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Decavanadate induces mitochondrial membrane depolarization and inhibits oxygen consumption

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Abstract

Decavanadate induced rat liver mitochondrial depolarization at very low concentrations, half-depolarization with 39 nM decavanadate, while it was needed a 130-fold higher concentration of monomeric vanadate (5 µM) to induce the same effect. Decavanadate also inhibits mitochondrial repolarization induced by reduced glutathione *in vitro*, with an inhibition constant of 1 µM, whereas no effect was observed up to 100 µM of monomeric vanadate. The oxygen consumption by mitochondria is also inhibited by lower decavanadate than monomeric vanadate concentrations, i.e. 50% inhibition is attained with 99 nM decavanadate and 10 µM monomeric vanadate. Thus, decavanadate is stronger as mitochondrial depolarization agent than as inhibitor of mitochondrial oxygen consumption. Up to 5 µM, decavanadate does not alter mitochondrial NADH levels nor inhibit neither F_OF_1-ATPase nor cytochrome *c* oxidase activity, but it induces changes in the redox steady-state of mitochondrial b-type cytochromes (complex III). NMR spectra showed that decameric vanadate is the predominant vanadate species in decavanadate solutions. It is concluded that decavanadate is much more potent mitochondrial depolarization agent and a more potent inhibitor of mitochondrial oxygen consumption than monomeric vanadate, pointing out the importance to take into account the contribution of higher oligomeric species of vanadium for the biological effects of vanadate solutions.
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1. Introduction

Most of the toxicological studies of vanadate in biological systems do not take in consideration the contribution of vanadate oligomers, such as the decameric vanadate species (V_{10}). Decameric vanadate, composed by fused V(V) octahedra, has an ellipsoid like structure with a major and minor axis of approximately 7 and 6 Å, respectively [1]. In previous studies [2–8], we have demonstrated that once formed in aqueous solutions decameric vanadate decomposition is slow enough to allow studying differential effects between decameric and monomeric vanadate not only *in vitro* but also *in vivo*. Furthermore, decameric vanadate is stabilized upon interaction with cytoskeleton and membrane proteins, while it affects calcium translocation by the sarcoplasmic reticulum calcium pump and it prevents actin polymerization [9].

Vanadate is known to affect mitochondrial respiration by altering electron transfer between complexes III and IV [10]. Several studies have associated vanadium toxicity with its capacity to induce the formation of reactive oxygen species (ROS), probably by interacting with mitochondrial redox centres [11,12]. Along this line of thinking, DeMaster and Mitchell [10] pointed out that vanadate blocked electron

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transfer through the respiratory chain between cytochrome b_1 and cytochrome c, which caused inhibition of the oxidation of NADH-linked substrates and succinate [13,14]. Mitochondrial succinate dehydrogenase had also been reported to be inhibited by vanadium (V) ions [15], as well as the ATP-dependent succinyl-CoA synthetase (A-SCS) from rat brain mitochondria [16,17]. Studies involving other vanadate species, instead of only monomeric vanadate (V_1), have shown that decavanadate: (i) stimulates oxidation of NADH by rat erythrocyte plasma membrane [18,19] and by rat liver microsomes [20,21]; (ii) enhances cytochrome c reduction [22]; (iii) exhibits α-adrenergic agonist activity in rat aortic rings [23]; and (iv) is reduced by $NADP^+$-specific isocitrate dehydrogenase (IDH) [22].

As the effects of decameric vanadate on mitochondrial membrane potential and oxygen consumption have not been described, its contribution to the impairment of mitochondrial function associated with vanadium citotoxicity (Soares et al., unpublished results) is still an unresolved issue. This work addresses these points using rat hepatic mitochondria, aiming to elucidate the relative sensitivity of mitochondrial functions to decameric and monomeric vanadate. It is shown for the first time that decameric vanadate is a very strong mitochondrial depolarizing agent and also a potent inhibitor of mitochondrial oxygen consumption.

2. Materials and methods

2.1. Vanadate solutions

Metavanadate stock solution (50 mM, pH 6.7) was prepared from ammonium metavanadate (NH_4VO_3). Decavanadate stock solution was obtained by adjusting the pH of the former solution to 4.0 [24]. Vanadate oligomers composition of decavanadate and metavanadate solutions upon dilution into the mitochondrial respiration buffer (0.2 M sucrose, 5 mM KH_2PO_4, 10 mM KCl, 5 mM $MgCl_2$ and 10 mM Tris–HCl, pH 7.4 plus 5 mM pyruvate and 0.5 mM malate), in the absence or in the presence of 5 mM glutathione (GSH), was measured by ^{51}V nuclear magnetic resonance spectroscopy in a Bruker AM-400 spectrometer at 105.2 MHz equipped with a 5-mm multinuclear inverse probe, by using a 90° pulse Fourier transform technique, as previously reported [4,7,8].

