Pressure Effects on the Interactions of the Sarcoplasmic Reticulum Calcium Transport Enzyme with Calcium and *para*-Nitrophenyl Phosphate

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The effect of hydrostatic pressure on calcium dependent *p*-nitrophenyl phosphate hydrolysis of the sarcoplasmic reticulum calcium transport enzyme has been investigated at different degree of enzyme saturation by calcium and Mg-*p*-nitrophenyl phosphate to distinguish between activation and binding volumes. The enzyme saturated by both ligands displays a significant dependence of the activation volume on pressure, rising from 20 ml/mol at atmospheric pressure (0.1 MPa) to 80 ml/mol at 100 MPa. At subsaturating concentration of Mg-*p*-nitrophenyl phosphate an activation volume of 35 ml/mol prevails between 0.1 and 40 MPa. At subsaturating concentration of calcium the activation volume approximates 80 ml/mol in the same pressure range. The binding volume for both substrates is likewise pressure dependent falling from 20 ml/mol to 0 ml/mol for Mg-*p*-nitrophenyl phosphate and rising from 67 ml/mol to 155 ml/mol for calcium. The pressure dependence of activation and binding volumes is analysed on account of a simplified reaction scheme yielding activation volumes and rate constants for individual reaction steps.

Introduction

The reaction cycle of the calcium transport enzyme in the sarcoplasmic reticulum membranes is initiated by calcium binding to the enzymes' high affinity binding sites [1–6]. Calcium enters the cycle relatively slowly because either a slow transition of low to high affinity binding sites precedes the binding reaction or the rapidly formed calcium protein complex is only slowly converted to the reaction intermediate which can accept the phosphoryl group from ATP or other phosphate donating substrates [7–10]. Subsequently phosphorylation leads to the transposition of calcium binding sites from the membrane's cytoplasmic to its luminal surface. As first pointed out by Dupont [11, 12] phosphorylation dependent changes of the enzyme's calcium affinity should be accompanied by changes in the calcium ion's hydration state. According to Dupont's suggestion the loss of hydration water during high affinity binding is compensated in a following reaction step by phosphorylation when the enzyme's calcium affinity is reduced. Since sizable volume changes were reported for the complexation of calcium ions with various ligands a marked pressure dependence of the enzyme's activity can be expected [13].

In a previous study we have attempted to trace such volume changes by determining the activation volume of the enzymes' calcium activated *p*-nitrophenyl phosphate splitting which like the hydrolysis of ATP is stoichiometrically connected with calcium translocation [14].

Yet the large water movement proposed by Dupont and Pougeois [11] could not be detected as a pressure sensitive volume change of calcium dependent *p*-nitrophenyl phosphate hydrolysis [15]. This finding is in line with the rather small activation volume observed for ATP hydrolysis by the Na^+-K^+ transport ATPase [16]. In contrast large volume changes were reported for the ATP dependent potassium transport and anion exchange by red cell membranes [17, 18]. Yet the absence of great activation volumes does not exclude extensive hydration changes because in complex reactions secondary volume changes might volumetrically compensate for the primary hydration dependent volume changes (*cf.* [19]).

In the present study we focus on volume changes related to enzyme activation by its ligands calcium, magnesium, *p*-nitrophenyl phosphate to specify the volume changes associated with distinct reaction steps. It will be shown that calcium binding is con-

Abbreviations: *p*-NPP, *p*-nitrophenyl phosphate.

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nected with a quite large positive reaction volume. The volume changes recorded for calcium binding by far excede the volume increase resulting from the loss of the ions' hydration shell. On the other hand the ligand couple Mg-*p*-nitrophenyl phosphate, which was identified as the energy yielding substrate-complex, enters the reaction cycle with a minor volume increment, being the small difference between much greater activation volumes for complex formation and dissociation. Thus the state of the enzyme itself either its structural or its hydration state must essentially contribute to the large volume changes associated with the reaction cycle.

Materials and Methods

Enzyme preparation

The sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as described by de Meis and Hasselbach [20]. The protein content of the vesicular suspension were determined by the Biuret procedure calibrated by Kjeldahl nitrogen determinations.

Enzyme assays

p-Nitrophenyl phosphatase activity was measured in media containing 20 mM NaCl, 6 mM histidine, 14 mM sodium glycerophosphate pH 7.0, 1 μM of the calcium ionophore A 23187 and 0.2 mM dithioerythritol. Temperature and the concentration of the reactants, *p*-nitrophenyl phosphate, magnesium and calcium are specified in the legends of figures and tables. The concentration of the vesicles in the assay media were chosen between 0.05 mg/ml and 0.25 mg/ml depending on the enzymatic activity under the respective conditions. $T = 30$ °C.

For measuring the dependence of *p*-nitrophenyl phosphate hydrolysis on calcium, magnesium and *p*-nitrophenyl phosphate at atmospheric pressure the reaction was terminated with sodiumdodecylsulfate 1% final concentration at appropriate times. Absorption was measured after dilution with 0.1 M Na_2HPO_4 – using 15,000 M^{-1} cm^{-1} as molar extinction coefficient. Calcium dependent ATP hydrolysis was monitored by measuring phosphate production as previously discribed [21]. The calcium independent activity was measured in the presence of 2–4 mM EGTA.

Pressure application

The assay media were brought to the respective temperatures prior to the addition of the enzyme. The suspension was subsequently filled into the pressure cell equipped with sapphire windows, Nova Swiss Switzerland, Effretikon. Pressure was generated by a hand-driven pump. The pressure was transmitted by silicon oil which was separated from the cell by a two-parted piston, sealed with a silicon paste plug in the middle. The pressure was monitored electronically. The pressure cell was surrounded by a thermostatically controlled water jacket and installed in a Philips double beam photospectrometer. The reaction was followed by monitoring the appearance of *para*-nitrophenol at 420 nm using 7000 for its molar absorbance at pH 7.0. The effect of pressure on the calcium independent enzyme activity was measured in the presence of 2 mM EGTA. The calcium independent activity was substracted from the total activity for each pressure value. All pressure values are given as gauge pressure and expressed as MPa.

