Journal of Fluorescence, Vol. 12, No. 1, March 2002 (© 2002)

Quenching of Myosin Intrinsic Fluorescence Unravels the Existence of a High Affinity Binding Site for Decavanadate

Teresa Tiago,[1,3] Manuel Aureliano,[1] and Carlos Gutiérrez-Merino[2]

Received September 27, 2001; revised December 20, 2001; accepted December 21, 2001

Decavanadate, one of the aggregated species of vanadate, is a potent inhibitor of several enzymes, including skeletal muscle myosin. However, its putative binding sites in myosin are largely unknown. Titration of the intrinsic fluorescence of myosin, purified from rabbit skeletal muscle, have been carried out in 0.3 M KCl, 5 mM $CaCl_2$ and 25 mM Tris-HCl (pH 7.0), with 0.1 mg/ml myosin. In the 0–200 μM total vanadate concentration range, decavanadate produced approximately 25% quenching of the intrinsic fluorescence of myosin, with an apparent dissociation constant in the micromolar range. This effect was found to be specific of decavanadate, because titration with metavanadate up to 200 μM did not produce a significant quenching of the intrinsic fluorescence of myosin. This quenching was accompanied by a parallel decrease of the accessibility of myosin tryptophans to the water-soluble collisional quencher KI, with an apparent dissociation constant also in the micromolar range. It is concluded that the binding of decavanadate to high-affinity sites in myosin produces local conformational change(s) near the tryptophans more accessible to water in the three-dimensional structure of this protein.

KEY WORDS: Decavanadate; skeletal muscle myosin; intrinsic fluorescence; Stern-Volmer.

INTRODUCTION

Vanadate has been shown to have pleiotropic biological effects, behaving either as a toxic agent for muscle function [1] or a beneficial agent by reversing drug resistance [2] or increasing glucose metabolism [3]. Vanadate solutions contain different vanadate species that can occur simultaneously in equilibrium [4], each interacting differently with several enzymes [5]. Decavanadate, one of the aggregated species of vanadate is a covalently closed polyanionic molecule of ellipsoidal shape that is formed in mildly acidic medium [6]. It is known to act as a potent inhibitor of several enzymes, such as phosphorylase a and b, fructose 1,6-bisphosphate, aldolase, and some specific kinases [7]. More recently, it has been shown that this molecule also interacts and inhibits skeletal muscle myosin [8], a highly specialized protein involved in the process of muscle contraction that converts the chemical energy of ATP hydrolysis to mechanical work [9].

Fluorescence properties are very sensitive to small local conformational changes in proteins [10]. On binding of MgATP or MgADP the intrinsic fluorescence intensity of myosin changes [11]. The major steady-state intermediate during ATP hydrolysis by myosin is M**Mg.ADP.Pi, where M** denotes myosin tryptophan fluorescence enhancement. Because this intermediate is too labile, a stable analogue has been synthesized by Goodno, who replaced Pi with the analogue Vi [12]. Vi is usually

[1] CMQA, Dept. Química, Faculdade de Ciências e Tecnologia, Universidade do Algarve, 8000 Faro, Portugal.
[2] Depto. Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Extremadura, 06071-Badajoz, Spain.
[3] To whom correspondence should be addressed. E-mail: ttiago@ualg.pt

1053-0509/02/0300-0087/0 © 2002 Plenum Publishing Corporation

attributed to monomeric vanadate species, which has been widely used as a tool in myosin kinetic and structural studies [13–15]. However, little is known about putative binding sites in myosin for other vanadate oligomers.

Myosin has been crystallized and its three-dimensional structured resolved by X-ray diffraction [16]; and thus, this protein can become a good model system to gain a deeper understanding of the basic structural requirements for high-affinity protein binding sites to decavanadate. In the present work we report the effect of decavanadate titration on myosin intrinsic fluorescence and the effect of decavanadate on the accessibility of myosin tryptophans to the water-soluble collisional quencher potassium iodide (KI). The results have provided evidence for decavanadate high-affinity binding sites in myosin, which are probably the cause of local conformational change(s) near the tryptophans of myosin more accessible to water in the three-dimensional structure of this protein.

