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Competition between decavanadate and fluorescein isothiocyanate on the Ca^{2+}-ATPase of sarcoplasmic reticulum

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The binding of vanadate and fluorescein isothiocyanate to the Ca^{2+}-transport ATPase of sarcoplasmic reticulum (EC 3.6.1.3) was analyzed. Monovanadate binds to the Ca^{2+}-transport ATPase at a single high affinity site (site 1), that is presumably related to the binding site for inorganic orthophosphate, and to one of the two sites for decavanadate. Binding of vanadate to this site stabilizes the enzyme in the E_2 conformation, with inhibition of ATPase activity and the formation of crystalline arrays of Ca^{2+}-ATPase. Decavanadate also binds with high affinity to a second site on the Ca^{2+}-ATPase (site 2), that is blocked by fluorescein isothiocyanate and may be part of the binding site for ATP.

Crystallization of Ca^{2+}-ATPase in sarcoplasmic reticulum, labeled with fluorescein isothiocyanate, by either monovanadate or decavanadate implies that occupation of site 1, but not of site 2, by vanadate is required for the conformational change of Ca^{2+}-ATPase leading to the formation of crystalline arrays.

Fluorescein isothiocyanate (FITC) inhibits the ATPase activity and ATP-dependent Ca^{2+}-transport of sarcoplasmic reticulum [1], by reaction with a lysine residue near the T_1 tryptic cleavage site of the Ca^{2+}-ATPase [2]. It appears that FITC specifically blocks the ATP binding site of the Ca^{2+}-ATPase, since the phosphorylation of the enzyme by inorganic orthophosphate and the transport of calcium with acetyl phosphate as energy donor were less affected [3]. Furthermore, ATP protects against inhibition of the Ca^{2+}-ATPase by FITC [1].

Vanadate inhibits ATPase activity and ATP-dependent Ca^{2+} transport by forming a kinetically stable intermediate with the Ca^{2+}-ATPase in the E_2 conformation [4, 5]. As a result, vanadate promotes the crystallization of Ca^{2+}-ATPase in sarcoplasmic reticulum membranes and in purified Ca^{2+}-ATPase preparations [6–9]. In contrast to FITC, vanadate inhibits the phosphorylation of the enzyme by inorganic phosphate, but has no effect on the formation of the phosphoenzyme intermediate from ATP [4]. These observations suggest that vanadate occupies the inorganic phosphate binding site of the Ca^{2+}-ATPase, without direct interference with ATP binding. Competition between vanadate and inorganic phosphate for a single site on the Ca^{2+}-ATPase is consistent with this interpretation.

Surprisingly, decavanadate, a highly charged vanadium (V) decamer, very effectively promotes the crystallization of Ca^{2+}-ATPase at concentrations as low as 10 μM [10], and mimics other effects of monovanadate on the Ca^{2+}-ATPase as well. Therefore it may be an oversimplification to view monovanadate simply as an analogue of inorganic phosphate. Equilibrium binding studies indicate that there are probably two binding sites for decavanadate on the Ca^{2+}-ATPase [10]. Both sites must be spacious enough to accommodate the large decavanadate polyanion. The occupancy of site 1 by decavanadate may be responsible for the stabilization of E_2 conformation that leads to the crystallization of the Ca^{2+}-ATPase. Site 1 is presumably related to the binding site for inorganic phosphate or monovanadate.

Competition of decavanadate with FITC implies that site 2 may overlap with the ATP binding site of the Ca^{2+}-ATPase. Since FITC-labeled Ca^{2+}-ATPase readily crystallizes in the presence of vanadate in Ca^{2+}-free medium, occupancy of site 2 is not required for the crystallization of the Ca^{2+}-ATPase.

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Abbreviation. FITC, fluorescein 5′-isothiocyanate.

Enzyme. Ca^{2+}-ATPase or ATP phosphohydrolase (EC 3.6.1.3).

EXPERIMENTAL PROCEDURES

Sarcoplasmic reticulum vesicles were isolated from predominantly white skeletal muscles of rabbits according to Nakamura et al. [11]. The animals were fasted for one day before the isolation of microsomes to reduce contamination by glycogen and phosphorylase.

