Formation and Decay of the Vanadate Complex of the Sarcoplasmic Reticulum Calcium Transport Protein

Pankaj Medda and Wilhelm Hasselbach

Max-Planck-Institut für medizinische Forschung, Abteilung Physiologie, Jahnstraße 29, D-6900 Heidelberg, Bundesrepublik Deutschland

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The calcium free sarcoplasmic reticulum calcium transport ATPase incorporates in the presence of magnesium ions approx. 8 nmol monovanadate per mg protein, indicating the formation of a complex containing one vanadate residue per enzyme molecule. On ligand-removal or dilution, the saturated enzyme complex displays biphasic decay kinetics, while the unsaturated complex slowly dissociates monophasically. – Ligand competition by raising the concentrations of unlabeled vanadate results in a progressive decrease of the dissociation rate of the unsaturated enzyme. The complicated dissociation kinetics indicate a sequential mode of interaction between two ligand binding sites. The one to one stoichiometry of the complex suggests that the two sites are located at adjacent ATPase molecules. – It appears unlikely that the decay of the enzyme, vanadate complex is retarded by the formation of a stable quaternary complex between the enzyme, magnesium, mono- and polyvanadate.

Introduction

The phosphate analogue vanadate interacts with the calcium-free, calcium transport ATPase of the sarcoplasmic reticulum in the presence of magnesium ions forming protein-magnesium-vanadate complex [1–3]. In contrast to the corresponding magnesium-phosphate complex, the vanadate residue cannot be trapped by acid quenching [3]. Yet, the slow formation of the complex and its relatively high stability allow to study the kinetics of complex formation as well as the characteristics of the complex under equilibrium conditions [1–5].

Vanadate binding has been measured either by applying radioactive vanadate or by monitoring calcium dependent phosphoprotein formation or ATP hydrolysis of the vanadate free protein fraction [3–6]. Vanadate binding completely shifts the enzyme in its E_2 conformation, having no high but only low affinity calcium binding sites, which are exclusively located on the luminal surface of the vesicular membranes [3, 6].

Their occupation by calcium results in a slow back transition of the enzyme into its E_1 conformation which binds calcium with high affinity and can be phosphorylated by ATP in the presence of calcium and magnesium ions *c.f.* scheme. The stability of the enzyme-vanadate complex allows to explore the properties of the enzyme's low affinity calcium binding state. In the present study we attempt to analyse the properties of the enzyme vanadate complex as an intermediate analogue in the physiological reaction cycle. The time course of the decay of the vanadate complex indicates that the ATPase molecule forms a dimer in the membrane when its vanadate binding sites are saturated.

Abbreviations: SR, sarcoplasmic reticulum.

Reprint requests to Prof. Dr. W. Hasselbach.

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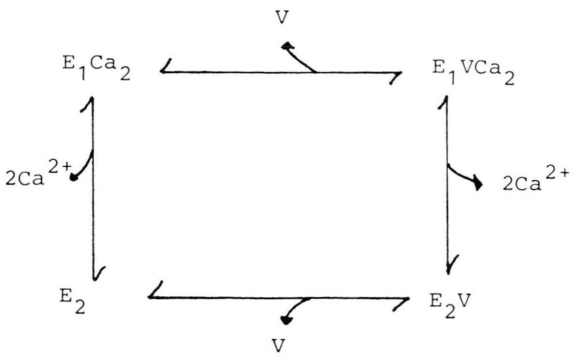
Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle according to the procedure described previously [7, 8]. Equilibrium vanadate bindings to the native vesicles were carried out by incubating 1 mg protein/ml with [^{48}V] vanadate at room temperature in standard buffer solution containing 40 mM KCl, 20 mM imidazole, pH 7.0, 5 mM $MgCl_2$ and 1 mM EGTA unless otherwise mentioned. After 30 min an aliquot of the reaction mixture containing 0.2 mg protein was filtered through 0.45 μm Millipore filter and subsequently washed once with 2 ml ice-cold water. The amount of vanadate bound to the enzyme was determined by measuring the radioactivity remaining on the filter by liquid scintillation counting (740 ml Toluene; 260 ml Triton X-100 and 4 g Omnifluor, New England). Filters rinsed with protein-free media and 2 ml ice cold H_2O respectively were used as blanks. The preparation and handling of stock solution of vanadate as



