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Decavanadate as a biochemical tool in the elucidation of muscle contraction regulation

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Abstract

Recently reported decameric vanadate (V_{10}) high affinity binding site in myosin S1, suggests that it can be used as a tool in the muscle contraction regulation. In the present article, it is shown that V_{10} species induces myosin S1 cleavage, upon irradiation, at the 23 and 74 kDa sites, the latter being prevented by actin and the former blocked by the presence of ATP. Identical cleavage patterns were found for meta- and decavanadate solutions, indicating that V_{10} and tetrameric vanadate (V_4) have the same binding sites in myosin S1. Concentrations as low as 50 μM decavanadate (5 μM V_{10} species) induces 30% of protein cleavage, whereas 500 μM metavanadate is needed to attain the same extent of cleavage. After irradiation, V_{10} species is rapidly decomposed, upon protein addition, forming vanadyl (V^{4+}) species during the process. It was also observed by NMR line broadening experiments that, V_{10} competes with V_4 for the myosin S1 binding sites, having a higher affinity. In addition, V_4 interaction with myosin S1 is highly affected by the products release during ATP hydrolysis in the presence or absence of actin, whereas V_{10} appears to be affected at a much lower extent. From these results it is proposed that the binding of vanadate oligomers to myosin S1 at the phosphate loop (23 kDa site) is probably the cause of the actin stimulated myosin ATPase inhibition by the prevention of ATP/ADP exchange, and that this interaction is favoured for higher vanadate anions, such as V_{10}.

Keywords: Decavanadate; Myosin; Photocleavage; NMR

1. Introduction

Interest in the interaction of vanadate oxoanions with biological systems has increased gradually since it was demonstrated to have a variety of physiological effects acting either as a phosphate analogue in the monomeric form ($H_2VO_4^-$) [1] or through oligomeric vanadate species. Although it is often disregarded which of the oxovanadates is the active species, most of the studies are conducted at such low concentrations that the major species in solution is the monomer. Among the oligomeric species, probably the most interesting in biochemical terms is the decamer ($V_{10}O_{28}^{6-}$), although it has been reported that dimeric ($H_3V_2O_7^-$) and cyclic tetrameric ($V_4O_{12}^{4-}$) forms may also influence the activity of several enzymes [2,3]. Vanadate has also proved to be a valuable tool to trap Mg^{2+} nucleotides in the catalytic site of ATPases, particularly for mapping the active site of myosin.

Myosin is the major muscle ATPase, which along with actin converts the chemical energy of ATP hydrolysis into mechanical work [4,5]. Vanadate inhibits myosin ATPase activity through the formation, in the absence of actin, of a long-lived complex with MgADP that is believed to mimic the transition state of myosin**-ADP-Pi [6]. In the

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presence of actin, the rate of vanadate release increases by 10^5 compared to that of myosin.ADP.V_1 alone (5×10^{-6} s^{-1}), and consequently the inhibition is reverted [7]. Nevertheless, it has been described that as long as excess Vi is present (denoting a mixture of monomeric and polymeric vanadate ions), the formation of the myosin.ADP.V_1 complex is favoured even with the "destabilizing" influence of actin [8,9]. Indeed, recently it was demonstrated that the decameric vanadate (V_{10}) inhibits the actin-stimulated myosin ATPase activity, noncompetitively with actin or with ATP upon interaction with a high-affinity binding site in myosin subfragment-1 (S1) [10]. In the work described by Tiago et al. [10], the interaction of V_{10} with myosin S1 was monitored using fluorescently labelled S1 at Cys-707 (SH1) and Cys-697 (SH2), and the results pointed out that the binding of V_{10} to myosin is very close to Cys-697. The flexible loop between Cys-707 and Cys-697 is believed to act as an energy transduction element through which intersite communication between ATP and actin binding sites is transmitted [11], and therefore could be related to the mechanism by which V_{10} (and probably other vanadate oligomers) impairs the actin-stimulated myosin ATPase activity. Moreover, it was found that V_{10} binding is competitive with the γ-phosphate modified nucleotide analogue, AP_5A [10]. The γ-phosphate of ATP lies adjacent to Ser-180 and Ser-243, two residues demonstrated to be photomodified by tetrameric vanadate ($V_4O_{12}^{4-}$) upon UV irradiation [12]. On this basis, it was suggested that V_{10} binds to the phosphate loop (amino acids 178–185), that forms part of the consensus ATP binding site on myosin, affecting the actin stimulated myosin ATPase activity. However, the analysis supporting this hypothesis has not yet been reported, as the residues implicated in V_{10} interaction with myosin were not clearly identified. Moreover, it is still unknown whether the other vanadate oligomers, particularly the tetrameric vanadate, inhibit the actin stimulated myosin ATPase activity through the same mechanism as V_{10}. Thus, in the present study, we further explore the mode of action of decameric vanadate (V_{10}) on myosin, by analysing the following aspects: (i) V_{10} binding sites on myosin by showing unambiguously the residues undergoing photo-cleavage due to the presence of decameric vanadate; (ii) competition between decameric vanadate and other vanadate oligomers for the binding sites on myosin under the influence of natural ligands, such as actin and/or ATP, as analysed by ^{51}V NMR spectroscopy.