EXPERIMENTAL

Protein Preparation

Myosin was prepared from leg and dorsal white rabbit skeletal muscle, as previously described [17]. Protein concentration was determined by the biuret method, using bovine serum albumin as a standard.

Vanadate Solutions

Metavanadate stock solution (50 mM, pH 6.0–7.0) was prepared from ammonium metavanadate, purchased from Riedel-de-Häen. Decavanadate stock solution was obtained by adjusting the pH of the former solution to 4.0. Both solutions were stored at 4°C [18].

Fluorescence Measurements

Intrinsic fluorescence measurements were performed at 25°C in a continuously stirred cuvette with a Perkin-Elmer 650-40 fluorimeter, using excitation and emission wavelengths of 280 and 340 nm, respectively. The experiments were carried out in a medium containing 0.3 M KCl, 5 mM $CaCl_2$, and 25 mM Tris-HCl, pH 7.0, with 0.1 mg/ml myosin and "vanadate" solutions ranging from 0–200 μM total vanadate concentration. The pH change of the solutions during titration with decavanadate or metavanadate were always less than 0.05 pH units. Triplicate experiments were carried out with three different preparations of myosin and two different vanadate solutions.

Inner filter effects (excitation side) due to the absorbance at 280 nm (A_{280}) of decavanadate or metavanadate solutions were corrected using tryptophan (Trp) solutions with the same A_{280} and fluorimeter settings. Except for decavanadate concentrations higher than 50 μM, the correction factors ($C_{ex} = F_{corrected}/F_{observed}$) obtained following this approach gave values less than 5% different than those that can be obtained through the equation C_{ex} = antilog ($A_{280}/2$) [10], with an extinction coefficient for decavanadate and metavanadate at 280 nm of 2.12 ± 0.05 $mM^{-1}\cdot cm^{-1}$ (measured for the solutions used in this work). For the highest decavanadate concentrations used in this work (200 μM), the C_{ex} was 14% higher than the value of C_{ex} that can be calculated using the above referred equation, therefore suggesting the use of this approach to achieve a better inner filter correction on the excitation side. The values for the correction factor on the emission side (C_{em}) were calculated using the equation C_{em} = antilog ($A_{340}/2$) [10], where A_{340} was the absorbance at 340 nm of decavanadate solutions (experimentally measured extinction coefficient 1.00 ± 0.05 $mM^{-1}\cdot cm^{-1}$), because A_{340} was 0.2 for the highest decavanadate concentration used in this work. The absorbance at 340 nm of metavanadate solutions is negligible. The overall inner filter correction factors ($C_T = C_{ex}\cdot C_{em}$) used for decavanadate and metavanadate fluorescence titration data reported in this work have been:

- C_T values for decavanadate concentrations (C_T given in parenthesis): 10 μM (1.07), 20 μM (1.12), 50 μM (1.28), 100 μM (1.58), 150 μM (1.915), and 200 μM (2.34).
- C_T values for metavanadate concentrations (C_T given in parenthesis): 10 μM (1.04), 20 μM (1.07), 50 μM (1.15), 100 μM (1.29), 150 μM (1.465), and 200 μM (1.69).

RESULTS AND DISCUSSION

Decavanadate (V_{10}) is known to bind at polyphosphate binding sites of enzymes or receptors located either in the substrate or in allosteric effector sites [19]. These studies are usually followed by crystallographic studies [20], ^{51}V NMR spectroscopy [21], or cleavage induced by UV irradiation [22]. In the present work a fluorimetric study is used for the first time to monitor putative V_{10} binding sites in myosin by measuring the quenching of the intrinsic fluorescence of myosin upon decavanadate titration in the 0–200 μM total vanadate concentration

range. As can be seen in Fig. 1, decavanadate solutions produced approximately 25% quenching of the intrinsic fluorescence of myosin (after correction for inner filter effects). An apparent dissociation constant (Kd) between 2 and 5 μM decavanadate was obtained. Similar constant values were also found for the inhibition of myosin ATPase stimulated by actin (unpublished results). The slight differences observed for the Kd constants are probably due to different oxidation states of the protein from one myosin preparation to another.