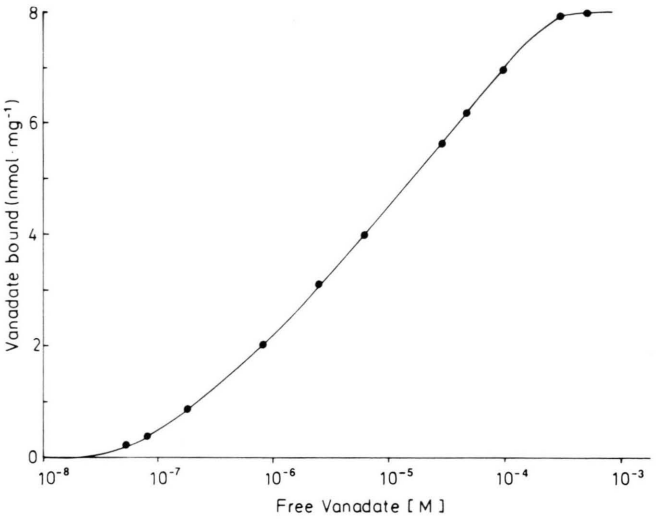


Fig. 1. Equilibrium binding of vanadate to sarcoplasmic reticulum vesicles.
The binding was measured by Millipore filtration technique as described in Materials and Methods at room temperature in standard media.

well as [^{48}V] vanadate from [^{48}V] $VOCl_3$ were described earlier [3, 6]. Vanadate release from enzyme-magnesium-vanadate complexes was studied by chelating the free ligands and by ligand competition. For the removal of free ligands the vanadate reacted incubation mixtures were diluted tenfold into a buffer solution containing EDTA which chelates not only Mg^{2+} but also vanadium(V) [9].

In case of ligand competition the reaction mixture was diluted tenfold into a buffer solution containing one of the ligands in excess. The semilogarithmic plots of $[100(V_t-V_\alpha)/V_o-V_\alpha)]$ *versus* time are applied to evaluate dissociation of the enzyme-vanadate complex. V_o is bound vanadate prior to dissociation, V_t bound vanadate at time *t*, and V_α at equilibrium. V_α was determined two hours after the addition of unlabelled vanadate or EDTA.

Results and Discussion

The dependence of vanadate binding to sarcoplasmic reticulum membranes at room temperature on the concentration of free vanadate shows that the preparation incorporates approx. 8 nmol/mg protein at 0.3–0.5 mM vanadate (Fig. 1). Thus, the vanadate membrane complex contains one vanadate residue per ATPase molecule, assuming an ATPase content of the preparation of 80% and a molecular weight of the enzyme of 100 kDa [10]. This number of binding sites well agrees with the figure given by Varga *et al.* [11] for maximum monovanadate binding. Polyvanadate does scarcely contribute to vanadate binding since under the prevailing conditions nearly exclusively monovanadate exists [11, 12]. A similar stoichiometry for the interaction of phosphate with the enzyme is only observed when the reaction is performed in the presence of 20–30% DMSO [10, 13]. In the absence of this modifier, maximal phosphorylation usually does not exceed 4 nmol/mg protein [14]. The depicted vanadate binding isotherme further reveals that the binding reaction extends over a concentration range of 3 decades indicating either negative binding cooperativity or the presence of two independent vanadate binding sites. Since each ATPase molecule appears to react only with one vanadate residue, both binding modes require to assume the interaction of adjacent enzyme molecules in the binding reaction. This conclusion based on equilibrium binding studies can further be substantiated by studying dissociation kinetics of the protein vanadate complex. Fig. 2 shows that the kinetics of the decay of the vanadate protein complex initiated by the removal of free magnesium and free vanadate by EDTA depends on the extent of enzyme saturation. At a lower degree of saturation (0.8–4 nmol/mg) the complex displays a linear first order decay. In contrast, at a high degree of saturation (6–8 nmol/mg) two kinetic components can be recognised. A fraction of the complex decays rapidly with a time constant of a few seconds, while the remaining fraction decays as slowly as the unsaturated enzyme (T = 15 min). This kinetic behaviour is consistent with the existence of two binding sites. The alternative between the occupation of two independent or of two

