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# THE ACTIVATION OF RABBIT SKELETAL MUSCLE PHOSPHORYLASE KINASE REQUIRES THE BINDING OF 3 $Ca^{2+}$ PER δ SUBUNIT

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SUMMARY : Rabbit skeletal muscle phosphorylase kinase (EC 2.7.1.38) binds 12 $Ca^{2+}$, i.e. 3 per δ subunit; among these three Ca-binding sites, one has a high affinity ($K_{diss}$ = 0.31 μM) and two have a lower affinity ($K_{diss}$ = 2.15 μM). Thus, the binding of calcium to the enzyme-bound calmodulin, called δ subunit, is different from the binding to free calmodulin. The activation of phosphorylase kinase occurs when three $Ca^{2+}$ are bound to the δ subunit.

Rabbit skeletal muscle phosphorylase kinase has the subunit structure $(\alpha\beta\gamma\delta)_4$ (1). The enzyme activity requires micromolar concentration of calcium, which directly binds to the protein (2). The δ subunit has been shown to be identical with calmodulin (3) and claimed to be the $Ca^{2+}$-binding subunit of the enzyme (4). However there is disagreement about the capacity and affinity of the enzyme for $Ca^{2+}$ (2, 4, 5, 6), apparently because different ionic strengths were used. Moreover a correlation between $Ca^{2+}$-binding to phosphorylase kinase and the $Ca^{2+}$-dependent activity is lacking. Such a relationship was recently worked out in this laboratory for the calmodulin-mediated activation of bovine brain phosphodiesterase (7) and of adenylate cyclase (8). It was of particular interest to investigate this correlation in the instance of phosphorylase kinase as the latter is the only known enzyme which contains calmodulin as an intrinsic subunit.

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Abbreviations: TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N' tetraacetic acid; HEDTA, N'-(2-hydroxyethyl)-ethylenediamine N,N',N'-triacetic acid.

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## MATERIALS AND METHODS

Proteins. Rabbit skeletal muscle glycogen phosphorylase *b* was purified by the procedure of Krebs *et al.* (9) and stored at -20° C in 60 mM TES, pH 7.0, 15 mM mercaptoethanol, 50% (w/v) glycerol.

Non-activated phosphorylase kinase was prepared from rabbit skeletal muscle according to Cohen (10). The specific activity of the enzyme measured as recommended by Shenolikar *et al.* (1), was 10 ± 2 U/mg at pH 8.2, and the activity ratio at pH 6.8 over pH 8.2 was 0.008 ± 0.002 (mean of 5 different preparations). The enzyme (ca 40 mg/ml) was stored at -20° C in 50 mM glycerophosphate, pH 7.0, 2 mM EDTA, 15 mM mercaptoethanol, 50% (w/v) glycerol.

The enzymes were homogeneous as seen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11). Protein concentrations were determined by optical density, using absorption coefficients, $A^{1\%}_{280\ nm}$, of 13.1 for phosphorylase (12) and 12.4 for phosphorylase kinase (10).

$Ca^{2+}$-dependent activity of phosphorylase kinase. The assay mixture (100 µl) included 60 mM TES, pH 7.0, 8 mg/ml phosphorylase *b*, 1.61 µg/ml phosphorylase kinase, 50 µg/ml bovine serum albumin, 4.5 mM mercaptoethanol, 10% (w/v) glycerol 3.6 mM ATP, 12 mM $MgCl_2$. Free $Ca^{2+}$ concentrations were controlled as described below. The reaction was carried out at 30° C and initiated by the addition of ATP and $MgCl_2$. After 5 min, 10 µl aliquots were diluted 20-fold with ice-cold 0.1 M sodium maleate, pH 6.5, 15 mM mercaptoethanol, 1 mg/ml bovine serum albumin, and assayed for phosphorylase *a* according to Hedrick and Fischer (13); 70 µl aliquots were diluted 10-fold with a 0.5% (w/v) EDTA solution (14) to measure the total $Ca^{2+}$ concentration.

$Ca^{2+}$ binding to phosphorylase kinase. The binding was measured by gel filtration according to Hummel and Dreyer (15). A column (31 x 0.9 cm) of Sephacryl S-200 was equilibrated at 4° C with 60 mM TES, pH 7.0, 8.0 mM $MgCl_2$, 1 to 2 µCi/ml $^{45}Ca^{2+}$. The free $Ca^{2+}$ concentrations were controlled as described below. Two to three mg of phosphorylase kinase diluted in 200 µl of the column buffer were applied to the gel and eluted at a rate of about 120 µl/min. Fractions of 1 ml were collected and measured for phosphorylase kinase concentration. The concentration of bound $Ca^{2+}$ was measured by liquid scintillation counting. Neither the specific activity of phosphorylase kinase nor its subunit pattern on sodium dodecyl sulfate-polyacrylamide gel electrophoresis were changed by the chromatography. Total $|Ca^{2+}|$ was measured after a two-fold dilution with bidistilled water. The amount of bound $Ca^{2+}$ was calculated per 1.3 x $10^6$ g protein (10, 16).