2.2. Animals

Adult (3-month-old) male rats (*Rattus norwegicus*), weighing 200–250 g, from an inbred Wistar strain were obtained from the Animal Services of the University of Extremadura, Badajoz (Spain). Animals were provided within a standard chow with free access to water and food. Animals were fasted overnight with a free access to water before sacrificed. All the experiments on animals follow the National Research Council's guide for the care and use of laboratory animals.

2.3. Experimental procedures

2.3.1. Mitochondria isolation

Mitochondria were isolated from rat liver by differential centrifugation, as described by Johnson and Lardy [25]. Mitochondria samples were aliquoted and stored at $-80\,°C$, immediately after their preparation in order to prevent *in vitro* aging, and used within 2 weeks. Protein concentration was determined with the Bradford's assay [26], using bovine serum albumin as protein standard. Rat hepatic mitochondria have succinate dehydrogenase and F_OF_1-ATPase activities of 2120 ± 453 and 1115 ± 64 nmol/min/mg protein, respectively, values that falls within the range reported for these enzymes activities in mammalian mitochondria [27].

2.3.2. Mitochondrial enzyme activities

Succinate dehydrogenase (SDH) activity was measured at $37\,°C$ from potassium ferricyanide reduction [28]. F_OF_1-ATPase activity was measured spectrophotometrically using the coupled enzyme pyruvate kinase/lactate dehydrogenase assay [29]. Vanadate solutions effects on mitochondrial F_OF_1-ATPase activity was assayed, with decameric (V_{10}) or monomeric (V_1) vanadate species up to 20 μM (total vanadium), in order to avoid the non-enzymatic NADH oxidation, observed in the presence of vanadium concentrations above 50 μM (data not shown).

Decavanadate and metavanadate effects on mitochondrial cytochrome c oxidase activity was analysed by the spectroscopic method of Smith [30]. The rate of oxidation of cytochrome c was measured by following the decrease in the absorbance of its α-band at 550 nm [31]. The standard reaction mixture containing 10 mM phosphate buffer (pH 7.0) plus 14% ferrocytochrome c and the absorbance change at 550 nm was measured at $37\,°C$, in the absence or in the presence of vanadate, after addition of 200 μg mitochondria protein per ml.

2.3.3. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was monitored using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1), a fluorescent indicator of membrane potential [32].

Mitochondria (15 μg protein/ml) were loaded with 0.6 μM JC-1 by incubation at $37\,°C$ for 15 min [33] and fluorescence experiments were performed under continuous stirring with a Perkin-Elmer 650–40 fluorescence spectrophotometer (Perkin–Elmer, Foster City, CA, USA). JC-1 monomer (green) fluorescence was measured by excitation at 490 nm and recording the emission near 532 nm. JC-1 aggregate (red) fluorescence was measured by excitation at 490 nm and recording the emission at 595 nm. The effect of vanadate solutions on mitochondrial membrane potential was determined by adding different vanadate concentration up to 50 μM (total vanadium). Assays were standardized with a known

potent uncoupler of oxidative phosphorylation, 1 mM 2,4(α)-Dinitrophenol (DNP). The ratio of JC-1 aggregate to monomer intensity was calculated and the degree of depolarization estimated (Eq. (1)). The value of the ratio (F595/F532) for monomeric JC-1, which corresponds to totally depolarized mitochondria, was 1.52 ± 0.47. An increase in this ratio is indicative of increase in membrane potential, whereas a decrease in the ratio is indicative of decrease in membrane potential [34]

$$Depolarization(\%) = \frac{R_t - R_0}{R_0 - R_{+DNP}} \times 100 \qquad (1)$$

R_t = ratio of JC-1 aggregate to monomer intensity after incubation (60 min)
R_0 = initial ratio of JC-1 aggregate to monomer intensity (time = 0 min)
R_{+DNP} = ratio of JC-1 aggregate to monomer intensity 1 h after DNP addition

