Pressure Effects on the Interactions of the Sarcoplasmic Reticulum Calcium Transport Enzyme with Calcium and Dinitrophenyl Phosphate

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The effect of hydrostatic pressure on the calcium-dependent hydrolysis of dinitrophenyl phosphate by the sarcoplasmic calcium transport enzyme has been studied. The magnesium dinitrophenyl phosphate complex is the true substrate of the enzyme (K = 7000 M⁻¹) by which it is hydrolyzed at 20 °C with a turnover rate of 4 s⁻¹. Activation by calcium ions occurs between 0.1 and 1 μM as observed for ATP hydrolysis. The activation volume of the enzyme saturated with both ligands exhibits pronounced pressure-dependence, rising from 25 ml/mol at atmospheric pressure to 80 ml/mol at 100 MPa. The apparent binding volumes for magnesium dinitrophenyl phosphate and calcium are likewise pressure-dependent. The volume changes connected with the binding of magnesium dinitrophenyl phosphate is quite small approaching zero at 100 MPa. The apparent binding volume for calcium greatly increases with pressure from 35 ml/mol at atmospheric pressure to 150 ml/mol at 70 MPa. A nearly constant binding volume of approximately 40 ml/mol results if the effect of pressure on the respective rate constants that contribute to the apparent binding constant, is taken into account. The pressure-dependence of enzyme activity at subsaturating calcium concentrations yields an activation volume of 250 ml/mol related to the rate of calcium binding indicating the occurrence of a transient large volume expansion of the enzyme complex. The volume changes observed for the calcium-dependent interaction of the enzyme with magnesium dinitrophenyl phosphate well agree with that found for magnesium p-nitrophenyl phosphate (W. Hasselbach and L. Stephan, Z. Naturforsch. 42e, 641–652 (1987)) indicating that the found volume changes are intrinsic properties of the transport enzyme, independent of the respective energy donor.

Introduction

We have recently described the effect exerted by hydrostatic pressure on the calcium-dependent hydrolysis of p-nitrophenyl phosphate by the sarcoplasmic reticulum calcium transport protein [1, 2]. For two reasons p-nitrophenyl phosphate was used as substrate of the transport enzyme.

1. p-Nitrophenyl phosphate is a pseudo-substrate of the sarcoplasmic reticulum calcium pump, which calcium-dependently supports a slow calcium transport [3, 4].

2. The colour development resulting from p-nitrophenyl phosphate hydrolysis allows directly to monitor enzyme activity in the pressure cell.

Like temperature, pressure dependence of enzymatic reactions can yield interesting information on various reaction parameters. Yet the volume changes resulting from the pressure dependence of the reaction velocity are composites of a number of different molecular events. In spite of this inherent limitation the volume changes can be assigned to specific reaction steps, provided, that the reaction conditions are appropriately chosen.

In the preceding paper [1] we have deduced 1) activation volumes from measurements near substrate saturation and 2) binding volumes for the two ligands, calcium ions and magnesium p-nitrophenyl phosphate. The latter were obtained by comparing the effect of pressure on enzyme activity exhibited by the saturated and the unsaturated enzyme. Most remarkably we found that the activation volumes for the saturated enzyme was not constant, but increased from 20 to 80 ml/mol with pressure. As to the binding volume of the two ligands, the binding of Mg-p-nitrophenyl phosphate was accompanied by only a small volume change, while calcium binding gives rise to a substantial pressure-dependent volume increase of the system from 60 ml to 120 ml at 100 MPa.

If the deduced reaction volumes, in fact, represent intrinsic properties of the enzyme mechanism, one should expect that these parameters do not depend...
on the energy yielding substrate. We, therefore, have applied in this study dinitrophenyl phosphate which is 10 times more rapidly consumed by the enzyme than p-nitrophenyl phosphate and which also effectively supports calcium transport [5, 6]. It is shown here that the reaction volumes obtained for calcium-dependent dinitrophenyl phosphate hydrolysis well agree with the volumes found for calcium-dependent p-nitrophenyl phosphate hydrolysis, thus demonstrating their direct association with the reaction mechanism.

Materials and Methods

Enzyme preparation

The sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as described by de Meis and Hasselbach [7].