The data were analyzed by a nonlinear least-squares curve-fitting procedure with a computer program.

$Ca^{2+}$-concentration measurements. In both $Ca^{2+}$-binding experiments and activity tests, free $|Ca^{2+}|$ was controlled by means of EGTA (1 mM or 0.2 mM) or HEDTA (1 mM or 0.2 mM). Total $|Ca^{2+}|$ was measured by atomic absorption using Titrisol standards (Merck, Darmstadt, Germany) diluted in the same conditions as the sample, i.e. in 0.5% (w/v) EDTA or 9% (w/v) glycerol. Free metal ion concentrations were calculated with a computer program adapted from Perrin and Sayce (17) taking into account the interactions of $H^+$, $Ca^{2+}$ and $Mg^{2+}$ with EGTA, HEDTA and ATP. For these calculations the association constants of Martell and Smith (18) were used for complexes with EGTA, those of Cohen (4) for complexes with HEDTA, and those of Donaldson and Kerrick (19) for complexes with ATP.

The distribution of the complex species $\delta.Ca_n$, incorporated in the holoenzyme, was calculated as a function of free $|Ca^{2+}|$ as previously described (7).

## RESULTS

Calcium dependence of phosphorylase kinase activity. The activation of phosphorylase kinase by free $Ca^{2+}$ occurs in the micromolar range (Fig. 1). The

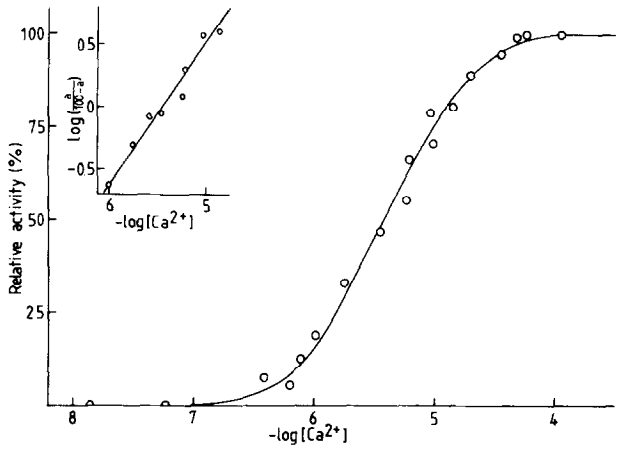


Figure 1. $Ca^{2+}$-dependence of phosphorylase kinase activity. The enzyme activity at 100 μM $Ca^{2+}$ is taken as 100%. The inset represents the corresponding Hill plot ($n_H$ = 1.1).

free calcium concentration at half maximal activation, $|Ca^{2+}|_{0.5}$, is 3.8 ± 0.6 μM (n=6). The enzyme is virtually inactive below 0.1 μM $Ca^{2+}$. The Hill plot of the activation curve yields a coefficient $n_H$ = 1.1 (inset Fig. 1).

Calcium binding to phosphorylase kinase. Phosphorylase kinase binds 12 $Ca^{2+}$ ions per $(\alpha\beta\gamma\delta)_4$ as shown by the saturation curve (Fig. 2). The data could be analyzed in terms of two types of independent sites: 3.9 sites with a dissociation constant ($K_{diss}$) for $Ca^{2+}$ of 0.31 μM and 8.4 sites with a $K_{diss}$ of 2.15 μM. Considering that δ is the calcium-binding subunit of phosphorylase kinase (1), a maximal binding of 3 $Ca^{2+}$ per δ is observed. The existence of a fourth $Ca^{2+}$-binding site with much lower affinity could not be demonstrated because of the aggregation of phosphorylase kinase in buffers containing more than 100 μM $|Ca^{2+}|_{free}$. Hence the calcium-binding properties of calmodulin incorporated as δ subunit in phosphorylase kinase are definitely distinct from those of free calmodulin, which has 3 $Ca^{2+}$-binding sites with a $K_{diss}$ of 6 μM and one with a $K_{diss}$ of 200 μM (7).

Comparison between calcium binding and phosphorylase kinase activity. From the intrinsic binding constants obtained by computer analysis, the three stoichiometric constants were calculated as previously described (7); they

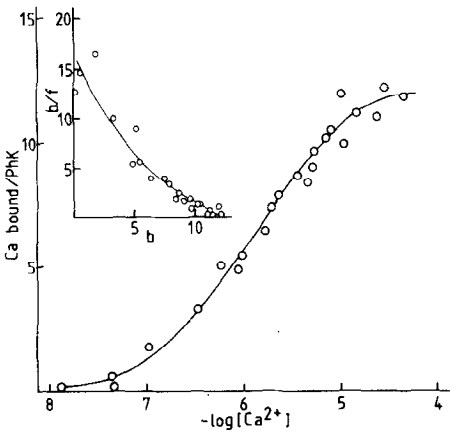


Figure 2. Calcium binding to phosphorylase kinase (PhK) and the corresponding Scatchard plot (inset). The solid lines represent the theoretical curves calculated by computer. b/f is the ratio of bound $Ca^{2+}$ to free $Ca^{2+}$ (μM).