Reaction buffers and stability constants

In the histidine – glycerophosphate buffered assay media, the absorbance of *p*-nitrophenol proved to be pressure independent [15]. This empiric finding is in line with the pressure dependences of the ionization constants for the used buffers and *p*-nitrophenol [22]. Furthermore activity measurements at pH 6.5, 7.0, 7.5 revealed that in this pH range the enzyme's *p*-nitrophenylphosphatase activity is nearly constant. Thus, even if pressure induced pH changes would occur, they would not affect substrate hydrolysis. In control experiments 40 mM imidazole was used as medium buffer. Pressure changes caused instantly considerable absorbance changes which required resetting of the recorder. No significant difference between the activity pressure relation of *p*-nitrophenyl phosphate hydrolysis in the two buffer systems could be detected.

The stability constant of the respective calcium and magnesium complexes of EGTA and EDTA were taken from Schwarzenbach [23] and used according to Blinks *et al.* [24]. Magnesium binding to *p*-nitrophenyl phosphate was determined at pH 7.0 with a divalent cation sensitive electrode (Orion Research Cambridge Mass. USA) at 20 °C. Inorganic phosphate was used as standard reagent of known

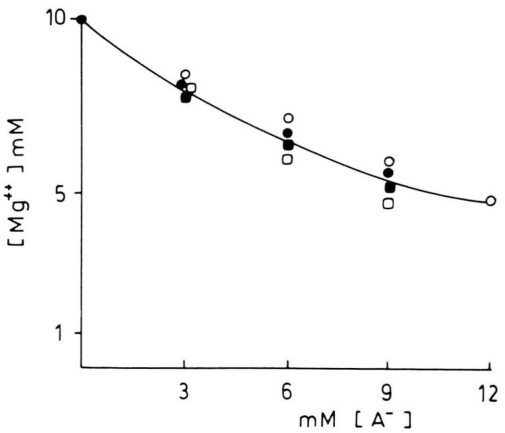


Fig. 1. Titration of magnesium with inorganic phosphate and *p*-nitrophenyl phosphate using a divalent ion selective electrode (Orion). Titrations were performed in 40 mM Mops buffer, pH 7.0. (●, ○) or in the reaction medium containing 20 mM histidine, 20 mM glycerophosphate and 0.1 M NaCl (■, □). Open symbols *p*-nitrophenyl phosphate, closed symbols inorganic phosphate. The electrode slope in Mops buffer and in reaction medium were 26 and 10 mV respectively.

magnesium affinity [23]. As shown in Fig. 1 complexation of magnesium by equal concentrations of *p*-nitrophenyl phosphate and inorganic phosphate identically reduced magnesium activity showing that at pH 7.0 inorganic phosphate and *p*-nitrophenyl phosphate exhibit the same affinity for magnesium ions. We therefore applied the reported stability constant of magnesium phosphate to Mg-*p*-nitrophenyl phosphate yielding 30 M^{-1} at pH 7.0 [25].

Determination of activation and reaction volumes

The volume changes were evaluated by plotting the logarithm of the reaction rates *versus* pressure. According to the relation

$$\Delta V^* = \frac{R \cdot T}{P_2 - P_1} \ln \frac{V_2}{V_1}$$

v_2 and v_1 are the rate observed at P_2 and P_1 respectively. R is the gas constant 8.3 ml MPa/K mol.

Activation volumes were obtained from the slopes of activity pressure profiles. Each pressure profile was registered at least three times. Below 60 MPa the SEM does not excede 5% and is smaller than symbols used in Fig. 5 and 6. At higher pressure values SEM increases as indicated by bars. Binding volumes were evaluated by applying the most simple reaction scheme consisting of a binding step followed by hydrolytic substrate cleavage.

$$[EL] + M \leftrightharpoons (ELM) \rightarrow E + P$$

For the evaluation of the binding volume for one of the ligands (M) the other ligands (L) should be present at saturating – at least near saturating – concentrations.

The binding volume of one ligand can be obtained from the apparent activation volume determined at different non-saturating ligand concentrations according to the following relation [19, 26].

$$\Delta V^* = \Delta V_A - \frac{1}{1 + K_m \cdot L} \Delta V_B \qquad (1)$$

ΔV_A is the true activation volume observed at saturating ligand concentrations; ΔV^* is the activation volume when the enzyme is only partly activated at nonsaturating substrate concentrations. ΔV_B is the binding volume for the corresponding ligand. K_m represents the apparent affinity constant of the enzyme for the ligand and must be considered to be pressure dependent [19, 26]. This effect can be allowed for because the expression $(1 + K_m \cdot L)$ in Eq. (1) corresponds to the nonactive enzyme fraction, the magnitude of which can directly be obtained from the enzyme activities measured at different enzyme saturations and pressure values.

Volume changes associated with ion complex formation

Since the ligands form ionic complexes in solution further pressure dependent equilibria might interfere with enzyme activity. To establish calcium concentrations $\lesssim 1$ μM as required for partial enzyme activations calcium buffers must be applied. Pressure can be expected to increase the free calcium concentration in the media because complex dissociation is favoured by pressure on account of the electrostrictive effect of the free ions. The concentrations of magnesium, *p*-nitrophenyl phosphate and of the Mg-*p*-nitrophenyl phosphate complex could likewise be affected by pressure. To take into account the pressure dependence of the respective complex dissociation constants we measured the volume changes accompanying complex formation in Tris-HCl puffered solutions at 20 °C with a two-lobe dilatometer as described by Linderström-Lang [27]. The data in Table I show that the volume increase accompanying complex formation of CaEGTA, MgEDTA,

Table I. Volume changes associated with the formation of various magnesium and calcium complexes. Measurements were performed in 0.2 M TrisHCl pH 8.0, $T = 20$ °C in a Linderström/Lang [27] two lobe dilatometer. The apparent affinities of the various ligands for magnesium and calcium at pH 8.0 were taken into account for the calculation of the amounts of the formed complexes. The listed volumes are the observed volume change devided by the calculated number of the moles of the respective complexes. The molar volume change of Tris + H^+ = $TrisH^+$ is negligible [22].