The microsomes were used either fresh after isolation or were stored at −70 °C in a medium of 0.3 M sucrose, 10 mM Tris-maleate, pH 7.0.

Reaction of microsomes with fluorescein isothiocyanate (FITC)

The sarcoplasmic reticulum vesicles (2 mg protein/ml) were incubated at 25 °C in 0.3 M sucrose, 5 mM $MgCl_2$, 50 mM Tris-maleate, pH 7.4 and 0.1 mM EGTA with fluorescein isothiocyanate (usually 15–30 μM) for times indicated in the figure legends. The rate of FITC reaction with microsomes was similar in 0.1 M KCl, 10 mM imidazole, pH 7.4, 5 mM $MgCl_2$ and 0.5 mM EGTA. FITC stock solution was dissolved in dimethylformamide and added to the reaction mixture in a volume not exceeding 0.5% of the total volume.

Measurement of FITC binding to microsomes

After the reaction with FITC was completed, the microsomes were collected by centrifugation at 80000 × *g*. The bind-

ing of FITC to the microsomes was determined from the supernatant, from the sediment, or usually from both.

Supernatant assay. The absorbance of the centrifuged supernatant was measured at 496 nm and compared with the absorbance of a FITC solution in buffer at identical starting concentrations. The difference between the two sets of measurements allowed the calculation of the concentration of bound FITC using an absorption coefficient of 80000 M^{-1} cm^{-1} [1, 2].

Sediment assay. The microsomal pellet (after occasional rinsing with buffer) was suspended in 1.5 ml of 10 mM imidazole, pH 7.4, and solubilized with 0.5 ml of 4% sodium dodecylsulfate. The absorbance of the clear solution was measured at 496 nm, using the absorbance at 550 nm for light-scattering correction. The amount of bound FITC was calculated using an absorption coefficient of 80000 M^{-1} cm^{-1} [1, 2].

Preparation of vanadate solution. Stock solutions of monovanadate (50 mM) were prepared by boiling freshly made aqueous solutions of Na_3VO_4 at pH 10.0 for 15 min. Decavanadate solutions were prepared by adjusting the pH of a monovanadate stock solution to 4.0 and after several hours to 7.4. The decavanadate solutions were stored at 2 °C for no longer than two weeks. Due to the temperature-dependent and pH-dependent decay of decavanadate [10], special care was taken to use only freshly diluted, ice-cold decavanadate solutions in each experiment.

Analysis of monovanadate and decavanadate binding to sarcoplasmic reticulum vesicles

The binding of vanadate was measured by incubation of microsomes (1 – 2 mg protein/ml) for 2 h at 4 °C in a medium of 0.1 M KCl, 10 mM imidazole, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA and monovanadate or decavanadate concentrations ranging between 5 μM and 5 mM. Occasionally the incubation medium was 0.3 M sucrose, 50 mM Tris-maleate, pH 7.4, 5 mM $MgCl_2$, and 0.1 mM EGTA.

The vesicles were removed by centrifugation at 80000 × *g* for 20 min and the concentration of vanadate in the supernatant and in the sediment were measured, as described earlier [10].

For the assay of monovanadate, the method of Goodno [12] was used. Decavanadate concentration was determined either by measuring the absorbance of decavanadate at 400 nm, or by converting the decavanadate to monovanadate by heating at 90 °C for 30 min as described earlier [10]. There was no detectable reaction of vanadate with FITC. The Lowry method [13] was used for the assay of protein.

Measurement of ATPase activity

The $Mg^{2+} + Ca^{2+}$-activated ATPase activity was measured by incubation of microsomes (0.05 – 0.1 mg protein/ml) in 0.1 M KCl, 10 mM imidazole, pH 7.4, 5 mM $MgCl_2$, 5 mM ATP, 0.5 mM EGTA and 0.5 mM $CaCl_2$, with further additions as indicated in the figure legends, at 25 °C for 5 – 10 min; the reaction was stopped with 2% trichloroacetic acid and the inorganic phosphate was determined according to Fiske and Subbarow [14]. In control experiments the Ca^{2+} was omitted from the medium to obtain the Ca^{2+}-insensitive basal ATPase activity. All ATPase data presented in this report were corrected for basal ATPase activity.