2.3.4. Determination of mitochondrial oxygen consumption

Oxygen consumption of mitochondria was measured polarographically with a Hansatech Oxygen Electrode equipped with a Clark-type oxygen electrode and a potentiometric recorder. Respiration of freshly isolated rat hepatic mitochondria at a final concentration 2 mg/ml was measured at 37 °C, in the standard reaction mixture contained (in mM): 250 sucrose, 5 KH_2PO_4, 10 KCl, 5 $MgCl_2$, 10 Tris–HCl (pH 7.4) and supplemented with the following substrate combination: 0.5 mM malate plus 5 mM pyruvate. Respiration rates were measured in the absence and presence of 200 μM ADP. V_{10} effects on mitochondrial respiration were assayed upon incubation with vanadate concentrations of both vanadate stock solutions below 25 μM (total vanadium). At the end of each run, an inhibitor of the mitochondrial electron transport chain – 40 μM rotenone – was added and the new rate of respiration measured. In the presence of rotenone, no oxygen consumption was obtained. The oxygen concentration in buffer was 219 nmol of O_2 per ml after standard calibration in water at 37 °C. Oxygen consumption rates were expressed as nmol O/min/mg protein. Respiratory control ratio, as described by Chance and Williams [35], and ADP/O ratio were calculated as previously reported [36].

2.3.5. Measurement of NADH content in mitochondria

NADH content in isolated mitochondria exposed to vanadate concentrations up to 20 μM (total vanadium), was determined through fluorimetric measurement of NADH in the neutralized alkaline mitochondrial extracts [37]. Fluorescence signals were recorded (λ excitation: 340 nm; λ emission: 460 nm) in a medium containing: 0.1 M phosphate plus 0.1 M KCl (pH 7.5), 1 mM pyruvate and 1.375 IU lactate dehydrogenase, and the NADH concentration was calculated using a fluorescence calibration curve obtained with known concentrations of this pyridine nucleotide.

2.3.6. Reactive oxygen species production

Vanadate effects on reactive oxygen species (ROS) production, namely superoxide anion (O_2^-) production, was measured at pH 7.4 and 37 °C using the fluorescent dye dihydroethidium (DHE), as in Budd et al. [38]. The effect of an electron donor (NADH) on ROS production was tested separately in the presence and absence of vanadate (concentrations up to 50 μM total vanadium). O_2^- production was calculated from 10 μM DHE fluorescence observed (λ excitation: 473 nm; λ emission: 600 nm) in presence of 0.5 mM malate plus 5 mM pyruvate or 250 μM NADH.

2.3.7. Cytochrome b redox state

The overall spectrum of mitochondria was acquired by differential spectroscopy in a Hewlett Packard 8451 A diode array spectrophotometer, a single-beam, microprocessor-controlled spectrophotometer. The absorbance readings at all wavelengths of the overall spectrum (every 2 nm from 400 to 600 nm) were obtained simultaneously. The incubation medium contained mitochondrial respiration buffer (Section 2.1), 0.5 mM malate plus 5 mM pyruvate and 1 mg mitochondria protein per ml. The electron chain spectrum and its redox changes upon incubation with decavanadate or metavanadate solutions were compared with a control situation (in the absence of vanadate).

2.4. Statistical analysis

All parameters studied are present as average and standard deviation of measurements taken at least in three separate experiments. Statistical significance of the data was calculated by Mann–Whitney non-parametric test. Differences from control were considered significant at $P < 0.05$.

3. Results and discussion

3.1. Effect of decavanadate on mitochondrial membrane potential

In the absence of GSH the membrane potential remains stable for 10–15 min and then slowly decays (Fig. 1a), thus, allowing to determine the effects of decameric (V_{10}) and monomeric (V_1) vanadate on mitochondrial membrane potential. Fig. 1b shows that 10 min exposure to increasing vanadate concentrations leads to increasing mitochondrial depolarization. V_{10} induced mitochondrial depolarization in rat hepatic mitochondria at very low concentrations with an IC$_{50}$ of 38.7 ± 10.2 nM, while 5.4 ± 2.5 μM V_1 was needed to induce a 50% depolarization in rat liver mitochondria.