2. Materials and methods

2.1. Protein preparation

Myosin was prepared from leg and dorsal white rabbit skeletal muscle, as previously described by Pires et al. [13]. The isolated myosin was then digested with α-chymotrypsin to obtain subfragment-1 (S1) according to Margossian and Lowey [14]. F-actin was prepared from acetone powder of rabbit skeletal muscle by the method of Pardee and Spudich [15]. The complexes S1 · ADP · VO_4^{3-} and S1 · ADP · AlF_4^- for the photocleavage experiments were prepared according to Werber et al. [16]. Briefly, S1 previously dialyzed against 60 mM KCl, 25 mM Hepes (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid), pH 7.9 was incubated at 25° C for 5 min with 1 mM $MgCl_2$, 0.2 mM ADP and, for AlF_4^- with 5 mM NaF. Then, metavanadate solution (for VO_4^{3-}) or $AlCl_3$ (for AlF_4^-) was added to a final concentration of 0.2 mM and the incubation was continued for 90 min at 25° C. Finally, the samples were passed through a PD-10 column (Pharmacia) in order to remove excess of nucleotide analogues. Myosin and Subfragment-1 concentrations were determined by the Bradford assay using bovine serum albumin as a standard, while G-actin concentration was measured spectrophotometrically by using an extinction coefficient of $e_{290}^{1\%} = 11.5\ cm^{-1}$. Molecular weights of 500, 115, and 42 kDa were considered for myosin, S1, and actin, respectively.

2.2. Vanadate solutions

Metavanadate solution (50 mM, pH 6.0–7.0) was prepared from ammonium metavanadate (NH_4VO_3) purchased from Riedel-de-Haen. Decavanadate solution was prepared in a manner that maximized the proportion of vanadate that was present as the decamer, by adjusting the pH of the metavanadate solution to 4.0 [17]. The presence of decameric vanadate species was revealed by a characteristic orange colour. Both solutions were kept at 4 °C.

2.3. Photocleavage studies

Photoirradiation was carried out in a reaction mixture containing 4.5 μM myosin S1, 25 mM KCl, 2.5 mM $MgCl_2$, 25 mM Hepes, pH 7.0, in the presence or absence of 5–1000 μM vanadate solutions, 5 mM ATP and 10 μM actin. The mixture (2 ml) in quartz cell and cooled on a water bath was irradiated with a 400 W medium-pressure Hg lamp (Applied Photophysics) at an average distance of 5 cm. Radiation below 300 nm was removed (in order to prevent protein degradation by UV irradiation) by using a $CuSO_4$ solution, having a peak of transmittance at 365 nm. The sample was then subjected to electrophoretic analysis on a 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel.

2.4. NMR measurements

1D ^{51}V NMR spectroscopy measurements were performed on a Bruker AM-400 MHz spectrometer at

105.2 MHz equipped with a 5 mm multinuclear inverse probe. Spectra were acquired at 22 °C using 0.5 ml of samples containing 10% D_2O, under the following conditions: 90° pulse angle, spectral width 23923 Hz, acquisition time 0.086 s, number of transients 10 000 and relaxation delay 0.01 s. ^{51}V NMR chemical shifts are reported relative to an external reference of $VOCl_3$ (0 ppm). NMR spectra of metavanadate (2 mM) and polyvanadate (2 mM metavanadate + 5 mM decavanadate) solutions were obtained in a reaction medium containing 25 mM KCl, 2.5 mM $MgCl_2$, 25 mM Hepes, pH 7.0 in the absence and presence of myosin S1, actin or ATP as desired. The ^{51}V NMR line widths of the several free and bound vanadate resonances represent the widths at half-height after subtraction of 20 Hz used in line broadening. The concentrations of each vanadate species V_x were calculated from the fractions of the total integrated areas using the following equation: $[V_x] = (A_x/A_t) \times ([V_t]/n)$, where A_x corresponds to the area measured for the x vanadate species with n aggregation number (number of vanadium atoms), A_t the sum of measured areas, and $[V_t]$ the total vanadate concentration, as described elsewhere [17].