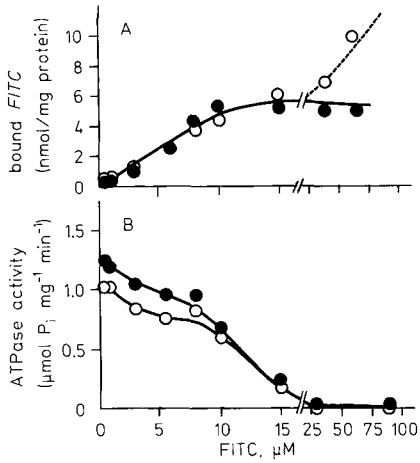

Fig. 1. *FITC binding and ATPase activity.* The binding of FITC to rabbit sarcoplasmic reticulum vesicles (2 mg protein/ml) was studied in 0.3 M sucrose, 50 mM Tris-maleate, 5 mM $MgCl_2$, 0.1 mM EGTA pH 7.4, and FITC in concentration indicated. The reaction was allowed to proceed for 60 min at room temperature, followed by centrifugation at 80000 × *g* for 20 min. The absorbance of the supernatant was measured at 496 nm. The sediment was washed twice with 5 vol. of buffer by repeated centrifugations, and the samples were divided into two portions for measurement of FITC binding (A) and of ATPase activity (B). (A) (○——○) Bound FITC determined from the difference between the absorbance of the supernatant and of the starting solutions; (●——●) bound FITC measured from the suspended pellet. (B) ATPase activity in the absence (○) or presence (●) or 2 μM A 23187. The relatively small stimulation of ATPase activity by A 23187 is attributable to the preincubation of microsomes with 0.1 mM EGTA for 1 h at room temperature that increases the permeability of the membrane for calcium

Electron microscopy

The vesicle suspensions were negatively stained with 1% uranyl acetate and viewed in a Siemens Elmiskop I microscope at an accelerating voltage of 60 kV. The extent of crystallization of Ca^{2+}-ATPase, denoted as the crystallization index, was determined by counting the vesicles with crystalline regions on their surface and expressing their number as a percentage of the total number of vesicles [8, 9, 15, 16]. Usually more than 600 vesicles were counted by several observers; the independent counts were generally in good agreement.

Materials

ATP, EGTA, fluorescein 5′-isothiocyanate, imidazole, $MgCl_2$, 4-(2-pyridylazo)-resorcinol and Tris-maleate were obtained from Sigma Chemical Co., St Louis, MO; KCl, Na_3VO_4, and uranyl acetate were purchased from Fisher Scientific Co., Rochester, NY; $CaCl_2$ was a Baker analyzed reagent, Philipsburg, NJ. A23187 was from Calbiochem, La Jolla, CA, and sucrose was from Mallinckrodt, Inc., Paris, KY. All the reagents used were of the best analytical purity.

RESULTS

The reaction of sarcoplasmic reticulum with fluorescein isothiocyanate

Reaction of sarcoplasmic reticulum vesicles with FITC at 25 °C for 60 min causes nearly complete inhibition of ATPase activity at an FITC concentration of about 15 nmol/mg protein (Fig. 1). Under these conditions about 6 nmol of FITC