equal 4.18 x $10^{6}$ $M^{-1}$, 7.76 x $10^{5}$ $M^{-1}$ and 2.17 x $10^{5}$ $M^{-1}$ for $K_1$, $K_2$ and $K_3$ respectively. From these constants, the percentage of each $\delta \cdot Ca_n$ species was calculated and compared with the activity (Fig. 3). The best correlation was obtained for the $\delta \cdot Ca_3$ species. The small difference between the two

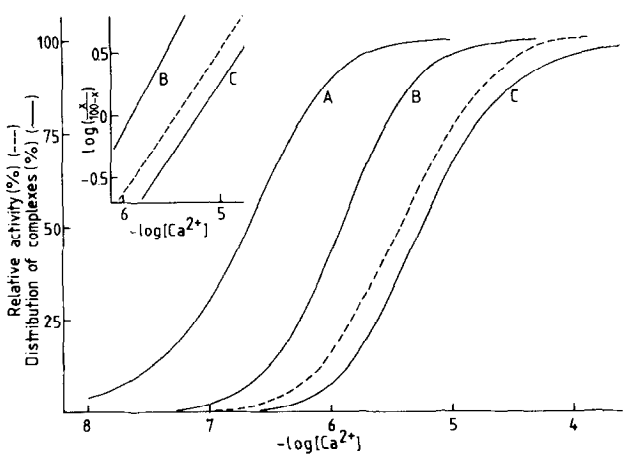


Figure 3. Distribution of the $\delta \cdot Ca_n$ complexes (expressed in percentage of the total amount of $\delta$ as a function of free $Ca^{2+}$ concentration. A is the distribution of $\delta \cdot Ca_1$ + $\delta \cdot Ca_2$ + $\delta \cdot Ca_3$, B of $\delta \cdot Ca_2$ + $\delta \cdot Ca_3$ and C of $\cdot Ca_3$. The dashed curve is the activity of phosphorylase kinase as in Figure 1. The inset shows the Hill plot of B ($n_H$ = 1.4), C ($n_H$ = 1.1), the dashed line the phosphorylase kinase activity ($n_H$ = 1.1).

curves is in the range of the experimental errors. Furthermore there is a good agreement between the Hill coefficients of the calcium-dependent activity ($n_H$ = 1.1) and of the $\delta \cdot Ca_3$ species ($n_H$ = 1.1). Hence it can be concluded that $(\alpha\beta\gamma\delta \cdot Ca_3)_4$ is the active species of phosphorylase kinase.

## DISCUSSION

At the first glance, the data concerning the $Ca^{2+}$-dependence of the enzymatic activity of phosphorylase kinase presented here disagree with those of Cohen (4). The latter author used a Tris-glycerophosphate buffer, pH 6.8, and apprently disregarded the affinities of ATP (19) and glycerophosphate (20) for $Mg^{2+}$, $Ca^{2+}$ and $H^+$. Therefore Cohen's results were recalculated taking into account all the chelates. Instead of 23 µM, a $|Ca^{2+}|_{0.5}$ of 7.3 µM was obtained, which is only two-fold higher than that found in this study where TES buffer, pH 7.0, was used. Considering the differences in the experimental conditions, we feel that both results are not significantly different.

The binding data presented here are not identical to those of Kilimann and Heilmeyer (5), which were obtained at high ionic strength in the presence of $Mg^{2+}$. These authors found 6.8 high affinity $Ca^{2+}$-binding sites ($K_{diss}$ = 0.3 µM) and about 4 sites with a lower affinity ($K_{diss}$ = 2.9 µM). Although the $K_{diss}$ are very similar, the number of $Ca^{2+}$-binding sites within each class is not the same; this discrepancy might have disappeared if a mathematical treatment of the data had been performed. Such an analysis is indeed required when a protein possesses two independent classes of sites with rather close dissociation constants (21).

The difference observed between the $Ca^{2+}$-binding parameters of free calmodulin, which shows the same affinity for its three high affinity sites, and those of the δ subunit of phosphorylase kinase may be due to the interaction of calmodulin with the catalytic γ subunit (22, 23). Differences between the $Ca^{2+}$-binding properties of a free $Ca^{2+}$-binding protein compared to those of the same $Ca^{2+}$-binding protein as part of a protein complex have al-

ready been described for troponin C: the latter also shows an increase in affinity for calcium of one order of magnitude when incorporated in the troponin complex (24).

The activation of phosphorylase kinase by $\delta \cdot Ca_3$ is reminiscent of results obtained in this laboratory for other enzymes: regulation of the $Ca^{2+}$-dependent activities of bovine brain phosphodiesterase (7) and adenylase cyclase (8) is achieved by calmodulin$\cdot Ca_3$.

To conclude, this work shows that calmodulin changes its affinity for calcium when incorporated in phosphorylase kinase as an intrinsic subunit, but still requires the binding of at least 3 $Ca^{2+}$ for the activation of the enzyme.

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