Enzyme assays

Dinitrophenyl phosphatase activity was measured in media containing 0.1 M NaCl, 6 mM histidine, 14 mM sodium glycerophosphate pH 7.0; 0.1 M sucrose, 1 μM of the calcium ionophore A 23187 and 2 mM dithioerythritol. The concentration of the reactants, dinitrophenyl phosphate, magnesium and calcium are specified in the legends of figures and tables. The concentrations of the vesicles in the assay media were chosen between 0.01 mg/ml and 0.05 mg/ml depending on the enzyme activity under the respective conditions. T = 20 °C.

For measuring the dependence of dinitrophenyl phosphate hydrolysis on calcium, magnesium and dinitrophenyl phosphate at atmospheric pressure the reaction was terminated with sodium dodecylsulfate, 1% final concentration, at appropriate times. Activity was calculated by using an \( \varepsilon_{412} \) value of 6600 m\(^{-1}\) cm\(^{-1}\), determined with 2,4-dinitrophenol under the same conditions.

Pressure application

The pressure was stepwise applied to the reaction medium in a pressure cell from Nova Swiss, Effretikon, Switzerland. The reaction was followed by monitoring the appearance of dinitrophenol at 412 nm. The effect of pressure on the calcium-independent enzyme activity was measured in the presence of 2 mM EGTA. The calcium-independent activity was subtracted 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 dinitrophenol proved to be pressure-independent [1]. The stability constants of the respective calcium and magnesium complexes of EGTA and EDTA were taken from Schwarzenbach [8] and used according to Blinks et al. [9]. Magnesium binding to dinitrophenyl phosphate was determined at pH 7.0 with a divalent cation-sensitive electrode (Orion Research Cambridge, Mass., U.S.A.) at 20 °C and a stability constant of 30 m\(^{-1}\) was obtained.

Determination of activation and reaction volumes

Activation volumes \( \Delta V^* \) were evaluated according to the relation:

\[
\Delta V^* = \frac{R \cdot T}{P} \cdot \ln \frac{v_p}{v_o}.
\]  

(1)

\( v_o \) and \( v_p \) are the rates observed at \( p \) (MPa) and atmospheric pressure, respectively. \( R \) is the gas constant 8.3 ml MPa/°K·mol. Activation volumes were obtained from the slope of the activity pressure profiles \( \log v \) versus pressure. For non-linear \( \log v \)-pressure relations, activation volumes were derived from the \( \log v \) increments of successive pressure values. The binding volume of a ligand was deduced from the apparent activation volume according to the following relation (cf. [8]).

\[
\Delta V^* = \Delta V_A^* + \frac{1}{1 + K_m \cdot L} \cdot \Delta V_B.
\]  

(2)

\( V_A \) is the activation volume observed at saturating ligand concentrations; \( \Delta V^* \) is the activation volume when the enzyme is only partly activated at non-saturating ligand concentration. \( \Delta V_B \) is the binding volume for the corresponding ligand, \( L \). \( K_m \) represents the apparent affinity constant of the enzyme for the ligand and must be considered to be pressure-dependent. The term \( (1 + K_m \cdot L) \) in Eqn. (2) corresponds to the non-active enzyme fraction, the magnitude of which can directly be obtained from the enzyme activities measured at different enzyme saturation and pressure values. Reaction volumes and relative rate constants were estimated by manual approximation and by using a computer program (Enz-
Reagents

Lutidine dinitrophenyl phosphate was synthesized by Mr. H. Gaugler according to [10] in our laboratories. A 23187 was purchased from Calbiochem GmbH, Frankfurt, F.R.G., other reagents were bought from E. Merck, Darmstadt, F.R.G., Serva, Heidelberg, F.R.G. and Sigma, Deisenhofen, F.R.G.

Results

In order to assign the observed reaction volumes to either ligand binding steps or transformation steps in the reaction sequence, the respective ligand species as well as the dependence of the reaction rate on the ligand concentration should be known [9]. Fig. 1 illustrates the activation of the dinitrophenyl phosphate hydrolysis by rising calcium concentrations at high concentrations of Mg-dinitrophenyl phosphate. Activation becomes discernible at 0.1 μM and reaches its maximum at 1 μM. Thus the hydrolysis of dinitrophenyl phosphate is activated at significantly lower calcium concentration than that of p-nitrophenyl phosphate. The concentration range in which dinitrophenyl phosphate hydrolysis is activated by calcium coincides with that of ATP hydrolysis. The steep activation profile of calcium-dependent dinitrophenyl phosphate splitting gives rise to a Hill coefficient of 2.1, like that found for calcium-dependent ATP hydrolysis, which is, however, significantly greater than that observed for the calcium-dependent $p$-nitrophenyl phosphate hydrolysis [1, 11, 12]. The basic splitting of dinitrophenyl phosphate measured in the presence of 2 mM EGTA amounts to approximately 10% of the maximal observed splitting rate.