3.1.1. Decavanadate effect on GSH-induced mitochondrial membrane repolarization in respiration buffer

Addition of a physiological concentration of GSH (5 mM) to the mitochondria respiration buffer after 60 min, i.e. to depolarized mitochondria, induced mitochondrial repolarization *in vitro* with a half-time of ≈5 min, reaching
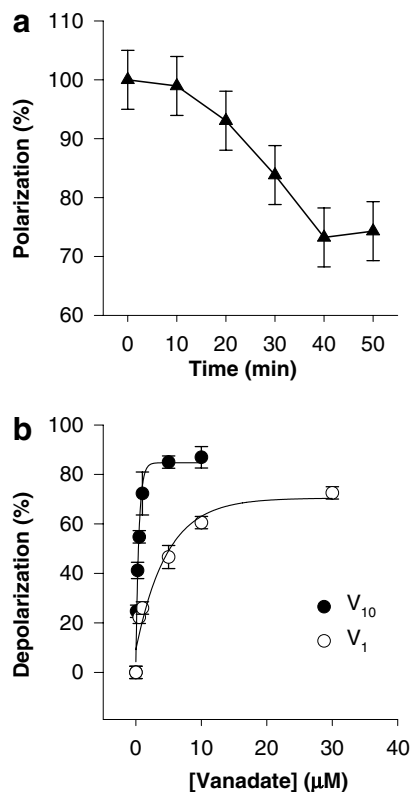
Fig. 1. Mitochondrial membrane depolarization by decameric (V_{10}) and monomeric (V_1) vanadate species. Time-induced mitochondrial membrane depolarization (a) in the absence of a physiological glutathione (GSH) concentration; V_{10}- (solid circles) and V_1-induced (open circles) mitochondrial membrane depolarization (%) on rat liver mitochondria (15 μg protein/ml), in the presence of 5 mM GSH (b). The assay was performed at 37 °C, in a reaction medium containing the corresponding mitochondrial isolation buffer (250 mM sucrose; 5 mM Tris pH 7.4 and 1 mM EGTA) supplemented with 150 mM KCl, 10 mM Tris pH 7.4, 0.5 mM malate plus 5 mM pyruvate and 0.6 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), a fluorescent indicator of membrane potential. Values are present as means ± SD ($n = 3$).
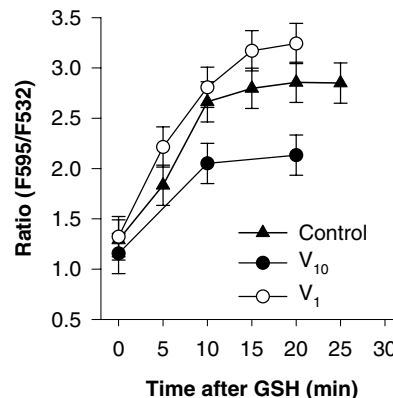

Fig. 2. Kinetics of mitochondrial membrane repolarization. Kinetics of mitochondrial membrane repolarization with physiological glutathione (GSH) concentration (5 mM) (solid triangles); GSH-induced repolarization after mitochondrial membrane depolarization by 1 μM decameric vanadate (V_{10}) (solid circles) or 100 μM monomeric vanadate (V_1) (open circles) in respiration buffer minus GSH. Repolarization was monitored at 37 °C in 250 mM sucrose, 15 mM Tris pH 7.4, 1 mM EGTA, 150 mM KCl, 0.5 mM malate plus 5 mM pyruvate, 15 μg hepatic mitochondria protein/ml and 0.6 μM JC-1 membrane potential fluorescent indicator. Values are present as means ± SD ($n = 3$).

a steady-state ≈10 min after GSH addition (Fig. 2). Besides inducing membrane repolarization, GSH confers stability of the mitochondrial membrane potential with a half-life time of ≈90 min (data not shown).

From steady-state values of the control (≈2.75), V_{10} (≈2.0) and V_1 (≈3.0) we determined the effects induced by the vanadate solutions on membrane repolarization, considering the approx. basal value of 1.25 observed at time zero, for the addition of GSH. In this sense, for 1 μM V_{10} we obtained an inhibition of almost 50%, whereas in the presence of 100 μM V_1 the mitochondrial repolarization was stimulated by ≈10% (Fig. 2).

3.2. Effect of decavanadate on mitochondrial oxygen consumption

With pyruvate plus malate, the basal respiration rate of our preparations of rat hepatic mitochondria was 39 and 20 ng atom O/min/mg protein for State 3 and 4, respec-

tively. It was observed that V_{10} inhibits mitochondrial respiration at State 3 (ADP-stimulated) as shown by a decrease in oxygen consumption measured in intact liver mitochondria (Fig. 3a). V_{10} is nearly 100-fold more potent than V_1 as inhibitor of oxygen consumption in hepatic mitochondria, as shown by their IC_{50} values, 98.5 ± 5.1 nM for V_{10} and 9.7 ± 1.4 μM for V_1 (Fig. 3a).

Both vanadate solutions promote mitochondrial respiration inhibition without uncoupling mitochondria, as the respiratory control ratio (RCR) was not affected (Fig. 3b). The RCR values for rat liver coupled mitochondria respiring on pyruvate and malate were 5.1 ± 0.1, in the absence or presence of both vanadates species (Fig. 3b). These results are in good agreement with those reported in [10], where there was found no uncoupling of oxidative phosphorylation in intact rat liver mitochondria and a small apparent increase in ADP/O rates, due to an inhibition of adenylate kinase activity by V_{10} (1 mM). It is to be noted that our results leads to ADP/O ratios for pyruvate and malate of 2.4 ± 0.7, both in the absence or presence of V_{10} or V_1, a value which is close to the theoretical value of 3 [36].

