connected to a decrease in the extracellular monomer signal, indicating its transport into the cell. The signal at −532 ppm did not belong to any known simple oxido-vanadate species, nor to a complex with any of the components of experimental medium. This signal was the only one present in spectrum of the mycelium washed 35 min after treatment, and the only one observed in mycelium cultivated on vanadate-contained medium. Therefore, its appearance can be attributed to intracellular complexation, and may represent an important detoxification mechanism of the cell exposed to a physiologically relevant concentration of vanadate. Experiments (^{51}V NMR and polarography) performed with Cd-pretreated mycelium (inhibitor of an enzyme responsible for V^{5+} reduction) and ferricyanide-preincubated mycelium excluded the possibility of V^{5+} tetramer's entry into the cell.

Keywords: ^{51}V NMR; *Phycomyces blakesleeanus*; Vanadate; Fungi

1. Introduction

The remarkable increase in investigation of vanadate (V^{5+}) behavior in biological systems in past decades has been associated with discoveries of its therapeutic effects [1–3], existence of vanadate-dependent enzymes [4] and a wide range of metabolic actions [5–7]. The most prominent vanadate effects are due to its structural and electronic analogy with phosphate, which enables V^{5+} to substitute for phosphates in phosphate-dependent physiological processes [4]. However, depending on the pH, the monomer undergoes condensation reactions, thus forming various anionic products, including dimer, tetramer, pentamer and decamer [8] where such an analogy is not always valid, but physiological action is still observed [7,9,10]. In micromolar concentrations and at neutral or alkaline pH, the monomer is the predominant form of vanadate [11]. An increase in concentration at similar pH conditions leads to the formation of a V^{5+} dimer (V2) and tetramer (V4). V4 represents the most abundant V^{5+} species under physiological conditions and concentrations above 1 mM. Further polymerization to decavanadate (V10) can be achieved in a more acidic environment [12].

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The activity of a large number of phosphate enzymes is closely correlated with the degree of vanadate oligomerization in a cell. It has been demonstrated that V2 is a potent inhibitor of glucose 6 phosphate dehydrogenase [13], glycogen phosphorylase isolated from rabbit muscle [14], fructose 1,6 bisphosphate aldolase [15] and glycerol 3 phosphate dehydrogenase [16], while V4 inhibits enzymes involved in the pentose-phosphate pathway [13–16] and glycolysis [13,17]. Various V^{5+} oligomers induce different cellular responses, i.e. V4 is a more potent inhibitor of 6-phosphoglycerate dehydrogenase than V1, although both forms exhibit inhibitory effects [18]. Also, V10 shows inhibitory effects upon several kinase enzymes, thus exhibiting the opposite action in regard to V1 influence upon the same group of enzymes [14]. The inhibitory effect of V10 on phosphoglucomutase is closely related to cessation of growth of *Leishmania tarentolae* [19]. Different characteristics of various V^{5+} species account for the more toxic effect of the dimer and decamer on yeast growth than that of the monomer [1,20]. Therefore, identification of species responsible for vanadate-induced effects is important, given the ability of V^{5+} to form complexes with many biologically relevant molecules, with coordination geometry far from ideal symmetry [12].

^{51}V NMR spectroscopy is a suitable technique for determination of different forms of V^{5+}, as the large chemical shift range contains a great deal of information on vanadate speciation [23]. It was established that many biologically important V^{5+} forms can be determined with a high signal to noise ratio [20].

In our previous investigations, we demonstrated that vanadate addition influences phosphate metabolism in the fungus *Phycomyces blakesleeanus* [24], without exhibiting a toxic effect on its growth [25], typically observed in yeast cells [20]. The significant increase in the level of glycolytic sugar phosphates (SP) was the result of V^{5+} action, in spite of its significant cellular reduction [25,26]. However, the exact form of V^{5+} that accumulates in the cell remains poorly understood. Here we attempt to discover the species responsible for the observed vanadate influence on glycolytic processes and the mechanism of V^{5+} detoxification of *P. blakesleeanus*.

2. Materials and methods

2.1. Mycelium cultivation and materials

The wild-type strain of the fungus *P. blakesleeanus* (Burgeff) (NRRL 1555(-)) was used. The mycelium was grown in standard minimal medium [27] in Erlenmeyer flasks shaken and aerated in the growth cabinet with continuous overhead white fluorescent light of 10 W/m^2, at a temperature of 20 °C and ca. 95% relative humidity. Stock solution of 200 mM sodium orthovanadate (Na_3VO_4) was prepared by the method of Gordon [28]. For some experiments, mycelium was cultivated for 24 h with addition of 2, 5, and 7 mM of sodium orthovanadate to standard minimal medium. All chemicals were of analytical grade and obtained from Sigma–Aldrich (Taufkirchen, Germany).

2.2. ^{51}V NMR spectroscopy

For NMR measurements, 24-h-old mycelium was collected by vacuum filtration and washed with experimental minimal medium (standard minimal medium without phosphates or microelements). The amount of 0.6 g of mycelia was suspended in aerated experimental medium and packed in small glass vials of 1.9 ml total volume. Sodium orthovanadate was added at a final concentration of 80 $\mu mol/g_{FW}$ (FW, fresh weight of mycelium). For experiments of vanadium uptake and reduction inhibition, phosphate (KH_2PO_4) was applied at a final concentration of 36.7 mM, and $CdCl_2$ or $K_3(Fe(CN)_6)$ at concentration of 40 $\mu mol/g_{FW}$. In all experiments with washed mycelium, the treated mycelium was washed 5 times with distilled water and vacuum filtration prior to spectra recording.

The measurements were performed on Apollo upgrade (Tecmag, USA), Bruker MSL 400 (Germany) spectrometer operating at 105.169 MHz for ^{51}V, using a Bruker static solid state probe, with horizontal sample position. Pulse duration was 20 μs (45°), the duration of spectrum recording from 1.6 min (vanadate in experimental medium), 3.6 min and 36 min for spectra collected from the unwashed and washed mycelium, respectively. Chemical shifts are given relative to external 1 M sodium metavanadate, pH 12 which, in aqueous solution, produces two signals at −535.7 ppm (used as auxiliary reference) and −560.4 ppm, relatively to $VOCl_3$ [12].

2.3. Polarographic measurements

Direct current polarography (DCP) curves were recorded by a Princeton Applied Research (PAR) 174A Polarographic Analyzer equipped with a Houston instrument, Omnigraphic 2000 X–Y recorder. A 30 ml electrolytic cell was supplied with a working dropping mercury electrode (DME) with capillary constant (m) 2.5 mg s^{-1} at mercury reservoir height of 75 cm. Drop time of DME in all experiments was programmed to 1 s. Saturated calomel electrode (SCE) and Pt-foil were used as the reference and auxiliary electrode. Polarographic curves were recorded with a scan rate of 10 mV/s. Current oscillations were damped with low pass filter instruments positioned at 3 s. Starting potentials as well as current sensitivities were selected according to requirements of each individual measurement. At the beginning of every experiment, buffer solution (10 mM HEPES, pH = 7.2) in the cell was de-aerated with nitrogen bubbling for 5 min. DCP experiments were performed on a mixture of 17 ml HEPES and 2 ml of mycelium suspension (about 20 mg mycelium dry weight), with addition of V^{5+} and $CdCl_2$ to a final concentration of 0.5 mM.

2.4. EPR

The mycelium was resuspended in HEPES buffer in order to maintain optimal pH conditions, treated with 0.5 and 2.5 mM sodium orthovanadate and, after 35 min, collected by vacuum filtration. Samples were placed on an open Teflon

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