At saturating concentration of calcium (0.2 mM) in the presence of 10 mM magnesium, the enzyme activity reaches high values already at a dinitrophenyl phosphate concentration as low as 0.5 mM. Fig. 2a illustrates in a double reciprocal plot the dependence of the hydrolysis of dinitrophenyl phosphate on its concentration at different constant concentrations of magnesium. At these low concentrations of both magnesium and dinitrophenyl phosphate, there is no significant difference between total and free concentrations of magnesium and dinitrophenyl phosphate.

The common intercept obtained at different magnesium concentrations, when the concentration of dinitrophenyl phosphate is changed and vice versa, indicates that the enzyme uses the complex Mg-dinitrophenyl phosphate as substrate. Fig. 2b shows the enzymatic activity related to the concentration of Mg-dinitrophenyl phosphate. The latter is calculated using 30 mM as dissociation constant of the complex. The enzyme activity follows simple Michaelis-Menton behaviour giving a dissociation constant of 0.14 mM for the enzyme substrate complex and a maximal activity of 1000 nmol/mg·min. This extrapolated rate yields a turnover number of 4 s$^{-1}$ assuming an active site concentration of 4 nmol/mg (cf. [1]).

The rate with which the calcium transport enzyme hydrolyses dinitrophenyl phosphate at 20 °C, and at saturating concentration of calcium and 0.5 mM magnesium dinitrophenyl phosphate, declines with rising pressure from 500 nmol/mg·min at 0 bar to 50 nmol/mg·min at 100 MPa. The effect of pressure on enzyme activity was measured by increasing the pressure in steps of 20 MPa. At each pressure, colour development was monitored for 2–5 min. At the
Fig. 2. a. Dependence on magnesium and dinitrophenyl phosphate of calcium activated substrate cleavage. The reaction medium given in Fig. 1 contained 0.2 mM calcium. Data are presented in double reciprocal plots of enzyme activity versus the concentrations of added dinitrophenyl phosphate at (•) 0.74 mM, (■) 1.1 mM and (▲) 2.2 mM magnesium.

b. Double reciprocal plot of enzyme activity versus the concentration of Mg-dinitrophenyl phosphate obtained from a. using 30 M⁻¹ as stability constant.

Fig. 3. Effect of pressure on the fully activated calcium dependent and calcium independent dinitrophenyl phosphate hydrolysis. The assay media given in Materials and Methods contained 1 mM magnesium-dinitrophenyl phosphate and either 0.2 mM calcium (•·•·•) or 2 mM EGTA (■—■). Calcium dependent activity corrected for irreversible activity loss occurring during pressure application (•·•·•). † recovery of activity at the end of the pressure cycle.

end of the cycle, pressure was released. Up to a pressure of 30 MPa the enzyme activity fully recovers. At higher pressure values, recovery becomes gradually incomplete falling to 70% after the application of 100 MPa. The dashed line in Fig. 3 shows the decline of enzyme activity with increasing pressure as observed in 11 experiments. The standard error of the means increases from 2% at 0 bar to 6% at 100 MPa. The smooth line is obtained when the experimental values were corrected for inactivation. The graphs of the data show that the relation between log ν and pressure is certainly not linear. The activation volume obtained from the slope of the curve thus increases from 22 ml to 78 ml/mol. An identical increase of the activation volume has been observed when the temperature is lowered to 10 °C (not shown) and reported for the much more slowly proceeding hydrolysis of p-nitrophenyl phosphate by the enzyme likewise measured under saturating conditions [1]. In contrast to the calcium-dependent, the calcium-independent hydrolysis of dinitrophenyl phosphate displays a much smaller and pressure-independent activation volume of 15 ml/mol. In order
to deduce the volume changes connected with substrate binding the effect of pressure on enzymatic activity at subsaturating concentrations must be related to the activity of the saturated enzyme [9]. Fig. 4 shows that the log v-pressure relation does not essentially change when at 0.2 mM calcium the concentration of magnesium dinitrophenyl phosphate is reduced from 0.5 mM to 0.02 mM. At low pressure values the curvature of the log v-pressure relation is less marked. In the lower pressure range the slope of the relation is somewhat greater for the unsaturated than for the saturated enzyme. In the higher pressure range > 50 MPa the log v-pressure relation becomes nearly identical for the three curves, indicating that the binding of magnesium dinitrophenyl phosphate is linked with only relatively small volume changes.