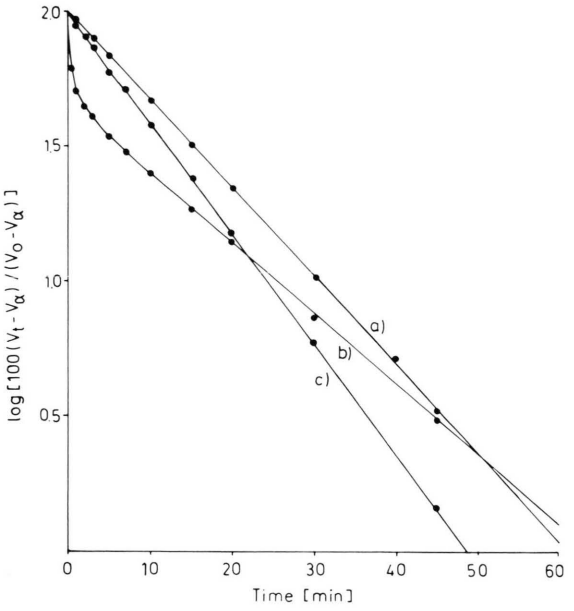


Fig. 2. Time course of vanadate release from protein-vanadate complexes after removal of free vanadate and Mg^{2+} with EDTA, as well as reduction of vanadate with norepinephrine.
1 mg protein/ml was incubated with (a) 1 μM or 10 μM; and (b) 300 μM [^{48}V] vanadate in standard buffer solution at 30 °C. After 30 min the assays were diluted 1:10 into a standard buffer solution containing 2 mM EDTA but no Mg^{2+} at 30 °C. (c) The protein was incubated with 1 μM [^{48}V] vanadate as described above. Complex dissociation was effected by dilution and addition of 1 mM norepinephrine. The suspensions containing 0.2 mg protein were then filtered to determine the bound [^{48}V] vanadate as described for equilibrium vanadate binding in Materials and Methods. The vanadate bound at time infinity was < 1% in the presence of EDTA.

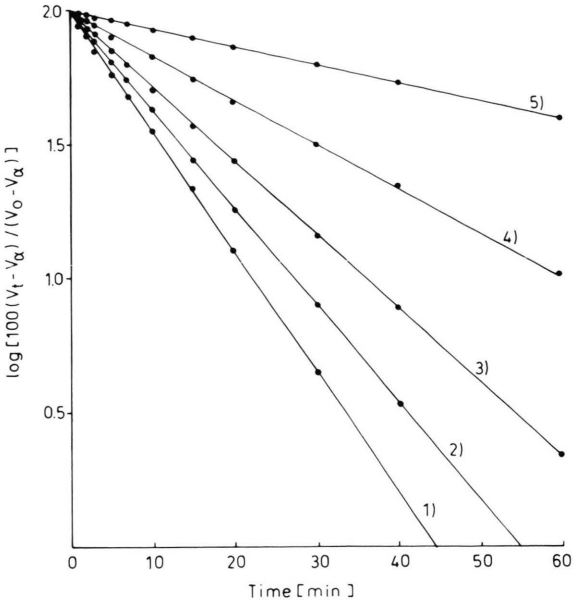


Fig. 3. Effect of isotopic substitution on the time course of vanadate release from the protein-vanadate complex.
1 mg protein/ml was incubated with 1 μM [^{48}V] vanadate as described in the legend of Fig. 2. Dissociation was effected by tenfold dilution in media containing 5 mM Mg^{2+} and varying concentrations of unlabeled vanadate.
(1) 100 μM, (2) 200 μM, (3) 300 μM, (4) 500 μM, (5) 1 mM.