3.3. Decavanadate effects on mitochondrial F_OF_1-ATPase activity, NADH levels and reactive oxygen species production

Rat hepatic mitochondria F_OF_1-ATPase exhibited a basal control activity of 1115 ± 64 nmol/min/mg protein. Up to 20 μM, no effects were observed for V_{10} or V_1 on this mitochondrial ATPase activity, in agreement with several studies using higher vanadate concentrations (100 μM total
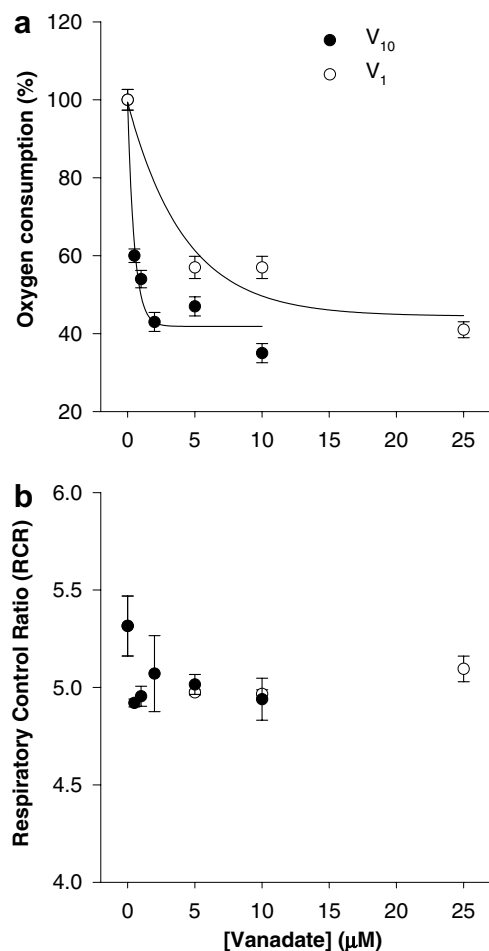
Fig. 3. Decameric vanadate (V_{10}) and monomeric vanadate (V_1) inhibitory effects on mitochondrial respiration measured with an oxygen electrode. Effect of V_{10} (solid circles) and V_1 (open circles) (up to 25 μM total vanadium, namely 2.5 μM V_{10}) on rat liver respiring mitochondria (2 mg protein/ml) on pyruvate plus malate. Oxygen consumption (%) (a) and respiratory ratio control (RCR) (b) on hepatic intact mitochondria were recorded in mitochondrial respiration buffer containing 0.2 M sucrose, 5 mM KH_2PO_4, 10 mM KCl, 5 mM $MgCl_2$ and 10 mM Tris pH 7.4, to a final volume of 2 ml (5 mM pyruvate plus 0.5 mM malate were added as substrate). Values are present as means $\pm$ SD ($n = 3$).

Fig. 4. Rate of reactive oxygen species (ROS) production, namely superoxide anion ($O_2^{\cdot-}$), upon exposure to decameric (V_{10}) and monomeric (V_1) vanadate species. Effect of V_{10} (solid circles) and V_1 (open circles) (up to 50 μM total vanadium) on $O_2^{\cdot-}$ production (%) minus NADH (A) and in the presence of NADH (B) in rat liver mitochondria (15 μg protein/ml). The assay was performed at 37 °C, in the presence of the corresponding mitochondrial isolation buffer (250 mM sucrose; 5 mM Tris pH 7.4 and 1 mM EGTA) supplemented with 150 mM KCl, 10 mM Tris pH 7.4, 0.5 mM malate plus 5 mM pyruvate (a) or 250 μM NADH (b) and 10 μM dihydroethidium (DHE) fluorescent probe. Values are present as means $\pm$ SD ($n = 3$).

vanadium) [39,40]. NADH levels in mitochondria were also not affected by the vanadate solutions.

Additionally, both V_{10} and V_1 had no effect on reactive oxygen species (ROS) production, namely $O_2^{\cdot-}$, on rat hepatic mitochondria (Fig. 4a), except when NADH is used as an electron transfer chain substrate (Fig. 4b). Under the latter experimental conditions, V_{10} decreases $O_2^{\cdot-}$ production by 40% (5 μM) whereas 10-fold V_1 (50 μM) is need to induce a similar effect. This decrease in $O_2^{\cdot-}$ formation is, at least in part, due to the observed inhibition in mitochondrial respiration, once it was proved that $O_2^{\cdot-}$ originating from respiring mitochondria is a stoichiometric precursor of mitochondrial hydrogen peroxide (H_2O_2).