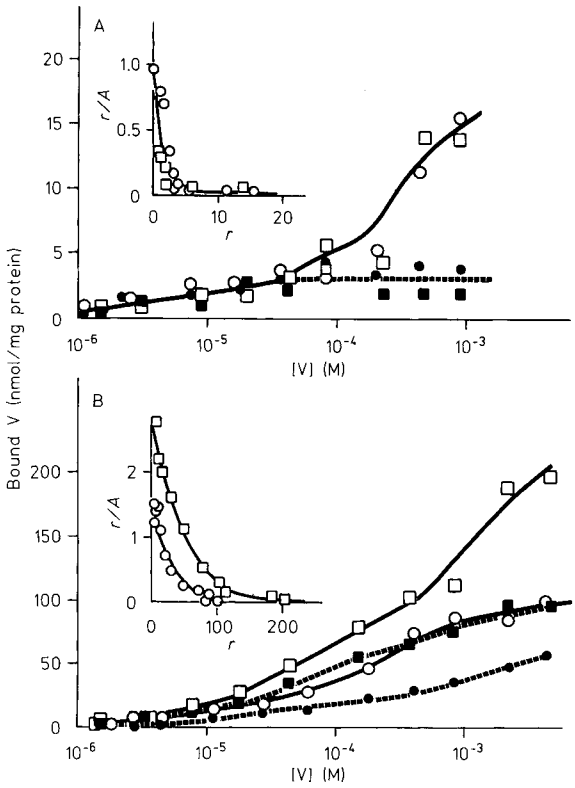

Fig. 2. *The binding of monovanadate and decavanadate to control and FITC-labeled sarcoplasmic reticulum vesicles.* Microsomes (2 mg protein/ml) were labeled with 15 μM FITC in 0.3 M sucrose, 50 mM Tris-maleate, 5 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4 for 60 min at room temperature. After centrifugation at 80000 × *g* for 20 min, the FITC-labeled and control vesicles were suspended in 0.1 M KCl, 10 mM imidazole, 5 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4 and incubated for 2 h at 4°C, with either monovanadate (A) or decavanadate (B) at the total V concentrations indicated. The vesicles were separated by centrifugation (80000 × *g*, 20 min) and were washed twice with 2.5 vol. of the incubation medium. The final pellet was suspended in 1.5 ml 10 mM imidazole pH 7.0 and solubilized with 0.5 ml 4% SDS. (A) monovanadate binding to FITC-labeled (○, ●) and control (□, ■) sarcoplasmic reticulum vesicles. (B) Decavanadate binding to FITC-labeled (○, ●) and control (□, ■) sarcoplasmic reticulum vesicles. The open symbols denote values based on the difference between the absorbance of the control and supernatant vanadate solutions; the filled symbols represent values measured from the pellets. The inserts are Scatchard plots of the 'supernatant' values. *A* is the free concentration of vanadate in μM; *r* denotes nmol of vanadate bound/mg microsomal protein. The data are average of two experiments

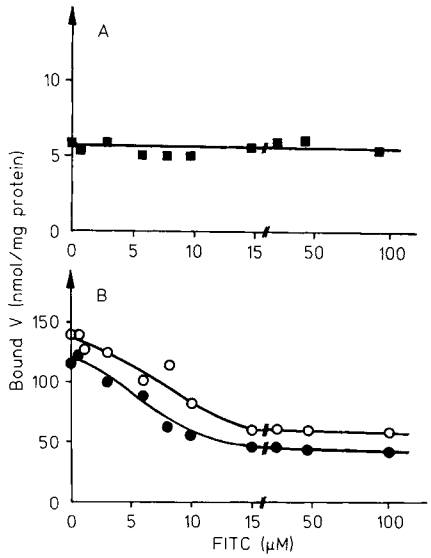

Fig. 3. *Monovanadate and decavanadate binding to sarcoplasmic reticulum labeled with different concentrations of fluorescein isothiocyanate.* Sarcoplasmic reticulum vesicles (2 mg protein/ml) were labeled with FITC at the concentrations indicated, for 60 min at room temperature in 0.3 M sucrose, 50 mM Tris-maleate, 5 mM $MgCl_2$, and 0.1 mM EGTA, pH 7.4. The vesicles were collected by centrifugation at 80000 × *g* for 20 min. The sediments were suspended in 0.1 M KCl, 10 mM imidazole, 5 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4 to final protein concentrations of 2 mg/ml for the monovanadate or 1 mg/ml for the decavanadate binding experiments. Monovanadate or decavanadate (5 mM) was added and the vanadate binding was measured as described under Experimental Procedure. (A) (■——■) Monovanadate; (B) (●——●) decavanadate measured by the difference in absorbance between 400 nm and 550 nm; (○——○) decavanadate measured after conversion to monovanadate. The data are corrected for the absorbance of bound FITC at 400 nm

are bound per mg protein, representing close to 1 mol of FITC per mol of Ca^{2+}-ATPase [2]. The rising phase of the binding curve above 25 μM FITC concentration derived from the supernatant data (Fig. 1A, O) is presumed to indicate low affinity noncovalent binding of FITC to the microsomes.