3. Results and discussion

Vanadate is photochemically active and irradiation of protein-bound vanadate species with UV light results in polypeptide photo-oxidation and cleavage making possible the identification of vanadate binding sites [18–21]. In the present work, a series of decavanadate-dependent photocleavage experiments were carried out in order to clearly identify decameric vanadate (V_{10}) binding sites on myosin. The electrophoretic patterns were analyzed and the results are summarized in Fig. 1. It was observed that irradiation of myosin S1 (4.5 μM) for 20 min, in the presence of 100 μM decavanadate (10 μM V_{10} species), caused the cleavage of the S1 95 kDa heavy chain at the 23 kDa- and, to a much lower extent, at the 74 kDa-sites, producing the 23 and 21 kDa cleavage products, respectively (Fig. 1(A), lane b). An additional product of 51 kDa also appears, likely derived from the further cleavage of the 74 kDa fragment. In presence of 2 mM ATP, the cleavage at the 23 kDa is inhibited leaving only the 74 kDa site available to photocleavage as can be seen from the appearance of a tenuous 21 kDa band (Fig. 1(A), lane c). The same observation was verified in the presence of S1-ADP-V_1 and S1-ADP-AlF_4 (Fig. 1(A), lanes g and i, respectively), two complexes known to mimic the intermediate activated myosin**-ADP-Pi state [16,22,23]. In presence of actin (10 μM), the cleavage at the 74 kDa site was totally protected while enhancing the cleavage at the 23 kDa site (Fig. 1(A), lane d). However, when ATP is added in the presence of actin, the 23 kDa site is no longer susceptible to photocleavage by decavanadate (Fig. 1(A), lane e).

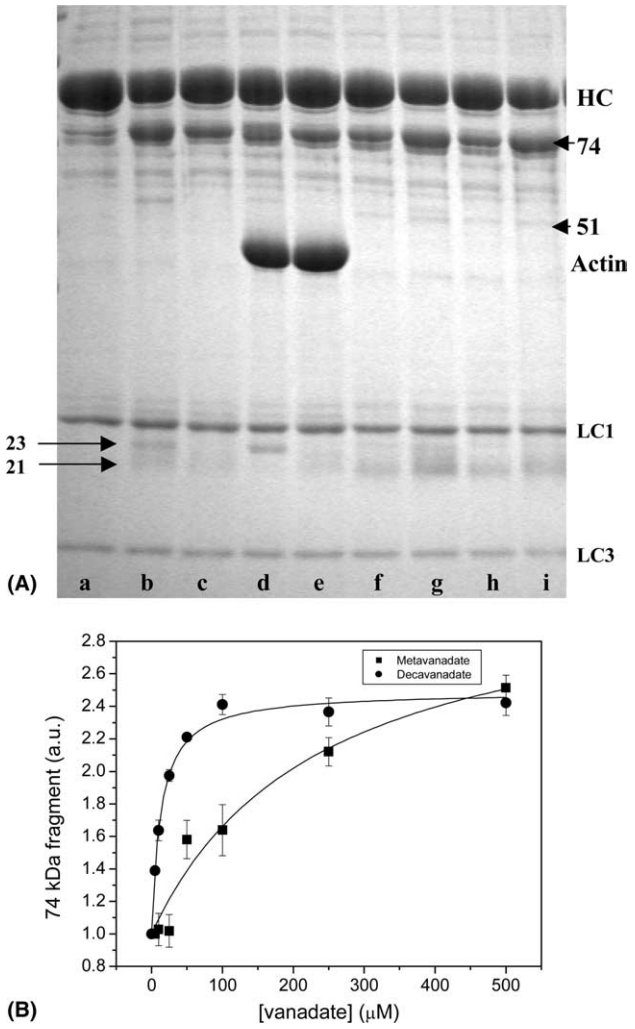

Fig. 1. SDS–PAGE analysis of the Vi-dependent photocleavage of myosin S1 (4.5 μM) in a medium containing 25 mM KCl, 2.5 mM $MgCl_2$, 25 mM Hepes, pH 7.0. Samples were irradiated for 20 min. as stated in Materials and Methods section and then run on a 12% acrylamide gel. (Panel A) SDS gel electrophoresis pattern of S1 following irradiation in the absence (a) and presence of 10 μM V_{10} (100 μM total vanadium added as decavanadate) with no additions (b); or with 5 mM ATP (c); 10 μM F-actin (d); and 5 mM ATP + 10 μM F-actin (e). Control S1–ADP–V_1 in the absence (f) and presence of 10 μM V_{10} (g). Control S1–ADP–AlF_4 in the absence (h) and presence of 10 μM V_{10} (i). (Panel B) Formation of the 74 kDa fragment estimated from the densitometry of the peptide bands upon increasing vanadate concentration up to 500 μM for metavanadate (squares) and decavanadate (circles) solutions. HC, LC1 and LC3 stands for S1 heavy chain, myosin light chain 1 and myosin light chain 3, respectively.

The electrophoretic patterns found for the decavanadate-dependent myosin S1 photocleavage in the present work are identical to the ones previously reported in the presence of metavanadate concentrations higher than 0.2 mM. Myosin S1 was shown to be photoirradiated by near UV-light in the presence of vanadate at three

specific sites: 23, 31, and 74 kDa from the N-terminus [24]. The cleavages at the 23 and 31 kDa sites, which were assigned to Ser-180 and Ser-243, respectively, are located in the consensus ATP binding site [25,26] while the 74 kDa site is near the Lys-rich 50/20 kDa junction which is believed to participate in an actin binding site [27]. Since ^{51}V NMR studies have shown that the tetrameric vanadate binds most strongly to myosin [12] and is favoured for vanadate concentrations higher than 0.2 mM, it was suggested that this species is the most likely responsible for the cleavage products.