Reactants	Reaction volume [ml/mol]	No. of determinations
Mg^{2+} + ATP	17.6	(2)
Ca^{2+} + ATP	17.0	(2)
Mg^{2+} + *p*-NPP	1.95 + 0.1	(4)
Mg^{2+} + EDTA	19.9 + 0.1	(4)
Ca^{2+} + EDTA	17.8 + 0.1	(5)
Ca^{2+} + Mg EDTA	3.6 + 0.7	(3)
Ca^{2+} + EGTA	20.4 + 0.72	(3)

CaEDTA, MgATP and CaATP are in the same range as observed by Rainford *et al.* [28] for MgATP. Surprisingly the formation of Mg-*p*-nitrophenyl phosphate only gives rise to such a small volume increase that the concentrations of Mg-*p*-nitrophenyl phosphate as well as of magnesium and *p*-nitrophenyl phosphate are scarcely affected by pressure.

Statistical Treatment

Three or more replicate samples were taken for each experimental conditions. Data are presented as mean ± standard error of the mean (SEM).

Reagents

Na-*p*-nitrophenyl phosphate was purchased from Serva Heidelberg FRG; phosphoenolpyruvate and pyruvate kinase from Boehringer Mannheim FRG; A 23187 from Calbiochem-Behringer Frankfurt FRG; all other reagents were bought from E. Merck, Darmstadt.

Results

In order to evaluate volume changes connected with the binding of specific reactants to the enzyme the interacting substrate species must be known. It has been reported that the hydrolysis of ATP and *p*-nitrophenyl phosphate are activated by similar concentrations of ionized calcium [14].

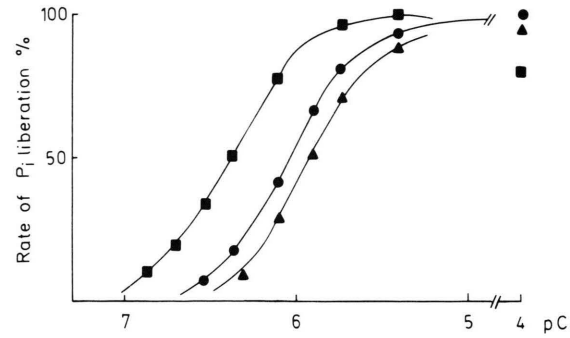


Fig. 2. Dependence on ionized calcium of the hydrolysis of ATP and *p*-nitrophenyl phosphate by the sarcoplasmic reticulum calcium transport enzyme at 30 °C. The medium contains 0.1 M NaCl, 20 mM histidine pH 7.0, 3 μM A 23 187 and ionized calcium as indicated on the abszissa. The calcium concentrations below pCa 5 were adjusted with EGTA. (■) ATP hydrolysis was measured in the presence of 11 mM Mg^{2+} and 2 mM or 0.1 mM ATP respectively. The concentrations of ATP were kept constant by ATP regeneration with pyruvate kinase 0.04 mg/ml and 3 mM PEP; (●) 10 mM total magnesium and 3 mM *p*-nitrophenyl phosphate, 100% activity = 40 μmol/mg·min; (▲) 16 mM total magnesium and 24 mM *p*-nitrophenyl phosphate, 100% activity = 70 μmol/mg·min; 100% ATPase activity 4.3 and 2.3 μmol/mg·min at 2 and 0.1 mM ATP, respectively.

Fig. 2 shows that for saturating concentrations of Mg-ATP and Mg-*p*-nitrophenyl phosphate (see below) the Ca^{2+}-concentration activity profiles for ATP and *p*-nitrophenyl phosphate hydrolysis – exhibit the same shape – yielding an Hill coefficient of 1.9.

Yet for the same degree of activation *p*-nitrophenyl phosphate hydrolysis requires significant higher Ca^{2+}-concentrations than ATP hydrolysis indicating that the apparent Ca^{2+}-affinity of the enzyme depends on the nature of the substrate. The experiments with both substrates were performed at the same excess of ionized magnesium excluding that the different apparent affinities of the enzyme for calcium might be the result of different concentrations of ionized magnesium in the assays.

Complete activation of hydrolysis is achieved for both substrates at Ca^{2+}-concentrations of 0.1–0.2 mM, which in the following are applied as saturating concentrations.

As to the role of Mg^{2+}-ions in the activation of the Ca^{2+}-dependent ATP hydrolysis by the enzyme, convincing evidence has been presented that magnesium enters the reaction cycle together with ATP as Mg-ATP [10, 29, 30].

Due to the quite high affinity of magnesium for ATP, Mg-ATP is the main ionic species in the reaction media even at low concentration, facilitating substrate assignment. To establish the enzyme's ligand species with *p*-nitrophenyl phosphate as reactant we have analyzed the dependence of the enzyme activity on the concentration of ionized magnesium and free *p*-nitrophenyl phosphate.

Due to the quite low stability constant $K = 30\ M^{-1}$ (pH 7.0) (Materials and Methods) the complex comprises under most conditions only a small fraction of the added ion species. Fig. 3 shows that reciprocal plotting of *p*-nitrophenyl phosphate hydrolysis versus ionized magnesium at constant concentrations of free *p*-nitrophenyl phosphate furnishes straight lines with intersect on the ordinate. The straight lines observed when the reciprocal activation at constant Mg^{2+}-concentrations are plotted versus the concentrations of free *p*-nitrophenyl phosphate also intersect on the ordinate. This indicates that the Mg-*p*-nitrophenyl phosphate complex is used as substrate by the enzyme. In fact the enzyme's activities observed at various concentrations of Mg-*p*-nitrophenyl phosphate and plotted against the respective Mg-*p*-nitrophenyl phosphate concentrations cluster around the same profile (Fig. 4). Double reciprocal plotting (inset) furnishes a dissociation constant of 3 mM for the Mg-*p*-nitrophenyl phosphate enzyme complex.