The binding of monovanadate and decavanadate to FITC-labeled microsomes

The binding of monovanadate (Fig. 2A) and decavanadate (Fig. 2B) to control and FITC-labeled sarcoplasmic reticulum vesicles was analyzed by equilibrium sedimentation. Monovanadate binds to one set of high-affinity sites with an approximate dissociation constant of about 2 μM, and a maximum amount approaching 5 nmol/mg protein (Fig. 2A). Inhibition of ATPase activity by FITC left the binding of monovanadate entirely unaffected. Vanadate bound to the high-affinity sites is not released by brief washing of the microsomes by buffer; therefore assays of the supernatant and of the washed sediment gave identical binding data. Vanadate bound to a set of low-affinity sites at vanadate concentrations in excess of 0.1 mM is readily removed from the microsomes by washing with buffer and therefore cannot be detected in the washed microsomal sediment (Fig. 2A).

The high-affinity decavanadate binding sites of control microsomes bind about 100 nmol of V/mg protein (Fig. 2B), or 10 nmol of decavanadate/mg protein. Assuming that the concentration of Ca^{2+}-ATPase is about 5 nmol/mg protein, this is equivalent to two decavanadate binding sites per mole of ATPase. In FITC-labeled microsomes the decavanadate binding is reduced to about one-half, suggesting that one of the two decavanadate binding sites was blocked by FITC (Fig. 2B).

The effect of FITC on the binding of monovanadate and decavanadate was further investigated as the function of FITC concentration (Fig. 3). The binding of monovanadate was essentially unaffected by FITC at concentrations as high as 100 μM (Fig. 3A). The decavanadate binding was reduced by about one-half after treatment of microsomes with 10–15 μM FITC and was not significantly affected by further increase in FITC concentration to 100 μM (Fig. 3B). These experiments support the existence of two distinct decavanadate binding sites. One of these, like the binding site

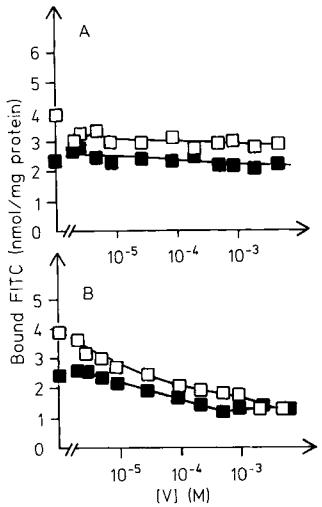

Fig. 4. *FITC binding to sarcoplasmic reticulum at different vanadate concentrations.* Sarcoplasmic reticulum vesicles were incubated with monovanadate or decavanadate at the different V concentrations indicated, in 0.1 M KCl, 10 mM imidazole, 5 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4 for 2 h at 4 °C. The microsomes were transferred into 0.3 M sucrose, 50 mM Tris-maleate, 5 mM $MgCl_2$, 0.1 mM EGTA pH 7.4, by maintaining the same vanadate concentration and 15 μM FITC was added. The samples were incubated for 60 min at room temperature. The binding of FITC was measured as described under Experimental Procedure. (A) FITC binding with monovanadate; (B) FITC binding with decavanadate. Open squares: binding data analyzed from the supernatants; filled squares: binding measured from the sediments

of monovanadate, is unaffected by FITC, while the second decavanadate binding site is inhibited by FITC.

The effect of monovanadate and decavanadate on the reaction of FITC with sarcoplasmic reticulum

In the experiments shown in Fig. 4, the reciprocal effect of monovanadate and decavanadate on the binding of FITC to the sarcoplasmic vesicles is analyzed. Up to 5 mM monovanadate had no influence on the binding of FITC to microsomes, while decavanadate even at 0.1 mM V concentration (1 μM decavanadate) significantly reduced FITC binding and this inhibition increased with decavanadate concentration up to 1 mM V. Complete inhibition of FITC binding was not achieved probably because the covalent reaction of FITC continually shifts the equilibrium in favor of the FITC-ATPase complex.