Recently, it has been described that in rat brain mitochondria even a very small decrease of mitochondrial membrane potential, which decreases mitochondrial res-
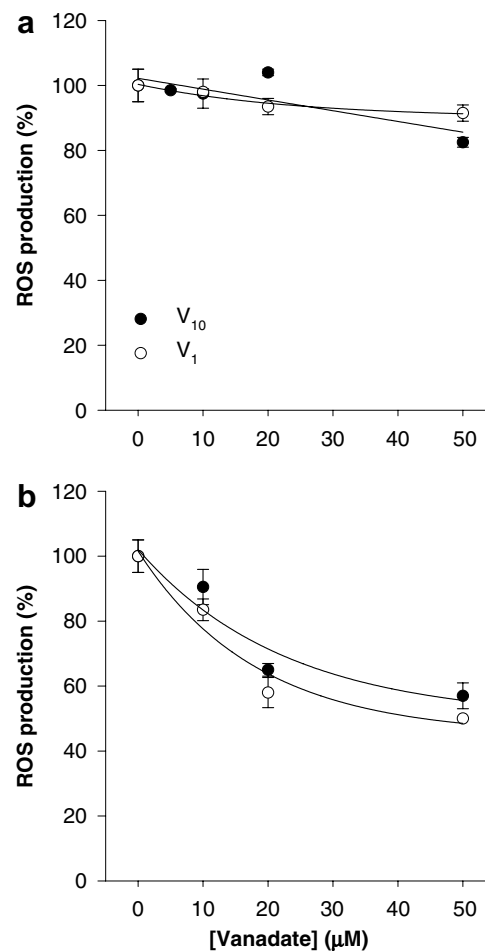
piration, results in a strong inhibition of ROS formation [41]. Therefore, ROS formation is also a function of mitochondrial membrane potential. Since V_{10} strongly depolarized mitochondria, the capacity to produce $O_2^{\cdot-}$ will be depressed and, in fact, the results obtained for V_{10} are fully consistent with this statement (Fig. 4b). Therefore, both V_{10}-induced mitochondrial depolarization and decrease in mitochondrial oxygen consumption can account for its antioxidant effect, monitored as partial inhibition of ROS production.

3.4. Characterization of vanadate solutions: NMR studies

In order to precisely define what vanadate species are promoting the observed effects in isolated rat liver

mitochondria, the vanadate species composition in vanadate solutions was analyzed by ^{51}V NMR spectroscopy. It was observed that the spectra of decavanadate solution, after dilution in the mitochondrial respiration buffer (Section 2.1) – 5 mM total vanadium, at pH 7.0 – contained only decameric species once the three signals from V_{10}, attributed to the three vanadium atoms of the decavanadate structure, as described elsewhere [24], are observed: V_{10A} at −516 ppm, V_{10B} at −499 ppm and V_{10C} at −425 ppm (Fig. 5a). Conversely, in the metavanadate solution (Fig. 5c), 5 mM total vanadium, were detected mono- (V_1), di- (V_2) and also tetrameric (V_4) vanadate species, respectively, at −561 ppm, −575 ppm and at −579 ppm. Two new vanadate signals were clearly identified in the ^{51}V NMR spectra of vanadate solution diluted in mitochondrial respiration buffer (Fig. 5c): these bound vanadate signals observed at −555 ppm (C1) and −540 ppm (C2) are due to the presence of sucrose in the medium, being C1 and C2 due to complexes of vanadate with geometric isomers of sucrose [42].

Millimolar GSH concentrations, the concentration range of GSH reported for most mammalian tissues, is known to reduce millimolar vanadate to vanadium(IV) at a physiological pH 7 [43]. In the presence of 5 mM GSH, used in the mitochondrial membrane potential studies, V_{10} signals suffer a decrease in intensity without broadening of the peaks (Fig. 5b). This decrease in the intensity of the decameric vanadate signals is due to V_{10} deoligomerization since it was observed that GSH induces it decomposition (not shown). In fact, a small peak at −561 ppm, can be observed upon addition of 5 mM GSH (Fig. 5d). The eventual presence of vanadyl paramagnetic species in solution would induce a broadening of NMR V_{10} signals besides an increase of the signal/noise ratio (that was not observed, see Fig. 5b). At physiological pH, vanadate NMR signals observed for the metavanadate solution remain unchanged upon addition of GSH to the medium (Fig. 5d), although it has been described that in the presence of GSH, vanadate is reduced to vanadyl and it becomes bound to haemoglobin [43].

In the presence of 1 mg/ml mitochondria, neither the concentration of the different vanadate species nor the ratio between vanadate oligomers in both solutions were changed (data not shown). Therefore, vanadate species do not disappear under the conditions used for the kinetic studies reported in this work, allowing correlating its in vitro effects on mitochondrial integrity and functionality. In contrast with metavanadate solutions, which contain labile oxovanadates interconverting each other within the milliseconds time scale, decameric vanadate present in decavanadate solutions has very slow decomposition kinetics upon dilution.