Given the rapid exchange rates between the labile oligovanadates (V_1, V_2, and V_4) [2] it is not possible to isolate a specific anion and consequently an equilibrium mixture must be examined, which may complicate studies aimed to determine the effect of a specific anion. Although vanadate decamer is a less labile oligomer, remaining relatively intact for studies of limited duration, in previous NMR studies it was shown that upon dilution to the reaction medium (pH 7.0), the decavanadate solution can contain approximately 10% of monomeric vanadate [17]. To ascertain whether the monomeric vanadate (V_1) added with decavanadate solutions, or eventually formed during irradiation due to decameric species decomposition, can account for the electrophoretic patterns observed in Fig. 1(A), we have carried out photocleavage reactions with 4.5 μM S1 over a wide range of meta- and deca-vanadate concentrations (5–1000 μM total vanadium). The formation of the 74 kDa fragment, as estimated from the densitometry of this peptide band, was followed and the results represented in Fig. 1(B). For decavanadate, cleavage starts to occur at vanadate concentrations as low as 5 μM (0.5 μM V_{10} species) increasing sharply up to 50 μM total vanadium (5 μM V_{10} species) and reaching 30% of protein cleavage. For metavanadate, cleavage at concentrations lower than 100 μM (containing mainly monomeric species) is modest and, only at concentrations favouring di- and tetra-meric vanadate species (500 μM), the extent of cleavage is comparable to the maximum decavanadate cleavage, ruling out the contribution of the monomeric vanadate to protein cleavage and indicating the vanadate oligomers as the photoactive species. However, for higher vanadate concentrations (1 mM), it was observed that while for decavanadate the extent of cleavage remains essentially constant never exceeding 30%, for metavanadate it exceeds 45% (results not shown).

The lower extent of cleavage found for decavanadate when compared to metavanadate solutions, at higher vanadate concentrations (1 mM), prompted us to monitor, by UV/VIS spectroscopy, the spectral changes in the 240–700 nm region of decavanadate solutions before and after exposure to irradiation, in order to examine the consequence of irradiation on V_{10} species stability. The results are presented in Fig. 2, as the difference

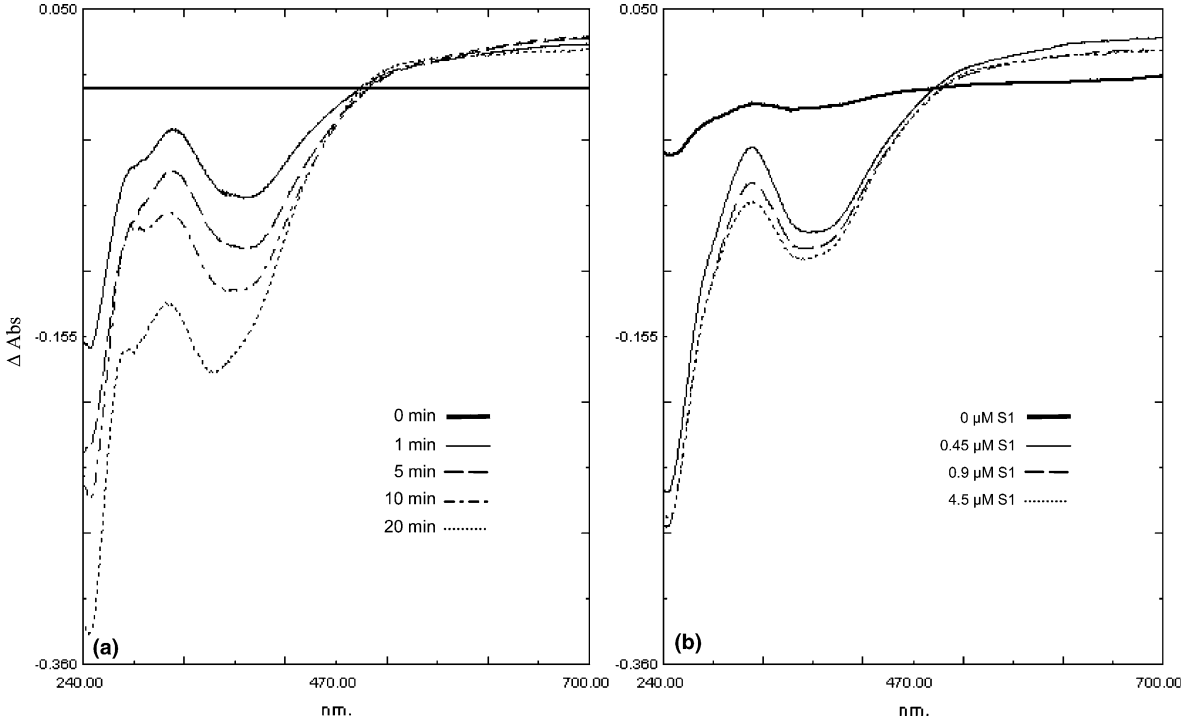

Fig. 2. UV–Vis differential spectra from 240–700 nm of 250 μM decavanadate solution in a medium containing 25 mM KCl, 2.5 mM $MgCl_2$, 25 mM Hepes, pH 7.0: (a) after different times of irradiation (0–20 min) in presence of 4.5 μM myosin S1; (b) in the presence of different myosin S1 concentrations (0, 0.45, 0.9 and 4.5 μM) at a fixed time of irradiation (20 min).