The protection of Ca^{2+}-ATPase from inhibition by FITC in the presence of decavanadate

Since decavanadate protects the Ca^{2+}-ATPase from reaction with FITC, it is expected that after incubation of microsomes with decavanadate and FITC together at inhibitory concentrations, the ATPase activity may be regained after removal of both reagents by dialysis. This expectation is borne out by the experiments shown in Fig. 5D, E, F, where significant protection of ATPase activity against inhibition by FITC was obtained at decavanadate concentrations in excess of 0.5 mM. Under similar conditions monovanadate was ineffective in protecting ATPase activity (Fig. 5A, B, C). The increased sensitivity of the native sarcoplasmic reticulum Ca^{2+}-ATPase to vanadate inhibition, observed in the presence of

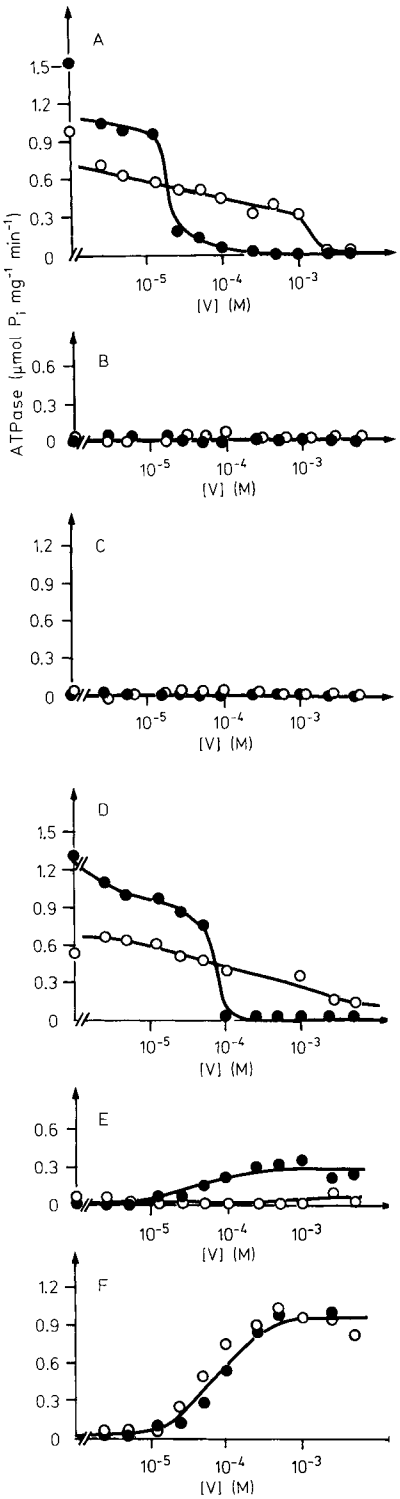

Fig. 5. *Protection of the Ca^{2+}-activated ATPase activities of sarcoplasmic reticulum vesicles by vanadate during FITC labeling.* The experiment was identical to the experiment described in Fig. 4, except that after the incubation with vanadate (A, D) and after the subsequent incubation with FITC (B, E) aliquots were taken for ATPase assay. After FITC labeling the pellets were suspended in 0.1 M KCl, 10 mM imidazole, 5 mM $MgCl_2$, 0.5 mM EGTA pH7.4, to a protein concentration of 1 mg/ml and dialyzed for 16 h against 250 vol. of the same buffer (C, F). (A – C) Ca^{2+}-activated ATPase activities after monovanadate treatment. (D – F) Ca^{2+}-activated ATPase activities after decavanadate treatment. Open symbols denote Ca^{2+}-activated ATPase activities without A23187, and the filled symbols with 2 μM A23187

Table 1. *Crystallization indices for Ca^{2+}-ATPase in control and FITC-treated microsomes after 24 h incubation with vanadate*
Control and FITC-labeled sarcoplasmic reticulum vesicles (1 mg protein/ml) were incubated with monovanadate or decavanadate at different V concentrations, in 0.1 M KCl, 10 mM imidazole, 5 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4, at 4°C. Samples were taken for electron microscopy after 4 h, 24 h and 4 days, and negatively stained with 1% uranyl acetate. Crystallization indices were determined by counting the vesicles with crystalline regions on their surface and expressing them as a percentage of the total number of vesicles [15, 16]. Data are crystallization indices ± standard errors after 24 h of incubation. Similar trends were observed after 4 h and 4 days

Total vanadate concentration	Crystallization index with			
	monovanadate		decavanadate	
	control	FITC-treated	control	FITC-treated
mM	%			
0.1	16.5 ± 6.1	15.9 ± 4.3	49.7 ± 4.3	53.8 ± 2.6
0.5	48.2 ± 5.1	42.6 ± 3.9	83.4 ± 3.3	86.1 ± 3.1
1.0	65.4 ± 2.6	63.2 ± 4.3	91.4 ± 1.2	90.0 ± 3.6
2.5	72.1 ± 6.0	70.0 ± 7.1	92.1 ± 3.6	89.7 ± 4.6

A23187 (Fig. 5A, D), is consistent with data in the literature [10, 17, 18].