The kinetics of deoligomerization, assessed by measurements of the absorption at 400 nm, is a first order kinetics [8], with a half-life time up to 3 h (measured at 37 °C with 100 μM decameric vanadate species in mitochondrial respiration buffer). Therefore, at the V_{10} concentrations used in the enzymatic studies the decavanadate solutions contain decameric species whereas metavanadate solutions up to 100 μM mostly contain monomeric vanadate species, as described elsewhere [42].

3.5. Decavanadate effects on complex III

3.5.1. Decavanadate effects on cytochrome c oxidase

To experimentally test the possibility that the mechanism of V_{10}-induced inhibition of the electron transport chain could be due to inhibition of complex III, at first we measured the effect of V_{10} on the redox state of ferrocytochrome c. It was observed that at pH 7.0, either V_{10} or V_1 did not induce changes on the redox state of purified ferrocytochrome c. Moreover, neither V_{10} nor V_1 alter the cytochrome c oxidase activity of rat liver mitochondria (measured as indicated in Section 2.1). These results are in good agreement with the major conclusion reached in Kalyani and Ramasarma [21], namely, that the oxidation of reduced cytochrome c during vanadate-stimulated NADH oxidation required the presence of both vanadate and H_2O_2, since the latter oxidation is produced by the hydroxyl radicals formed in vanadate/H_2O_2 mixtures.
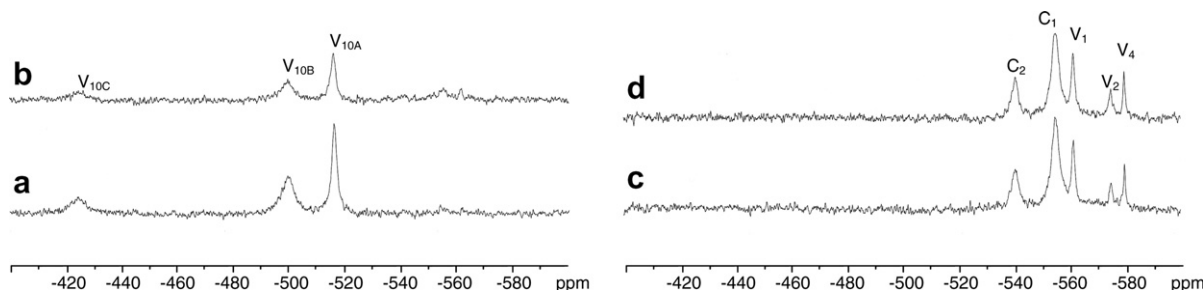

Fig. 5. 105.2 MHz ^{51}V NMR spectra, at room temperature, of decavanadate (pH 7.0) (a and b) and metavanadate (pH 7.0) (c and d) solutions (5 mM total vanadium) in mitochondrial respiration buffer (see Section 2.1) plus 5 mM pyruvate and 0.5 mM malate, in the absence (a and c) or in the presence (b and d) of 5 mM glutathione (GSH). All spectra were acquired in the presence of 10% D_2O. V_{10A}, V_{10B} and V_{10C} are signals of vanadium atoms from the decameric species. In the presence of GSH, new vanadate NMR signals, C_1 and C_2, are observed in metavanadate spectrum (C e D). V_1 and V_2 NMR signals correspond, respectively, to monomeric and dimeric vanadate species regardless of the protonation state, whereas V_4 corresponds to cyclic tetrameric vanadate species. C_1 and C_2 stand for sucrose:vanadate complexes.
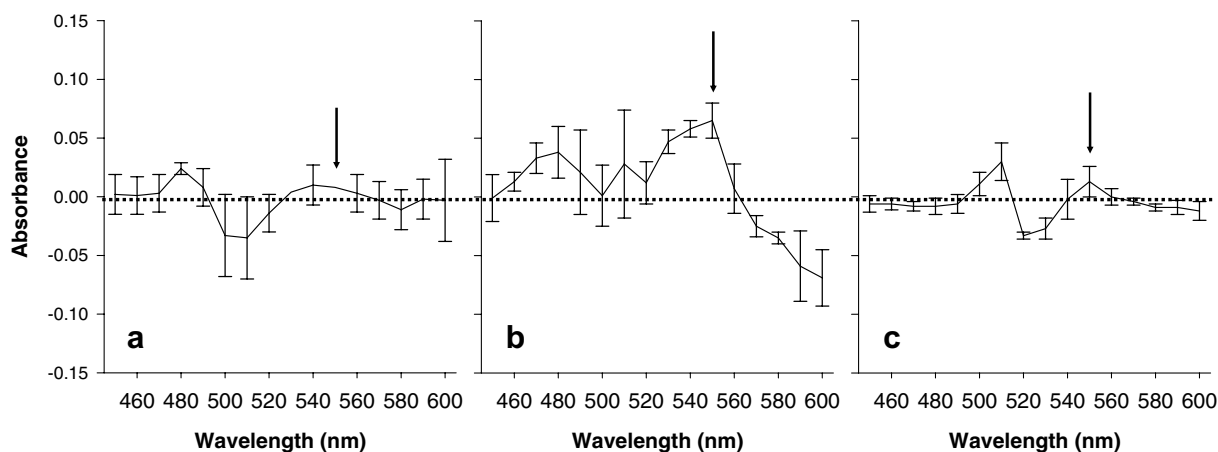
Fig. 6. Decameric vanadate (V_{10})-induced redox changes on cytochromes spectra of the respiratory chain. The figure shows the differential spectrum of rat liver mitochondria (1 mg protein/ml) in the presence of respiratory substrates (5 mM pyruvate + 0.5 mM malate) minus the spectrum in the same buffer in the absence of respiratory substrates. All the spectra were recorded on a split beam spectrophotometer upon incubation with 20 μM (total vanadium) V_{10} (b) or monomeric vanadate, V_1 (c) on mitochondrial respiration buffer (see Section 2.1). In the presence of V_{10} (B) there are large absorbance changes in the wavelength range that monitors the redox state of mitochondrial cytochromes b (500–550 nm) with respect to control (a). The arrows point at the wavelengths reported for the absorbance peaks of mitochondrial cytochromes b [46]. Values are present as means ± SD ($n = 3$).