between spectra after and before irradiation, showing a decrease of an absorption band centred at 390 nm (characteristic of decameric vanadate), corresponding to a decrease of the decamer concentration. In the presence of 4.5 μM S1, after 1 min irradiation, the absorption band at 390 nm of the decavanadate solution (250 μM) decreases almost 38%, and it reaches 80% after 20 min, with the concomitant increase of a broad band near 700 nm, indicative of vanadyl (V^{4+} species) formation during the process (Fig. 2(a)). Furthermore, the decrease of the decameric absorption band occurs only in the presence of myosin S1, with slight changes for S1 concentrations between 0.45 and 4.5 μM, pointing out that V_{10} decomposition is promoted upon protein irradiation (Fig. 2(b)). In fact, it is known that the mechanism of vanadate-promoted photocleavage reaction on myosin involves addition of molecular oxygen to a stabilized seryl radical generated by a light-catalyzed vanadate (V^{5+}) reduction to vanadyl (V^{4+}) [28]. It was also recently shown that V^{5+} of V_{10} is more easily reduced in comparison to V_1, V_2 and V_4 present in metavanadate solutions [29]. These observations could account for the rapid decomposition of the decameric vanadate (V_{10}), upon protein addition, into vandyl species after a short irradiation time as described above (Fig. 2), as well as for the higher cleavage efficiency of decavanadate for extremely low vanadate concentrations when compared to metavanadate (Fig. 1(B)).

Collectively, the results in Figs. 1 and 2 demonstrate that, besides tetrameric vanadate (V_4), also decameric vanadate (V_{10}) is photoactive and apparently is a better substrate for the myosin S1 photocleavage reaction than the former species. In addition, the identical cleavage patterns found for deca- and meta-vanadate solutions suggests that V_{10} and V_4 have the same binding sites in myosin S1. On this basis, we further evaluated the competition between decameric vanadate and other vanadate oligomers, particularly tetrameric vanadate, for the myosin S1 binding sites. For that purpose, we have monitored changes in the ^{51}V NMR signals upon protein addition to vanadate solutions containing both species (V_{10} and V_4) at several experimental conditions.

While it is possible, at pH 7.0, to obtain a decavanadate stock solution containing exclusively decameric species, only for metavanadate concentrations lower than 200 μM, monomeric species is mainly present [30,31]. As the vanadate concentration is raised, oligomeric forms start to be favoured. In the present NMR experiments, a 2 mM metavanadate solution was used containing approximately 766 μM monovanadate, 146 μM divanadate, and 236 μM cyclic tetravanadate calculated from the fractions of the total integrated areas of the ^{51}V NMR resonances, as stated in Materials and Methods section, at −561.0, −574.6 and −578.8 ppm, respectively (Fig. 3(A)). On the other hand, when a decavanadate stock solution is diluted into the reaction medium, decameric vanadate is partially converted into monomeric vanadate [32]. The monomer contribution increases with incubation time accompanied by the appearance of di- and tetrameric vanadates. Since the kinetics of dissociation of decameric vanadate is relatively slow, we have used a mixed solution containing 2 mM-meta and 5 mM-decavanadate, referred as polyvanadate solution, in order to have both V_4 and V_{10} species in the same solution. As can be observed in Fig. 4(A), in addition to the resonances of the metavanadate solution (Fig. 3(A)), the spectra presents three new NMR signals at −515.5 (V_{10A}), −499.2 (V_{10B}) and −425.2 ppm (V_{10C}) corresponding to the three different chemical environmental vanadate atoms in the decameric vanadate molecule structure. In this solution, the concentration of the vanadate oligomers is approxi-