interacting binding sites can be resolved by choosing appropriate dissociation conditions. Dissociation can be initiated either by dilution or removal of one of the two protein ligands or by ligand competition, *i.e.* by studying the decay of the complex in the presence of excess ligand. As shown above, the unsaturated enzyme complex dissociates monophasically when magnesium and vanadate are simultaneously removed by EDTA. A monophasic decay is likewise observed when vanadate is reduced with norepinephrine [5] (Fig. 2). The small difference between the time constants of the two dissociation reactions might be due to the quite different dissociation conditions. The complex also monophasically decays on addition of excess unlabelled vanadate (Fig. 3). However, Fig. 3 demonstrates that the decay rate declines progressively when the concentration of the competing ligand is raised from 0.1 mM to 1.0 mM. This progressive decrease of the dissociation rate by increasing the ligand concentrations cannot be explained by simple random dissociation from dependent or independent binding sites but rather favours a sequential mode of ligand interaction. This conclusion is based on the fact that ligand substitution at no mode of random interaction can furnish dissociation rates slower than the rate observed when the ligands were removed [15]. Since dissociation observed in the presence of 1 mM vanadate is at least four times slower than that shown in Fig. 2 random mechanisms are excluded. The observed progressive retardation of dissociation by excess ligands can be described by a most simple sequential interaction mode. It is assumed that the dissociation of the ligand bound first depends on the occupancy of the second binding site, *i.e.* the ligand which binds first to the enzyme, can dissociate only when the second binding site is empty [15]. Thus, rising concentrations of the competing

ligand will finally suppress dissociation completely. Since in the presence of saturating concentrations of vanadate the enzyme binds one vanadate residue, the observed ordered interaction of binding sites forced to assume that in the vanadate binding reaction at least two enzyme molecules have to interact with each other.

The interaction between neighbouring enzyme molecules in the membranes have repeatedly been disputed *cf.* [16–18]. In the resting membrane the ATPase molecules appear to be in close contact as shown by excimer formation between pyrene residues attached to the protein [19]. In fact, association of ATPase molecules in native membranes have been observed after treatment with high concentrations of vanadate or phosphate [20]. Furthermore, a number of kinetic differences in the kinetic behaviour of ATPase in either native vesicles or in detergent containing solutions have been reported [18, 21]. On the other hand, the detergent solubilized enzyme in spite of being monomerised can catalyse calcium-dependent ATP splitting. ATP synthesis and the ATP-dependent affinity change as required for calcium transport [22, 23]. Thus, the functional importance of the interaction between neighbouring sub-units has been questioned. Yet, the described retardation of the decay of the protein-vanadate complex produced by ligand competition indicates that at least the neighbouring molecules in the E_2 state functionally cooperate. Since the corresponding phosphate intermediate is thought to be directly involved in calcium translocation, it is tempting to assume that this kind of cooperation is a necessary requirement for calcium translocation across the membrane.

Recently Andersen *et al.* [5] have described a pronounced retardation of the decay of the enzyme vanadate by ATP. Since it can not be excluded that at higher concentrations of vanadate in our experiments small quantities of polyvanadate might exist and can act as ATP analogues, the retardation described in this report, could be produced by a corresponding mechanism namely the formation of a stable quaternary complex between enzyme, magnesium, mono- and polymeric vanadate. This mechanism appears to be less likely: The inhibition of vanadate release effected by ATP is observed only in the absence of magnesium while the inhibition produced by rising vanadate concentrations takes place in the presence of magnesium. Furthermore, an involvement of polyvanadate should result in binding numbers higher than observed in the present study.

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