It was observed by ^{51}V NMR spectroscopy that at the experimental conditions used (25°C, pH 7.0), less than 10% of the decameric species is converted to monomeric vanadate (results not shown). Moreover, the kinetics of dissociation of decameric species at 30°C (results not shown), assessed by measurements of the absorption at 400 nm (an absorption band that disappears upon vanadate dissociation into less aggregated species), conforms to a first-order kinetic process with half-time of 158 minutes at pH 7.0 and 30°C (measured with a V_{10} concentration of 10 μM). Therefore this oxoanion is perfectly stable during the period used in the experiments (10–15 minutes). Titration with metavanadate did not produce any quenching of the intrinsic fluorescence of myosin up to 200 μM total vanadate (see Fig. 1). In this concentration range (0–200 μM) and at the experimental conditions used, metavanadate solution contains essentially monomeric species (results not shown); it is concluded that

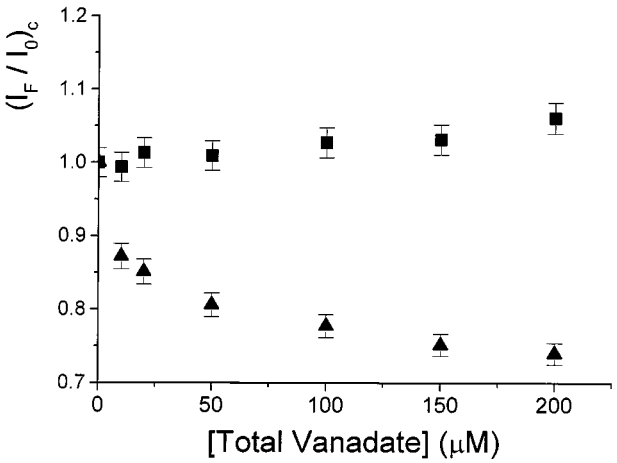

Fig. 1. Titration of myosin intrinsic fluorescence with decavanadate (▲) and with metavanadate (■). The concentrations of the abscissa are given as total vanadium concentration (e.g., 200 μM corresponds to 20 μM decavanadate or 200 μM vanadate). In the Y-axis is plotted the fluorescence relative to the fluorescence in the absence of vanadate (I_F/I_0). Experiments were performed at 25°C in a medium containing 0.3 M KCl, 5 mM $CaCl_2$, and 25 mM Tris-HCl, pH 7.0, with 0.1 mg/ml myosin. The results presented are the averages of the data obtained in, at least, triplicate series of measurements.

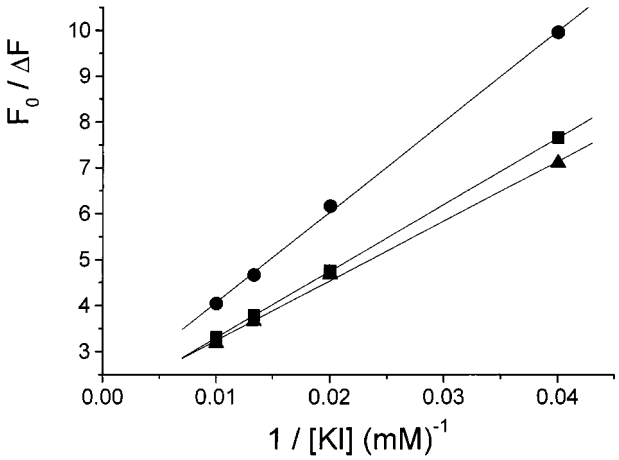

Fig. 2. Modified Stern-Volmer plot of the quenching by KI of myosin intrinsic fluorescence. Experiments were performed at 25°C in a medium containing 0.3 M KCl, 5 mM $CaCl_2$, and 25 mM Tris-HCl, pH 7.0, with 0.1 mg/ml myosin in absence (■) and in the presence of 50 μM metavanadate (▲) or 5 μM decavanadate (●). The results presented are the averages of the data obtained in, at least, triplicate series of measurements.