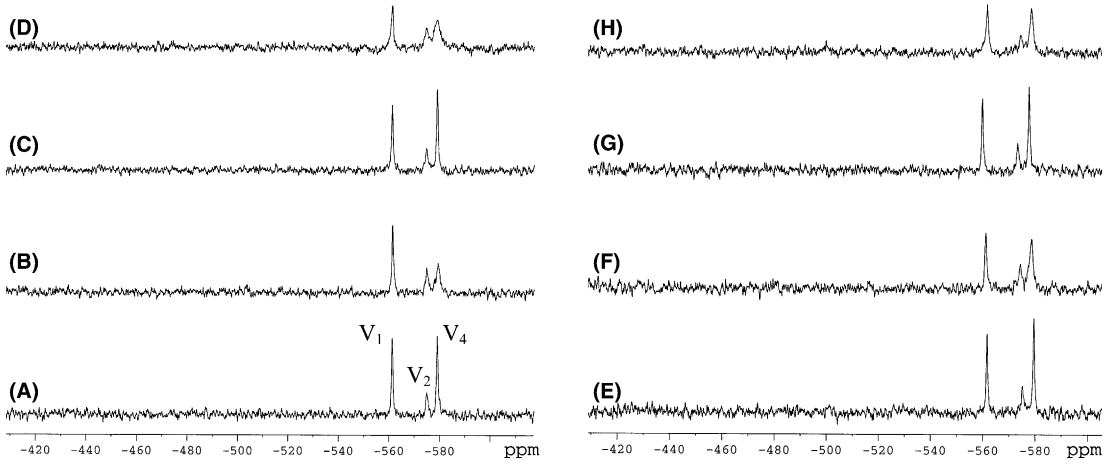

Fig. 3. ^{51}V NMR spectra of 2.0 mM nominal metavanadate solution in the absence (A,E) and presence of 30 μM S1 (B,F); 30 μM F-actin (C,G); 30 μM S1 + 30 μM F-actin (D,H) with (Right panel) or without (Left panel) 2 mM ATP. The spectra were collected in a medium containing 25 mM KCl, 2.5 mM $MgCl_2$, 25 mM Hepes, pH 7.0 at 22 °C. V_1, V_2 and V_4 are referring to monomeric, dimeric and cyclic tetrameric forms of vanadate, respectively.

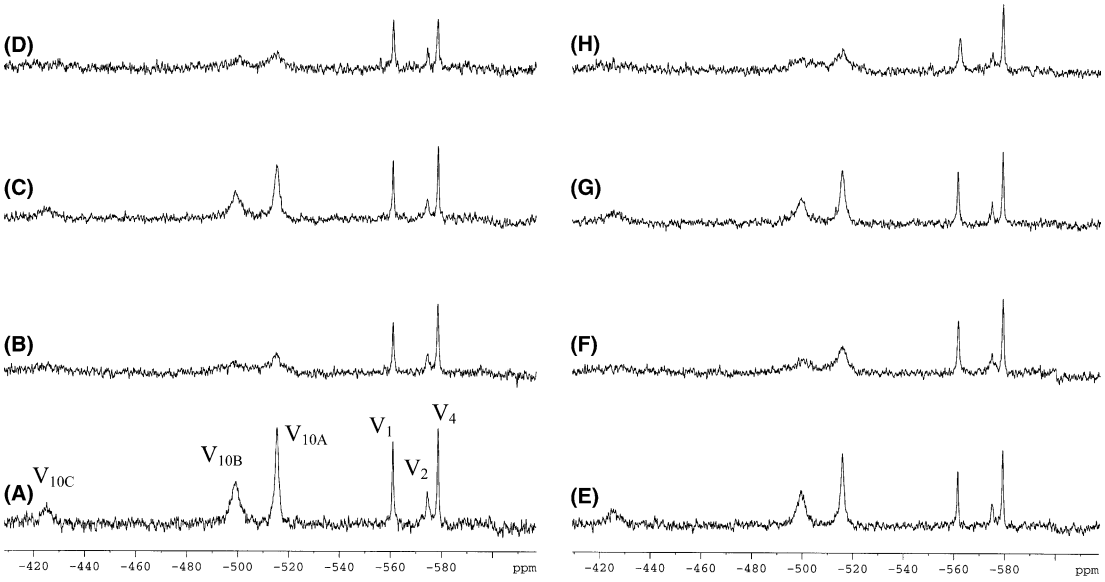

Fig. 4. ^{51}V NMR spectra of 2.0 mM nominal metavanadate + 5.0 mM nominal decavanadate solutions in the absence (A,E) and presence of 30 μM S1 (B,F); 30 μM F-actin (C,G); 30 μM S1 + 30 μM F-actin (D,H) with (Right panel) or without (Left panel) 2 mM ATP. The spectra were collected in a medium containing 25 mM KCl, 2.5 mM $MgCl_2$, 25 mM Hepes, pH 7.0 at 22 °C. V_1, V_2 and V_4 are referring to monomeric, dimeric and cyclic tetrameric forms of vanadate, respectively. V_{10A}, V_{10B}, and V_{10C} correspond to the three chemical environmental different vanadate atoms in the decameric vanadate structure.

Table 1
Chemical shifts (δ), line widths ($\Delta\nu_{1/2}$), and broadening factor of the line widths (F) of monomeric (V_1), dimeric (V_2), and tetrameric (V_4) forms of vanadate present in 2 mM nominal metavanadate (META) solution in different experimental conditions