The suboptimal calcium concentrations required for the evaluation of the volume changes linked to calcium binding were adjusted as previously described by Ca-Mg EDTA as Ca²⁺ buffer which largely avoids pressure-dependent changes of the calcium concentration [1].

In Fig. 5 the pressure effect on enzyme activity at two subsaturating calcium levels is compared with the activity profile of the saturated enzyme. The activities of the subsaturated enzyme much more steeply decline with rising pressure than that of the saturated enzyme. Identical activity profiles of increasing steepness have been observed at 10 °C. The apparent activation volumes thus increase from 50 ml to 250 ml/mol. If Eqn. (2), which allows the evaluation of the apparent binding volume, is applied to successive pressure intervals of 20 MPa, values of

![Fig. 4. Effect of pressure on calcium dependent dinitrophenyl phosphate hydrolysis at different concentrations of magnesium dinitrophenyl phosphate and 0.2 mM calcium. The profiles were obtained from 5 separate experiments. The bars indicate SEM. The reaction mixtures given in Materials and Methods contained (●) 10 mM magnesium, (▲) 2 mM magnesium, (■) 0.6 mM magnesium and 1 mM dinitrophenyl phosphate.](image)

![Fig. 5. Effect of pressure on calcium-dependent dinitrophenyl phosphate hydrolysis at different calcium concentrations and 0.5 mM magnesium dinitrophenyl phosphate. 0.2 mM calcium were applied as saturating concentration (●). Subsaturating calcium concentrations were adjusted by adding 1.5 mM Mg EDTA (▲) and 2.5 mM Mg EDTA (■) to the reaction media containing 0.05 mM calcium. (SEM of 5–10 experiments is indicated by the bars.)](image)
the binding volume are obtained which increase from 50 ml to 180 ml/mol. If these apparent binding volumes are plotted versus the respective pressure values, a linear relationship results (Fig. 6).

Discussion

The results of the present study demonstrate that the activation and binding volumes linked to the activity of the sarcoplasmic reticulum calcium transport system are intrinsic properties of the enzyme. Nearly identical volume changes were obtained which are connected with the calcium-dependent hydrolysis of dinitrophenyl phosphate and p-nitrophenyl phosphate, although the enzyme has a much higher affinity for di- than for p-nitrophenyl phosphate, and, furthermore, dinitrophenyl phosphate is hydrolyzed much more rapidly than p-nitrophenyl phosphate. All observed reaction volumes have the interesting common feature that, they exhibit pronounced pressure dependence. This pressure dependence of the activation volumes can be accounted for by assuming that at least two consecutive steps in the reaction chain are differently affected by pressure [9]. Hence, the following relation was used for assigning the two activation volumes and the two rate constants by using a computer program for the fitting procedure.

$$E_0 \frac{\exp \left( \frac{\Delta V_m^* \cdot P}{R \cdot T} \right)}{v} + \frac{\exp \left( \frac{\Delta V_n^* \cdot P}{R \cdot T} \right)}{k_m}$$

In the Table the resulting parameters, which fit the pressure dependence of the calcium-dependent hydrolysis of the two substrates under different conditions are compiled. The data for p-nitrophenyl phosphate are taken from our recently published measurements [1]. The data for the activation volumes obtained by the computer-supported fitting procedure are in general agreement with the reported volume data obtained by manual fitting.

Under saturating conditions for calcium and both nitrophenyl phosphates, the pressure dependence of substrate hydrolysis between 20 and 30 °C is determined by activation volumes of ~ 20 ml/mol in the lower pressure range, while in the higher pressure range, 5–6-fold larger activation volumes become predominant. Comparable volume changes were found for dinitrophenyl phosphate hydrolysis at 10 °C. The rather different pressure-independent terms $k_m^\prime$, $k_n^\prime$ account for the large difference in the maximal reaction velocity of the two substrates. A factor of 3.75 has to be taken into account for the difference of 10 °C in reaction temperature. In contrast to the activation volumes, the pressure-independent rate terms exhibit a marked temperature dependence. It is much larger for the rate constant related to the large than for the small activation volume (Table).