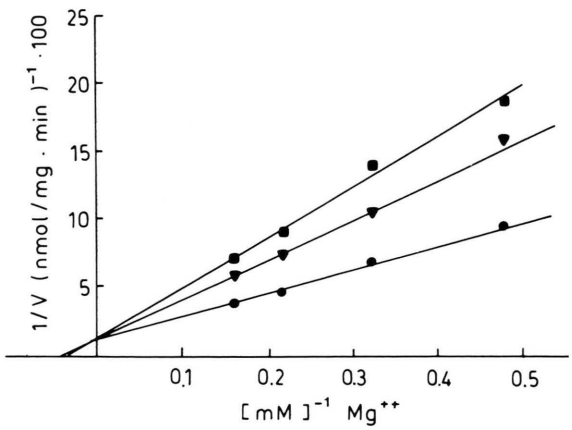


Fig. 3. Dependence on magnesium and *p*-nitrophenyl phosphate of calcium activated *p*-nitrophenyl phosphate hydrolysis. *p*-Nitrophenol production was measured in media containing 20 mM histidine, 20 mM NaCl, 0.2 mM $CaCl_2$ and 0.1–0.2 mg protein/ml as described in "Materials and Methods". The data are presented by double reciprocal plotting of enzyme activity versus the concentration of ionized magnesium applied at 6 (●), 3 (▼) and 2 (■) mM *p*-nitrophenyl phosphate. A stability constant for Mg-*p*-nitrophenyl phosphate of 30 M^{-1} at pH 7.0 was used to calculate the respective concentration of free magnesium and *p*-nitrophenyl phosphate from the added concentrations.

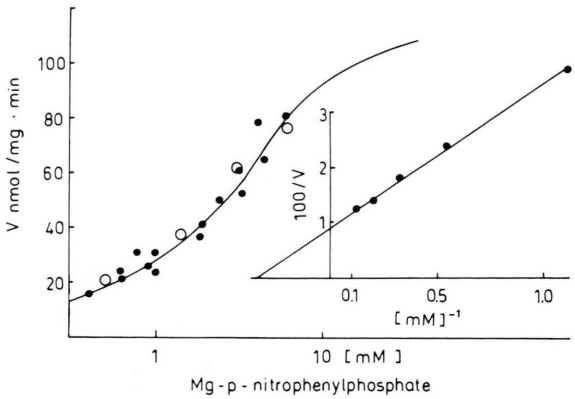
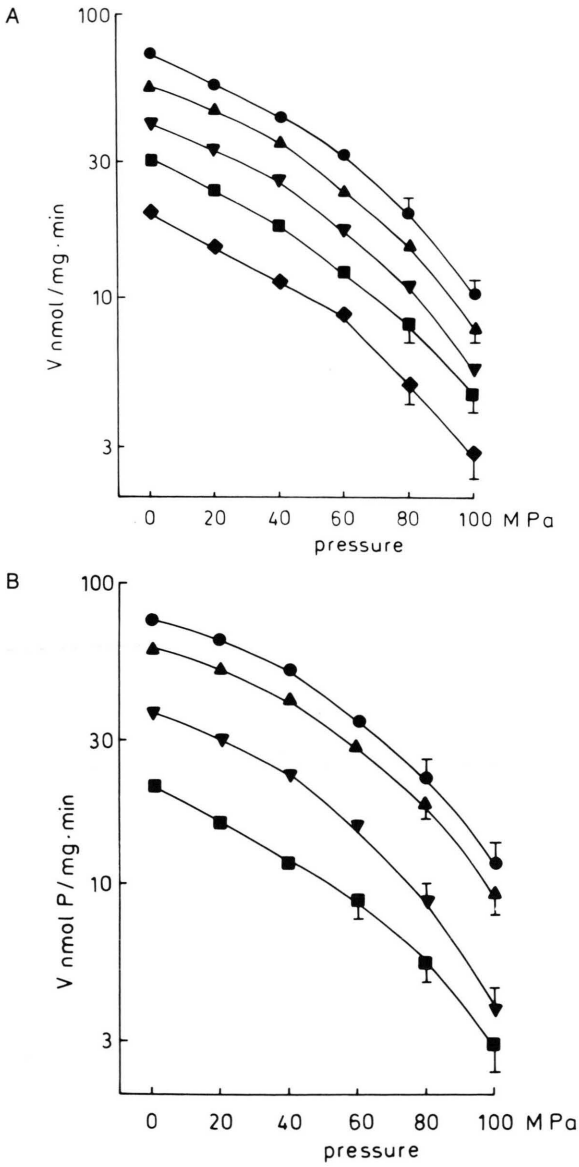


Fig. 4. Dependence of calcium activated *p*-nitrophenyl phosphate hydrolysis on the concentration of Mg-*p*-nitrophenyl phosphate. The assay media contains 20 mM histidine, pH 7.0, 0.1 M NaCl, 0.2 mM $CaCl_2$, pH 7.0. The concentrations of total magnesium and *p*-nitrophenyl phosphate were adjusted to give 10 mM free magnesium and the Mg-*p*-nitrophenyl phosphate concentrations indicated on the abscissa, $T = 30$ °C. (●) Single measurements; (○) means of 5–12 measurements. SEM corresponds to the size of the symbol. Inset: The curve drawn on the main figure was used for the construction of the double reciprocal plot.

In order to evaluate the volume changes connected with Mg-*p*-nitrophenyl phosphate binding, the pressure dependence of *p*-nitrophenyl phosphate hydrolysis was studied at different Mg-*p*-nitrophenyl phosphate concentrations ranging from 0.5 to 6 mM at saturating concentration of calcium (0.2 mM). The results are presented in the usual semilogarithmic plot of activity versus pressure (Fig. 5a and 5b). They demonstrate for several substrate concentrations in the presence of 10 and 30 mM free magnesium, firstly, that the shape of the activity profiles is very similar and, secondly, that the activity profiles appear to be composed of several sections, the slopes of which increase with pressure. The rising steepness of the profiles indicate that the activation volume of the calcium dependent *p*-nitrophenyl phosphate hydrolysis is pressure dependent. Under the reaction conditions applied in the experiments of Fig. 5a the activation volume changes relatively little between 0.1 and 40 MPa at the given Mg-*p*-nitrophenyl phosphate concentration. Yet a small but significant increase of the activation volume from 24 to 35 ml/mol