	Meta (2 mM)		
	V_1	V_2	V_4
No protein (A)			
δ (ppm)	−561.0	−574.6	−578.8
$\Delta\nu_{1/2}$ (Hz)	75.2	150.3	75.2
30 μM S1 (B)			
δ (ppm)	− 561.2	− 574.6	−579.1
$\Delta\nu_{1/2}$ (Hz)	75.2	150.3	225.6
F (relative to A)	(1.0)	(1.0)	(3.0)
30 μM F-actin (C)			
δ (ppm)	−561.0	− 574.5	−578.8
$\Delta\nu_{1/2}$ (Hz)	75.2	150.3	75.2
F (relative to A)	(1.0)	(1.0)	(1.0)
30 μM S1 + 30 μM F-actin (D)			
δ (ppm)	−561.1	−574.6	−578.8
$\Delta\nu_{1/2}$ (Hz)	112.8	225.6	376
F (relative to C)	(1.5)	(1.5)	(5.0)
No protein + 2 mM ATP (E)			
δ (ppm)	− 561.0	− 574.5	− 578.9
$\Delta\nu_{1/2}$ (Hz)	75.2	150.3	75.2
F (relative to A)	(1.0)	(1.0)	(1.0)
3 μM S1 + 2 mM ATP (F)			
δ (ppm)	−561.6	−574.8	−579.0
$\Delta\nu_{1/2}$ (Hz)	90.2	150.3	150.3
F (relative to E)	(1.2)	(1.0)	(2.0)
30 μM F-actin + 2 mM ATP (G)			
δ (ppm)	−561.0	−574.5	−578.9
$\Delta\nu_{1/2}$ (Hz)	75.2	150.3	75.2
F (relative to E)	(1.0)	(1.0)	(1.0)
30 μM S1 + 30 μM F-actin + 2 mM ATP (H)			
δ (ppm)	−562.0	−574.8	−578.8
$\Delta\nu_{1/2}$ (Hz)	112.8	150.3	188.0
F (relative to G)	(1.5)	(1.0)	(2.5)

mately 908 μM for monovanadate (V_1), 300 μM for divanadate (V_2), 219 μM for tetravanadate (V_4) and 461 μM for decavanadate (V_{10}). ^{51}V NMR spectra of meta- and poly-vanadate solutions were collected in the presence or absence of myosin natural ligands such as ATP and actin, and the relative order of vanadate signals line broadening upon protein addition (reflecting the interaction of vanadate species with myosin S1) was evaluated and presented in Tables 1 and 2 for metavanadate and polyvanadate solutions, respectively. The corresponding NMR spectra can be followed in Figs. 3 and 4. Myosin S1 (30 μM), selectively broadened by a factor of five and decreased the intensity of V_4 resonance in the metavanadate solution with no effects on the mono- or di-vanadate signals (Table 1, Fig. 3(A)). In the same way, V_{10} resonance in the polyvanadate solution was selectively broadened, by a factor of three, preventing any changes of the V_4 or the other vanadate NMR signals (Table 2, Fig. 4(B)). These results implied a preferential binding of higher vanadate oligomers to the protein and suggest either a competition between decavanadate and tetravanadate for the same binding site(s), in concordance with the photocleavage studies described above, or a localized structural change caused by decavanadate that prevents tetravanadate binding. Addition of either F-actin (30 μM) or ATP (2 mM) to myosin S1 free meta- and polyvanadate samples does not induced significant differences in the line widths and chemical shifts of the vanadate resonances (Table 1, Fig. 3(C), (E) and Table 2, Fig. 4C, E for meta- and poly-vanadate samples, respectively), indicating that neither F-actin or ATP associate with any of the forms of vanadate present in the reaction medium, in agreement with previous reports [10,24]. Moreover, actin appears not to affect the binding of either V_4 or V_{10} to myosin S1, as the increase in the line width broadening of the NMR signals upon myosin S1 addition is similar in the absence or presence of actin (Table 1, Fig. 3(D) and Table 2, Fig. 4(D) for V_4 and V_{10}, respectively). On the other hand, when ATP is added to the medium containing myosin S1 either in the absence (Fig. 3(F)) or presence (Fig. 3(H)) of actin, V_4 signal becomes narrow (broadening factor of 2) (Table 1) as shown previously by Ringel et al. [24] for myosin S1, in the

Table 2
Chemical shifts (δ), line widths ($\Delta\nu_{1/2}$), and broadening factor of the line widths (F) of monomeric (V_1), dimeric (V_2), tetrameric (V_4), and decameric (V_{10}) forms of vanadate present in 2 mM nominal metavanadate (META) + 5 mM nominal decavanadate (DECA) solutions in different experimental conditions