At subsaturating concentrations of both phosphate donors the activation volumes become less pressure-dependent as indicated by the straighter log v-pressure relations. A relatively good approximation can be obtained by a single exponential expression for fitting. The derived activation volumes are not significantly
Table. Reaction volumes and rate constants of calcium dependent dinitrophenyl phosphate and p-nitrophenyl phosphate hydrolysis.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Reaction volumes</th>
<th>Rate constants</th>
<th>Rate constants</th>
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<td></td>
<td>$\Delta V_m^*$</td>
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<td>Rate constants</td>
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<td>[ml/mol]</td>
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<td></td>
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<td></td>
<td>10 °C 106</td>
<td>20</td>
<td>2.8</td>
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<tr>
<td>Non-saturating Mg-p-NPP substrate concentrations</td>
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<td>(12); 20</td>
<td>$k_{+1}/k_2 \cdot K$</td>
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<tr>
<td>Mg-DiNPP</td>
<td>20 °C 108</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>Mg-DiNPP Ca$_{2+}^+$ = 0.6 mm</td>
<td>20 °C (200); 240 (85); 57</td>
<td>(30); 38</td>
<td>$4 \times 10^5$</td>
</tr>
<tr>
<td>Mg-DiNPP Ca$_{2+}^+$ = 0.2 mm</td>
<td>20 °C (250); 278 (43); 34</td>
<td>(25); 42</td>
<td>$10^6$</td>
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Reaction volumes were obtained from the respective activity pressure relations by either manual (in brackets) or computer supported data fitting. The rate constants ($k'$) were calculated from the rates measured at saturating and non-saturating ligand concentrations together with the ratios of the preexponential terms $k_{+1}/k_2$ and $k_{+1}/k_2 \cdot K$ respectively by applying either Eqn. (3) or Eqn. (5). The rate constants $k'$ for dinitrophenyl phosphate hydrolysis were calculated by using 4 nmol/mg as active site concentration. The rate constants for p-nitrophenyl phosphate hydrolysis were estimated with active site concentrations of either 4 nmol/mg or 2 nmol/mg ($a$) accounting for our previous assumption that the ligand reactive enzyme-form only constitutes a constant fraction of the total enzyme [1]. The data for p-nitrophenyl phosphate hydrolysis are taken from our previous report [1]. To compare the rates at different temperatures an activation energy of 21,000 cal/mol must be considered for both substrates (unpublished observations).

different (Table). They result from the product of two pressure-dependent terms $k_2$ and $K$ yielding the sum of the binding volume of the substrate and the activation volume of the following rate limiting step. This simple assignment is applicable, if at low substrate concentration the rate of hydrolysis can be described by Eqn. (4) where pressure dependence is indicated by bars.

$$ v = E_o \cdot \bar{K} \cdot \bar{k}_2 \cdot S; $$

$$ v_p = E_o \cdot K \cdot k_2 \cdot S \cdot \exp(\Delta V_{+1}^* + \Delta V_k) \cdot P/RT \tag{4} $$

This approximation results from the scheme

$$ E + S \overset{\bar{k}_{+1}}{\longrightarrow} ES \overset{\bar{k}_2}{\longrightarrow} p $$

under the assumption that $\bar{k}_{+1} \gg \bar{k}_2$; and $\bar{K} = \bar{k}_{+1}/\bar{k}_{-1}$. However the well discernible deviation from linearity of the log $v$-pressure relation indicates that the assumption $\bar{k}_{-1} \gg \bar{k}_2$ is not quite fulfilled. Without this limitation an expression containing two exponentials is obtained, which was used for fitting

$$ E_o \cdot S \cdot \frac{\exp(\Delta V_{+1}^* \cdot P)}{R \cdot T} \cdot \frac{\exp(\Delta V_{+1}^* + \Delta V_k) \cdot P}{k_{+1} \cdot K} \tag{5} $$