becomes detectable in this pressure range when the substrate concentration is reduced from 6 to 0.5 mM. From these data and the extent of enzyme activation a volume change of 13–22 ml/mol is computed for the formation of the Mg-*p*-nitrophenyl phosphate enzyme complex according to Eq. (1) (Table II). The tendency of the activity profile to depart from linearity appears to be more pronounced at high than at low substrate concentrations and is even more accentuated when the experiments are performed in the presence of 30 mM ionized Mg^{2+}. In these experiments total *p*-nitrophenyl phosphate concentration up to 36 mM must be applied (Fig. 5b). Corrections for the irreversible activity loss being more significant, (11 ± 3%), for the fully than the partially activated enzyme do not straighten the lines. The pressure dependence of the activation volume observed at high substrate concentrations indicate that at low pressure other reactions limit the rate of *p*-nitrophenyl phosphate hydrolysis than at high pressure. – At low substrate concentrations the activation volume appears to be less susceptible to pressure starting to increase only above 60 MPa.

As shown in Fig. 2 *p*-nitrophenyl phosphate hydrolysis is partially activated at calcium concentrations below 5.0 μM which were adjusted with Ca

Fig. 5. Effect of pressure on calcium dependent *p*-nitrophenyl phosphate hydrolysis by the sarcoplasmic calcium transport ATPase at different concentrations of Mg-*p*-nitrophenyl phosphate. The experimental protocol is described in "Materials and Methods". In Fig. A the free magnesium concentration was adjusted to 10 mM and in Fig. B to 30 mM. $T = 30$ °C. Each profile was obtained from 3–5 separate experiments. SEM is indicated by bars at high pressure values, SEM remained below the size of the symbols at low pressure values (3–5 experiments). A: 6 (●), 3 (▲), 1.4 (▼), 1.0 (■), 0.5 (◆) mM Mg-*p*-NPP; B: 6 (●), 3 (▲), 1.5 (▼), 0.5 (■) mM Mg-*p*-NPP.

Table II. Binding and activation volumes associated with calcium dependent *p*-nitrophenyl phosphate hydrolysis. The activation volumes were obtained from the activity pressure relations measured in media containing 6 mM Mg-*p*-nitrophenyl phosphate and either 10 mM or 30 mM ionized magnesium, 20 mM NaCl, pH 7.0, $T = 30$ °C. The binding volumes were calculated according to Eq. (1) from the activity pressure profile at different degrees of ligand saturation.

Ligands	Pressure range MPa	Binding volume Mg^{2+} 10 mM; Mg^{2+} 30 mM [ml/mol]		Activation volume Mg^{2+} 10 mM; Mg^{2+} 30 mM [ml/mol]	
Magnesium *p*-NPP	0.1 – 40	22 ± 2	13 ± 1.5	27 ± 0.5	19 ± 2.0
	>40	0	0	76 ± 4	–
	0.1 – 40	67 ± 5	–	27	19
Calcium	>40	107 – 155	–	58 – 86	

EGTA/EGTA. The application of this buffer system in pressure studies would make evaluations of the results rather difficult because of the marked pressure dependence of the buffer's stability constant (Table I). In order to avoid pressure dependent changes of the calcium concentration we adjusted the calcium concentrations with Mg EDTA/EDTA. The free calcium concentration is given by the expression:

$$Ca^{2+} = \frac{K^{Mg}}{K^{Ca}} \cdot \frac{[Ca\ EDTA]}{[Mg\ EDTA]} \cdot Mg^{2+}$$

which can be approximated by:

$$Ca^{2+} \cong \frac{K^{Mg}}{K^{Ca}} \cdot \frac{Ca_t}{Mg\ EDTA} \cdot Mg_t$$

since the free calcium concentration never excedes one per cent of the total concentration (Ca_t) (0.05 mM) and total magnesium (Mg_t) is Mg EDTA. The main advantage of the Ca-MgEDTA buffer lies in the fact that its binding constant for calcium is given by the ratio of the stability constant of Ca-EDTA and Mg-EDTA. Thus the effect which pressure exerts on the individual stability constants is largely eliminated because volume changes which accompany dissociation are similar for both complexes (Table I).

The calcium concentrations required to establish suboptimal calcium enzyme activity were adjusted with Mg-EDTA (1–1.5 mM) and 0.05 mM calcium. The corresponding free calcium concentrations are obtained by comparing the resulted activities with the activity profile which is observed when the free calcium concentration is adjusted with Ca-EGTA (Fig. 1). The thus found values agree quite well with the readings of a calcium sensitive electrode which however operates at its sensitivity limit under the prevailing conditions. Fig. 6 shows the pressure dependence of *p*-nitrophenyl phosphate hydrolysis at calcium saturation and two non-saturating concentrations. For saturating calcium concentrations the log activity pressure profile is identical with that of Fig. 5a. For suboptimal calcium levels the slopes of the log activity pressure profiles become considerably steeper. Both profiles also deviate from linearity. In Table II the activation volumes computed from adjacent sections of the log activity profiles are listed together with the corresponding values for the binding volumes obtained from Eq. (3). The listed data show that the volume change related to calcium binding is considerably larger than the activation volume connected with the cleavage of *p*-nitrophenyl phosphate at atmospheric pressure.