3.5.2. Decavanadate effect on cytochrome b

Once excluded that V_{10}-induced mitochondrial depolarization and inhibition of oxygen consumption can be rationalized in terms of inhibition of cytochrome oxidase, we considered the possibility that the redox state of mitochondrial cytochromes b could be affected by decavanadate. To this end we recorded the effects of V_{10} and V_1 (20 μM total vanadium) on the differential spectrum of mitochondria in the presence and absence of the respiration substrates (pyruvate plus malate). The changes in the 500–550 nm wavelength range induced by V_{10} pointed out that the cytochrome b redox state is altered by V_{10}, but not with V_1, suggesting that mitochondrial complex III (ubiquinol:cytochrome c oxidoreductase) is a target for decameric vanadate (Fig. 6). Therefore, V_{10} induces antimycin A-like inhibition (specific inhibitor of complex III) in mitochondria respiratory chain.

4. Concluding remarks

Previous *in vivo* studies allowed us to suggest that mitochondria is a subcellular target of vanadate when vanadium is administered as V_{10} [2,5–8]. In the present work, it is shown for the first time that V_{10} is 10 to 100-fold more potent than V_1 as mitochondrial depolarization agent and as inhibitor of oxygen consumption by mitochondria. Moreover, V_{10}-induced mitochondrial depolarization is observed at V_{10} concentrations lower than those required to produce a significant inhibition of oxygen consumption by mitochondria. As membrane hyperpolarization has been described to be an early mitochondrial response during apoptosis [44,45], these results suggest to explore the usefulness of V_{10} as an anti-apoptotic agent in future studies. These effects of V_{10} are likely due to V_{10}-induced changes in the redox steady-state of cytochrome b (complex III), as

neither the mitochondrial NADH level, nor cytochrome oxidase, nor F_OF_1-ATPase activities are significantly altered by V_{10}. Furthermore, it is concluded that decameric vanadate strongly affects mitochondria bioenergetics through different toxicity mechanisms than monomeric vanadate.

5. Abbreviations

DHE	dihydroethidium
DNP	2,4(α)-dinitrophenol
GSH	glutathione reduced form
H_2O_2	hydrogen peroxide
IC_{50}	concentration that produced 50% of the effect on mitochondria membrane potential, on oxygen consumption or that inhibits 50% an enzyme activity
IU	amount of enzyme that release 1 μmole of product per minute
JC-1	5,5′,6,6′-tetrachloro-1,1′,3, 3′-tetraethylbenzimidazolcarbocyanine iodide
NADH	β-nicotinamide adenine dinucleotide (reduced form)
$O_2^{\cdot-}$	superoxide anion
ROS	reactive oxygen species
SDH	succinate dehydrogenase
Tris	tris(hydroxymethyl)aminomethane
V_1	orthovanadate or monomeric vanadate species
V_{10}	decameric vanadate species
V_2	dimeric vanadate species
V_4	tetrameric vanadate species

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