Evaluation by hand and computer yielded two activation volumes for each of the two substrates which respectively well coincide. The activation volumes related to the on-rate of substrate binding $\Delta V_{+1}^* \sim 100$ ml/mol are three to six times larger than the volume changes connected with the following reaction step which is the composite of two reaction volumes $(\Delta V_{+1}^* + \Delta V_k)$. From the ratio of the pressure-independent constants $k_{+1}/k_2 K$ together with the rates at atmospheric pressure, the known affinity constant $K$, the respective substrate concentrations $S$ and the active site concentrations, first and second order rate constants were deduced. The $k_{+1}'$ values for both substrates are quite small and do certainly not represent diffusion controlled reaction rates. Dinitrophenyl phosphate is evidently more efficient in forming the initial transport complex than p-nitrophenyl phosphate as indicated by the 1000 times...
greater rate constant \( k'_{+1} \). The values for the first order rate constants derived from the measurements performed at low substrate concentration quite well agree with the values of the slow reaction step obtained near substrate saturation. The two reaction volumes \((\Delta V^2 + \Delta V_k)\) are difficult to separate.

The course of the log \( v \)-pressure profile at sub-saturating calcium concentration clearly differs from that obtained at low magnesium dinitrophenyl phosphate levels. The slopes are much steeper and they are markedly curved. Consequently the biexponential rate expression is dominated by a larger activation volume for the on-rate of calcium binding \( k'_{+1} \). Activation volumes of this magnitude have only been reported recently for the permeation of potassium ions through erythrocyte membranes [14]. The apparent volume changes of 240–280 ml/mol at low calcium levels indicate that the loss of hydration water from calcium can account only for a small fraction of approx. 40 ml of the volume change occurring when two calcium ions enter the active complex (comp. below). We, therefore, must assume that prior to the binding of the calcium ions an expanded transition complex must be formed. The expansion should subsequently be followed by a considerable constriction to account for the difference between the activation volume for the binding step and the binding volumes of 40 ml. Figures for the pressure-independent constants have been deduced as described for the binding of the nitrophenyl phosphate. The on-rates for calcium binding are quite high in agreement with previous estimates [13, 14]. Yet they appear to depend on the respective substrate, which indicates that the measurements at low calcium concentrations did not lead to a complete separation of the on-rate from the subsequent reaction steps.

The first order rate constants \( k_{+2} \), derived from the second term of Eqn. (5), exhibits the same substrate specific differences as observed at saturating calcium concentrations. Yet they are by a factor of 2 smaller than those observed at saturating calcium concentrations. The reason for this deviation presumably lies in the simplifying assumptions used to analyse the rather complex calcium binding sequence. The volume changes connected with substrate and calcium binding have been deduced from the apparent activation volumes determined at saturating and non-saturating substrate concentrations using the relation for successive pressure intervals of 20 MPa.

The binding volumes obtained by the application of this relation are apparent figures. That is because the true binding constants \( K \) appears in combination with various rate constants, depending on the assumed reaction sequence. The apparent binding volume for dinitrophenyl phosphate is relatively small in the lower pressure range and becomes insignificant above 40 MPa. For \( p \)-nitrophenyl phosphate the same behaviour was observed. The apparent binding volume for calcium on the other hand changes in the opposite way with pressure. From a value of approx. 40 ml/mol at 10 MPa it increases up to a value of 190 ml/mol at 70 MPa. In Fig. 6 the observed binding values, including those obtained with \( p \)-nitrophenyl phosphates, plotted linearly versus pressure, yield an apparent linear relationship.

This steep increase of the binding volume with pressure can in fact be referred to the effect of pressure on the rate constants which, in combination with the true binding constant \( K \), constitutes the apparent binding constant:

\[
K_{app} = \frac{\dot{k}_{+1} \cdot \overline{K}}{\dot{k}_{+1} + \dot{k}_2 \cdot \overline{K}}.
\]

Size and pressure dependence of \( \dot{k}_1 \) and \( \dot{k}_2 \) can be obtained from the data given in the Table. These data together with the pressure dependence of the apparent binding constant \( K_{app} \) yield values between 35–55 ml/mol, as the volume change connected with the binding step itself. Considering the involved uncertainties the obtained values might be considered to be deviations from a mean binding volume of approx. 40 ml/mol. This value is in accordance with the observed volume changes determined for the chelation of two calcium ions by EDTA or EGTA [1]. The application of a corresponding procedure for the deduction of the true binding volume for magnesium dinitrophenyl phosphate is made difficult by the relatively small and more complex effect of pressure on the binding constant.

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