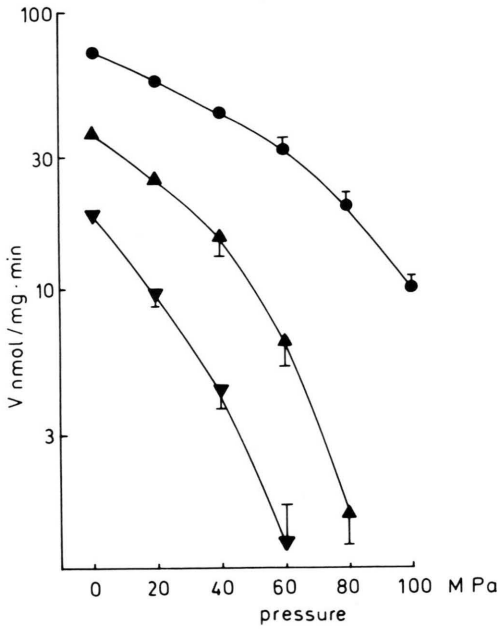


Fig. 6. Pressure induced inhibition of the calcium dependent *p*-nitrophenyl phosphate hydrolysis by the sarcoplasmic calcium transport enzyme at saturating substrate and at nonsaturating calcium concentrations. The assay media are described in "Materials and Methods" and contained magnesium and *p*-nitrophenyl phosphate to give 6 mM Mg-*p*-nitrophenyl phosphate and 10 mM ionized magnesium. 0.2 mM calcium were applied as saturating concentration (●). Subsaturating calcium concentrations were adjusted by adding 1.0 mM MgEDTA (▲) and 1.5 mM MgEDTA (▼). The prevailing free calcium concentrations were 1 μM and 0.6 μM respectively. The profiles are obtained from 5 experiments.

Discussion

The reevaluation of the interaction of magnesium and *p*-nitrophenyl phosphate with the calcium transporting enzyme of the sarcoplasmic reticulum has enabled us to redetermine the volume change the fully saturated enzyme undergoes and to separately measure the volume changes connected with the binding of calcium and magnesium *p*-nitrophenyl phosphate to the enzyme. The analysis of the pressure effect on the enzyme's activity in a previous study depending on the premise that the enzyme was saturated in the presence of 10 mM magnesium and *p*-nitrophenyl phosphate each [15]. This assumption relied on the

observation that in double reciprocal plots of enzyme activity versus total concentrations of magnesium or *p*-nitrophenyl phosphate straight lines were obtained which intersect on the abscissa to the left of the origin [31]. If the concentrations of the ligands remain below 10 mM we assumed that Mg-*p*-nitrophenyl phosphate formation could be neglected and that magnesium and *p*-nitrophenyl phosphate interact with the enzyme in random fashion. Doubts concerning this assignment arose when we observed that in the presence of activity modifiers such as dimethylsulfoxide other activity concentration relations seem to prevail (unpublished results). Furthermore when the activity measurements were performed at higher concentration of magnesium and *p*-nitrophenyl phosphate deviation from linearity in the double reciprocal plot occured. These inconsistencies could be resolved by considering Mg-*p*-nitrophenyl phosphate to be the true substrate for the enzyme.

Half saturation of the enzyme with Mg-*p*-nitrophenyl phosphate is obtained at a concentration of 3 mM which requires the application of quite high concentration of magnesium and *p*-nitrophenyl phosphate to approach saturation. The anticipated effect of pressure on the concentration of Mg-*p*-nitrophenyl phosphate could fortunately be neglected since the volumetrically determined reaction volume turned out to be much smaller for Mg-*p*-nitrophenyl phosphate than for most other magnesium complexes tested.

The pressure dependent activation volume at saturating substrate concentration rising from 20 ml/mol to approximately 80 ml/mol reflects the fact that the reaction chain of the calcium dependent *p*-nitrophenyl phosphate hydrolysis is composed of a series of reaction steps. The corresponding rate constants must differ considerably in their activation volumes.

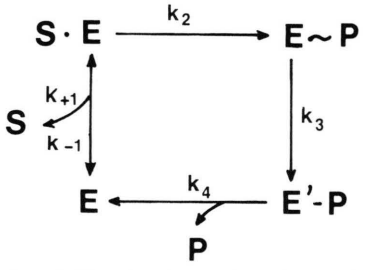


Fig. 7. Simplified reaction scheme for the sarcoplasmic calcium transport enzyme. The transition between the reaction intermediates E′ and E is included in reaction step 4.

From the simplified scheme of the reaction cycle (Fig. 7) containing only four steps – the following expression is obtained for the dependence of the unidirectional forward reaction rate on the concentration of one substrate *i.e.* the concentration of Mg-*p*-nitrophenyl phosphate at saturating calcium concentrations.

$$v = \frac{E_o \cdot k_1 \cdot k_2 \cdot k_3 \cdot k_4 \cdot S}{k_1 \cdot S\,(k_2 \cdot k_3 + k_2 \cdot k_4 + k_3 \cdot k_4) + k_3 \cdot k_4\,(k_{-1} + k_2)} \quad (2)$$

whereby the pressure dependence of the rate constants is given by:

$$k_n = \bar{k}_n \cdot \exp.\left(\frac{\Delta V_n^* \cdot P}{RT}\right);$$ k_n are the rate constants at atmospheric pressure.

A corresponding relation can be deduced for the dependence of the reaction rate at saturating concentration of Mg-*p*-nitrophenyl phosphate on the concentration of calcium, assuming that all calcium containing intermediates carry two calcium ions.

If saturating substrate concentrations are applied the resulting rate expression only contains three pressure dependent terms k_2, k_3 and k_4.

$$v_p = \frac{E_o}{\sum_{n=2}^{4} 1/\bar{k}_n \cdot \exp.\ \frac{\Delta V_n^* \cdot p}{RT}} \quad (3)$$

v_p = reaction rate at pressure p.

In Table III activation volumes and normalized rate constant are collected which were obtained by the application of this expression to the activity pressure relations shown in Fig. 5a, b for the highest substrate concentrations. In our previous study performed at Mg-*p*-nitrophenyl phosphate concentrations ~ 1 mM deviations from the log activity relation from linearity could not be ascertained and an activation volume of 30 ml was computed at 30 °C [15]. This finding fairly agrees with the results of this study also yielding straight lines at low substrate concentration for most of the log activity relations. The limiting apparent activation volume derived from Fig. 5a amounts to 35 ml/mol between 0.1 and 60 MPa. A constant activation volume at low substrate concentration could be expected under these conditions if the constant which describes the decay of the enzyme substrate complex (*cf.* Eq. (5)) is governed by one term yielding a pressure independent activation volume even if it contained different volume contributions.

Table III. Reaction volumes and normalized rate constants. The rate constants and volumes were introduced into Eqs (3), (4) and (6) to fit the activity pressure relation shown in Fig. 5 and Fig. 6 respectively.