decameric molecule is clearly the promoter of the observed quenching. Moreover, the decameric molecule induced the same quenching effect in the presence and in the absence of MgADP (2 mM) (not shown). Taking into account that monomeric vanadate has been shown to interact near or with the myosin ATP binding site [12], these results suggest that decavanadate binds to a different site in myosin.

Titration with the water-soluble collisional quencher iodide were also performed to measure the effect of decavanadate upon the accessibility of myosin tryptophans. We found that the extent of quenching of myosin tryptophans by iodide decreased in the presence of micromolar decavanadate concentration, whereas metavanadate was found to have no effect up to 50 μM (Fig. 2). From modified Stern-Volmer plots of the data obtained for myosin and tryptophan solutions in the absence and in the presence of metavanadate and decavanadate, we have calculated the data shown in Table I. The results show that micromolar decavanadate concentrations, decreased by about 33% the Stern-Volmer constant (K_{SV}) and also decreased approximately 20% the accessibility factor (f_a). The Stern-Volmer quenching constants obtained for accessible myosin tryptophans in the absence of decavanadate are almost identical to those obtained for L-Trp in the same experimental conditions and yields an average Stern-Volmer constant similar to those reported for subfragment 1 [23]. The decrease of the Stern-Volmer constants were not observed with tryptophan solutions in the presence of micromolar decavanadate concentrations, nor

Table I. Myosin Intrinsic Fluorescence Quenching Parameters Derived From Modified Stern-Volmer Plots: Effects of Decavanadate (V_{10}) and Metavanadate (V_1)

Sample	$[V_{10}]$ (μM)	$[V_1]$ (μM)	f_a	$K_{SV}(mM^{-1})$
L-Trp (5 μM)	0	0	0.97 ± 0.05	0.0125 ± 0.001
L-Trp (5 μM)	10	0	1.00 ± 0.05	0.0130 ± 0.001
L-Trp (5 μM)	0	100	0.97 ± 0.05	0.0125 ± 0.001
Myosin (0.1 mg/ml)	0	0	0.56 ± 0.03	0.012 ± 0.001
Myosin (0.1 mg/ml)	5	0	0.45 ± 0.05	0.009 ± 0.001
Myosin (0.1 mg/ml)	10	0	0.45 ± 0.05	0.008 ± 0.001
Myosin (0.1 mg/ml)	0	50	0.52 ± 0.03	0.015 ± 0.001

Note: Fluorescence measurements were carried out as indicated in the Experimental section. The data obtained with L-Trp solutions (in the same buffer) are included to show the lack of a direct effect from a putative complexation of vanadate with L-Trp. The data included for myosin are the average of, at least, triplicate experiments done with two different myosin preparations. K_{SV} and f_a are the Stern-Volmer constant and the accessibility factor derived from linear regression fit of the experimental data to the modified Stern-Volmer equation:

$$F_0/\Delta F = f_a^{-1} \cdot K_{SV}^{-1} \cdot [Q]^{-1} + f_a^{-1}$$

with myosin in the presence of up to 50 μM metavanadate (Table I and results not shown). Therefore we concluded that this is a specific effect of decameric vanadate binding to myosin.

CONCLUSION

Myosin fluorescence quenching studies show the presence of high-affinity site(s) in myosin for decavanadate, which are different to the monomeric binding site located near the MgATP binding site of myosin, and that the binding of decameric vanadate to myosin produces local conformational changes near the tryptophans more accessible to water in the three-dimensional structure of myosin.

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