	Meta (2 mM) + Deca (5 mM)			
	V_1	V_2	V_4	V_{10}
No protein (A)				
δ (ppm)	−561.1	−574.5	−578.8	−515.5
$\Delta\nu_{1/2}$ (Hz)	75.2	150.3	75.2	150.3
30 μM S1 (B)				
δ (ppm)	−561.2	−574.7	−578.7	−515.2
$\Delta\nu_{1/2}$ (Hz)	75.2	188.0	75.2	450.9
F (relative to A)	(1.0)	(1.2)	(1.0)	(3.0)
30 μM F-actin (C)				
δ (ppm)	−561.1	−574.5	−578.8	−515.4
$\Delta\nu_{1/2}$ (Hz)	75.2	150.3	75.2	225.6
F (relative to A)	(1.0)	(1.0)	(1.0)	(1.5)
30 μM S1 + 30 μM F-actin (D)				
δ (ppm)	−561.3	−574.6	−578.7	−515.7
$\Delta\nu_{1/2}$ (Hz)	75.2	112.8	75.2	676.4
F (relative to C)	(1.0)	(1.0)	(1.0)	(3.0)
No protein + 2 mM ATP (E)				
δ (ppm)	−561.1	−574.6	−578.8	−515.5
$\Delta\nu_{1/2}$ (Hz)	75.2	112.8	75.2	150.3
F (relative to A)	(1.0)	(0.8)	(1.0)	(1.0)
30 μM S1 + 2 mM ATP (F)				
δ (ppm)	−561.3	−574.6	−579.0	−515.6
$\Delta\nu_{1/2}$ (Hz)	75.2	188.0	75.2	451.2
F (relative to E)	(1.3)	(1.3)	(1.0)	(3.0)
30 μM F-actin + 2 mM ATP (G)				
δ (ppm)	−561.1	−574.6	−578.8	−515.4
$\Delta\nu_{1/2}$ (Hz)	75.2	150.3	75.2	225.6
F (relative to E)	(1.0)	(1.3)	(1.0)	(1.5)
30 μM S1 + 30 μM F-actin + 2 mM ATP (H)				
δ (ppm)	−561.8	−574.7	−578.8	−515.4
$\Delta\nu_{1/2}$ (Hz)	150.3	150.3	75.2	451.2
F (relative to G)	(1.0)	(1.0)	(1.0)	(2.0)

absence of actin, or by Aureliano and Madeira [17] for the sarcoplasmic reticulum Ca^{2+}-ATPase, which suggests that tetravanadate is displaced from the protein in presence of ATP either with or without actin. Contrarily to these observations, the presence of the substrate upon protein addition seems not to significantly affect the line width of V_{10} NMR signal in the absence, or to much lower extent in the presence of actin as can be seen from Fig. 4(F) and (H), respectively, and Table 2. When interpreting this finding we can not exclude, however, the fact that the line broadening of bound V_{10} is very large and may be masking possible ATP effects on V_{10} binding to myosin S1. In this regard, it may be that some displacement of decavanadate occurs although in less extent than tetravanadate. Moreover, it is known that the rapid exchange between bound and free decavanadate is likely to contribute to the disappearance of the signal even in the presence of excess vanadate [2].

In the present article, we have determined unambiguously the residues undergoing photocleavage on myosin by the decameric vanadate (V_{10}), demonstrating two putative binding sites: (i) one of higher affinity (23 kDa) located on the 178–185 sequence (loop-P) which forms the consensus ATP binding site of myosin; and (ii) another one of lower affinity (74 kDa) from residues 627–646 (loop-2), which is part of the actin-binding interface. These results came to confirm the hypothesis recently proposed by Tiago et al. [10] of the preference V_{10} binding to the Walker A motif or P-loop on myosin which forms part of the phosphate tube. Yount et al. [9] have previously postulated that actin binding promotes the movement of P-loop, containing Ser-181, which allows Pi from ATP to leave via a ''back-door'' in the 50-kDa fragment while ADP is still bound at the active site. It was also proposed by the same authors that in fibers, vanadate could enter the active site via the ''back-door'' to form the more stable MgADP.Vi.myosin complex inducing muscle relaxation. However, this is only possible if an excess vanadate is present which is described to favour vanadate oligomerization. In fact, it was shown that V_{10} binding produces non-competitive inhibition of the actin-stimulated S1-ATPase activity ($K_i = 0.27 \pm 0.05$ μM), without causing dissociation of the acto-S1 complex [10]. Altogether, these results suggest that the replacement of the γ-phosphate of ATP by V_{10} at the phosphate loop, forming the V_{10}-myosin-ADP-Mg complex during ATP hydrolysis, will probably block actin-stimulated S1-ATPase activity acting as a ''back-door stop'' by preventing ADP/ATP exchange.

Another important result is that V_{10} and V_4 bind to the same protein binding site(s), although its interactions are differently modulated by the myosin natural ligands (ATP and actin). While V_4 is highly affected by the products release during ATP hydrolysis, V_{10} appears to be affected at a much lower extent, as shown by the NMR line broadening experiments. In addition, V_{10} has a greater affinity for myosin binding sites than V_4, in the same way that, V_4 has a greater affinity than the lower vanadate oligomers, which can be explained if electrostatic forces play an important role in vanadate interaction with myosin. In this sense, it is concluded that besides V_{10}, other vanadate oligomers may contribute to the inhibition of the actin-stimulated myosin ATPase activity, through binding at the myosin phosphate loop, although the protein affinity is favoured for higher vanadate anions such as V_{10}.

Even if V_{10} is not likely to be present at significant concentrations under physiological conditions, it has revealed to be a useful biochemical tool not only for the description of the location and function of the phosphate binding sites on myosin, but also for the molecular interpretation of actomyosin interactions and consequently for muscle contraction regulation.

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