		Relative rate constants			Reaction volumes		
Conditions					[ml/mol]		
		k_2	k_3	k_4	ΔV_2^*	ΔV_3^*	ΔV_4^*
Saturating ligand concentration	10 mM Mg^{2+}	2.6	2.3	1	20	80	−10
	30 mM Mg^{2+}	5.0	3.0	1	20	80	−10
		k_1		$K\,k_2$	ΔV_{+1}^*		$\Delta V_2^* + \Delta V_K$
Nonsaturating ligand concentration	low Mg-*p*-NPP*	20		1	100		35
	low Ca^{2+}	30		1	200		85

[a] At 10 mM Mg^{2+}.

This would be the case if the rate constant k_2 for the processing of the enzyme substrate complex is much smaller than the rate constant k_{-1} for its dissociation. The rate equation would reduce to

$$v = \frac{E_o \cdot k_1 \cdot k_2 \cdot S}{k_{-1} + k_2} \rightarrow E_o \cdot K \cdot k_2 \cdot S; \qquad k_{-1} \gg k_2;\ K = k_1/k_{-1} \tag{4}$$

$$\frac{v_p}{v_o} = \exp.\left(-\frac{(\Delta V_K + \Delta V_2^*) \cdot P}{RT}\right) = \exp.\left(-\frac{\Delta V \cdot P}{RT}\right) \tag{5}$$

Below 60 MPa this relation appears to be valid. At pressure values above 60 MPa, however, deviation from activity pressure relation starts to deviate from linearity indicating that the condition $k_{-1} \gg k_2$ introduced above is not fulfilled. With $k_{-1} \sim k_2$ an expression can be derived from Eq. (4) containing an additional pressure depending term.

$$\frac{v_p}{v_o} = \frac{\bar{K} \cdot \bar{k}_2 + \bar{k}_1}{\bar{k}_1 \cdot \exp.\frac{\Delta V_K + \Delta V_2^*}{RT} P + \bar{K} \cdot \bar{k}_2 \cdot \exp.\frac{\Delta V_1^* \cdot P}{RT}} \tag{6}$$

The activity pressure relation at low substrate concentration (Fig. 5a) can well be fitted with the reaction volumes and the normalized rates given in Table III (Fig. 8). A new and quite large activation volume V_{+1} related to the on-rate of Mg-*p*-nitrophenyl phosphate binding has to be taken into account. This large activation volume contrasts to the small reaction volume observed for equilibrium binding of Mg-*p*-nitrophenyl phosphate (/). Since the binding volume is defined as the difference between the activation volumes for *p*-nitrophenyl phosphate binding (ΔV_{+1}) and dissociation (ΔV_{-1}), the latter must be only little smaller than V_1. The large activation volumes in conjunction with relatively small rate constants for k_1 indicate that the reactive enzyme substrate complex is formed not in just one but rather in a series of reaction steps.

At high pressure values the binding volume for Mg-*p*-nitrophenyl phosphate vanishes (Table II). This is qualitatively in line with the fact that the constant describing Mg-*p*-nitrophenyl phosphate binding

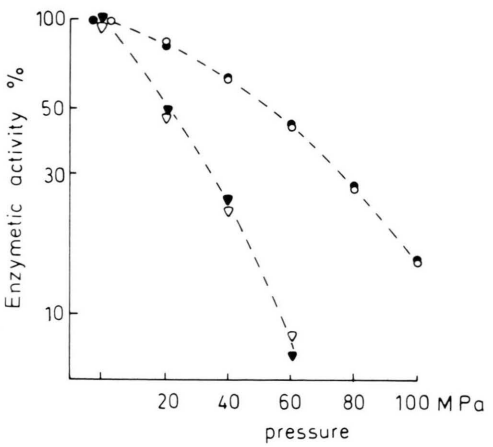


Fig. 8. Comparison of experimental and fitted relations between enzymatic activity and pressure. Eq. (3) was applied to fit the activity-pressure relation of the saturated enzyme (Fig. 5a) ○, ●. Eq. (2) was used to fit the activity pressure relation of the enzyme at subsaturating concentrations of calcium (Fig. 6) ▽, ▼. Closed symbols ●, ▼, experimental data; open symbols ○, ▽; values obtained by fitting.

is not sufficiently defined by $K_m = k_1/k_{-1}$ but includes contribution from the catalytic cycle. Hence a pressure dependent term appears in conjunction with the dissociation volume [19].

The activation of *p*-nitrophenyl phosphate hydrolysis by ionized calcium furnished an astonishing low apparent calcium affinity in agreement with results of Holguin [32]. It is considerably lower than the affinities observed either in binding studies or derived from calcium dependent ATP hydrolysis (*cf.* [33]). The different affinities the enzyme display for calcium can be explained if we assume that the enzyme resides in a state of high affinity (E) when it is either inactive or energized by ATP while during *p*-nitrophenyl phosphate supported hydrolysis the low affinity state (E′) from which calcium has been released during the reaction cycle prevails (*cf.* [4]). A value of 3 for the equilibrium between the two conformational states E′/E can account for the observed affinity shift. This equilibrium constant is considerably smaller than that derived by Tanford *et al.* [34] to explain calcium binding under equilibrium conditions. The low apparent affinity of the enzyme for calcium ions allowed us to adjust sub-saturating calcium concentration with the magnesium EDTA buffer system.