These observations provide conclusive proof that one of the binding sites occupied by decavanadate overlaps with the binding region of FITC, manifested in competition between these reagents, while the binding of monovanadate and FITC to the Ca^{2+}-ATPase involves independent binding sites.

Vanadate-induced crystals of Ca^{2+}-ATPase in control and FITC-labeled microsomes

Inhibition of the Ca^{2+}-ATPase by covalent attachment of FITC to lysine 3/190 did not interfere with the crystallization induced by either monovanadate or decavanadate. The crystal forms of the Ca^{2+}-ATPase obtained in the two systems were visually identical (not shown), and there was no significant difference in the extent of crystallization between control and FITC-labeled microsomes over a wide range of monovanadate and decavanadate concentrations (Table 1).

DISCUSSION

In agreement with earlier observations [17], monovanadate binds with high affinity to one site per Ca^{2+}-ATPase molecule in sarcoplasmic reticulum vesicles. Under similar experimental conditions decavanadate ($V_{10}O_{28}^{6-}$) binds to two sites per ATPase molecule, representing the binding of 20 atoms of vanadium (V) per Ca^{2+} transport ATPase [10].

The binding of monovanadate to the Ca^{2+}-ATPase inhibits the ATPase activity and Ca^{2+} transport of sarcoplasmic reticulum [18], blocks the phosphorylation of the enzyme by inorganic phosphate, but not with ATP [4], and inhibits the tryptic cleavage of the Ca^{2+}-ATPase at the T_2 site [7]. It is assumed that monovanadate serves as an analogue of inorganic phosphate, and interacts with the enzyme in the E_2 conformation at the P_i binding site; as a result the kinetically stable E_2 – V enzyme intermediate accumulates. The stabilization of the enzyme by monovanadate in the E_2 conformation leads to the crystallization of Ca^{2+}-ATPase dimers in the membrane [6–9].

Decavanadate also inhibits ATPase activity and very effectively promotes the formation of Ca^{2+}-ATPase crystals in sarcoplasmic reticulum [10]. These observations suggest that decavanadate mimics at significantly lower concentration the effects of monovanadate in stabilizing the E_2 conformation of the Ca^{2+}-ATPase.

Since binding data indicate the binding of 2 mol of decavanadate per mol of ATPase, as the simplest model, we assume that one of the two decavanadate binding sites (site 1) is common with the binding site for monovanadate or inorganic phosphate. Addition of Ca^{2+} in micromolar concentrations displaces vanadate from this site [17], either by direct interference with vanadate binding or through a shift in equilibrium in favor of the Ca^{2+}-stabilized E_1 conformation. This change is reflected in the disruption of vanadate-induced ATPase crystals by Ca^{2+} [8, 19].

The second vanadate binding site (site 2) is unique to decavanadate. Binding of decavanadate to this site prevents the reaction of FITC with a lysine residue at position 3/190; in turn the second vanadate binding site is missing in FITC-labeled sarcoplasmic reticulum. Since FITC inhibits the binding of ATP to the Ca^{2+}-ATPase [3], we presume that site 2 of vanadate binding overlaps with the ATP binding site labeled by FITC. The occupancy of site 1 by decavanadate appears sufficient for the crystallization of the enzyme, since inhibition of decavanadate binding by FITC at site 2 does not interfere with the decavanadate-induced crystallization of the Ca^{2+}-ATPase.

Site 1 and site 2 may be contiguous regions of the active site of the Ca^{2+}-ATPase. The higher affinity of decavanadate, as compared with monovanadate, for site 1 is presumably due to the unique coordination geometry and greater negative charge density of decavanadate [20]. The assumption that the same site is occupied by inorganic phosphate, monovanadate and the much larger decavanadate ions, implies a relatively open structure and considerable flexibility.

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