From the pressure dependence of *p*-nitrophenyl phosphate hydrolysis at sub-saturating calcium concentrations the reaction volume for calcium binding of 67 ± 5 ml/mol between 0.1 and 40 MPa has been obtained (Table II). Contaminations by effects which pressure might excert on the calcium buffer itself have been avoided by choosing magnesium EDTA/EDTA as buffer system which is nearly pressure independent. This is in contrast to the pressure sensitive stability constant of calcium EGTA which, for instance, gives rise to a 1.5-fold increase of the free calcium concentration when 50 MPa are applied. This counteracts the pressure dependent reduction of the enzyme's calcium affinity and thus diminishes the apparent binding volume [15]. If we consider that two calcium ions might completely loose their hydration water when they are bound to the enzyme a volume increase of approximately 40 ml/mol can be expected [35]. The observed volume increase of 67 ml/mol indicates that an additional volume expansion must take place. The enzyme might either loose hydration water or it might increase its internal volume [36]. The activity pressure profiles obtained at sub-saturating calcium concentrations are not only very steep, but they also considerably increase with pressure values above 40 MPa. The reaction volume for calcium binding computed in this pressure range increases from 100 to 160 ml/mol. A discussed for Mg-*p*-nitrophenyl phosphate binding, we have to consider that the rate determining step at sub-saturating calcium level is likewise shifted by pressure. The steep decline of enzymatic activity at low calcium levels and high pressure values indicates that the association of calcium with the enzyme is accompanied by a large volume change of the system. For enzyme activation by low calcium concentration at saturating concentration of Mg-*p*-nitrophenyl phosphate the approximation introduced for low Mg-*p*-nitrophenyl phosphate concentration at saturating calcium levels can be applied although the entry of two calcium ions per reaction cycle is needed (*cf.* Table IV). Fitting of the

Table IV. Absolute rate constants. The derivation of k_2 from rate measurements at subsaturated concentrations of Mg-*p*-nitrophenol phosphate according to Eq. (2): $v = \alpha \cdot E_o \cdot K \cdot L \cdot k_2$ is less affected by the uncertainties associated with the simplifying assumptions on which Eq. (2) is based. K are the ligands affinities as determined in the experiments of Fig. 2 and 4. The total enzyme concentration (E_o) was obtained from phosphorylation experiments which yielded 4 nmol/mg prot. under optimal conditions (*cf.* [6]). The coefficient α accounts for the fact that the ligand reactive form exists in equilibrium with a nonreactive enzyme species [34]. $\alpha = 1/3$ has been deduced from the different calcium affinities displayed by the enzyme when supported by either ATP or Mg-*p*-nitrophenyl phosphate (Fig. 1). This equilibrium is not specified in the simplified reaction scheme (Fig. 7). It is contained in reaction step 4. The measured maximal rate of 0.4 s^{-1} from Fig. 5a, b agrees well with the rate obtained from the constants derived by applying Eq. (6).

Ligands	Rate constants				
	k_1 M^{-1} s^{-1}	k_{-1} s^{-1}	k_2 s^{-1}	k_3 s^{-1}	k_4 s^{-1}
Ca^{2+}	$3-6 \times 10^7$	30–60	(0.5–1.0)*	–	–
Mg-*p*-NPP	7200	24	2.0	1.8	0.8

* Reaction rates at subsaturating concentrations of calcium are difficult to ascertain because enzymatic activity is a highly cooperative function of the calcium concentration (Fig. 2) and enzyme activities at subsaturating concentration of calcium are difficult to obtain. This uncertainty does not only affect the determination of the rate constant k_2 itself but also the transformation of the normalized rate constants (Table III) into absolute rate constants by applying Eq. (6). Yet the values obtained for k_1 (expressed as second order rate constants) and k_{-1} are in good agreement with the estimates reported by Tanford *et al.* [34] and Pickard and Jencks [5], respectively.

activity pressure relation at low calcium concentrations by applying Eq. (6) yielded data for activation volumes and rates given in Table III (Fig. 8). The entry of calcium into the reactive complex is associated with an activation volume of 200 ml/mol which is considerably greater than that for Mg-*p*-nitrophenyl phosphate. This activation volume of the binding step excedes the volume change for equilibrium calcium binding by a factor of three leaving 120 ml/mol for the activation volume of calcium dissociation below 40 MPa and only 50–90 ml/mol above 40 MPa, because of the increasing binding volume. The considerable increase of the binding volume for calcium with rising pressure shows that the calcium binding constant as discussed for Mg-*p*-nitrophenyl phosphate is affected by subsequent reaction steps.

The deduced absolute rate constants for calcium binding and dissociation presented in Table IV are in good agreement with the data reported for the calcium dependent interaction of the enzyme with ATP [6, 34]. Thus the calcium binding characteristic to the enzyme in its high affinity state (E) is not affected by the energy yielding substrate.

The volume change which is connected with calcium translocation is more difficult to disclose because it occurs inside the reaction chain. An assignment can be attempted by considering the normalized rate constants and the activation volumes obtained by approximating the activity pressure relation of the fully saturated enzyme. The application of Eq. (3) yielded three volumes and corresponding normalized rate constants of Table III. The quite simple activity pressure relation can even be fitted by only two pressure dependent rate constants accounting for a three step reaction cycle (not shown). Both fitting attempts have two features in common. First, the negative activation volume of 10 to 20 ml/mol combined with a small rate constant must be introduced to account for the relatively small activation volume found at low pressure values. Second, a positive activation volume of ~ 80 ml/mol combined with a somewhat larger rate constant has to take care of the increase of the activation volume when the pressure excedes 40 MPa. The step with a negative activation volume, being accelerated by pressure, is the slowest step in the reaction chain at atmospheric pressure. It presumably includes hydrolytic removal of the phosphate residue and the transition of the enzyme into its high affinity calcium binding state. The more complete four step reaction sequence yields an additional rather small positive activation volume which might be related to the first step in the processing of the enzyme substrate complex (k_2). This step most likely can be assigned to the transfer of the phosphoryl group from the substrate to the enzyme. In the most simple three step sequence, the latter step is lumped together with k_4. Thus by exclusion the most probable candidate for calcium translocation remains reaction step 3 with an activation volume of ~ 80 ml/mol. The absolute magnitude of k_3 derived from the normalized rate constant and the rates observed at subsaturating substrate concentrations is much smaller than that for the corresponding constants for ATP supported translocation [5, 6, 34]. Evidently calcium translocation is much more accelerated by ATP or ADP than by *p*-nitrophenol phosphate or *p*-nitrophenol. In contrast to the rather indirectly achieved assignment of calcium translocation with the activation volume of reaction (3), the large volume changes connected with calcium as well as Mg-*p*-nitrophenyl phosphate binding could directly be determined. Evidently the volume changes at the start of the reaction cycle when calcium and the energy yielding substrate are bound to the enzyme considerably excede the volume change associated with calcium translocation.

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