THE PROTEIN KINASE PROPERTIES OF CALSEQUESTRIN

Magdolna VARSÁNYI and Ludwig M. G. HEILMEYER, jr
Institut für Physiologische Chemie der Ruhr-Universität, Universitätsstrasse 150, 4630 Bochum 1, FRG

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1. Introduction

Vesicles of rabbit skeletal muscle sarcoplasmic reticulum contain protein kinase [1–3] which converts glycogen phosphorylase *b* to the *a* form. Antibodies against phosphorylase kinase inhibit the activity of this membrane-associated protein kinase only partially whereas that of soluble phosphorylase kinase completely [3,4].

Isolated sarcoplasmic reticulum from I-strain mice which lack soluble phosphorylase kinase [5,6] contains Ca^{2+}-dependent protein kinase activity which suggests the existence of a protein kinase different from phosphorylase kinase in the membranes.

Calsequestrin, a component of the sarcoplasmic reticulum [7], can be purified either from these membranes or from the protein–glycogen complex [8]. The latter material contains most of the glycogen metabolizing enzymes in addition to sarcoplasmic reticulum [9]. Following degradation of the carbohydrate the membranes can be sedimented; the enzymes are further purified by DEAE-cellulose chromatography and calsequestrin is obtained as the protein which is eluted at the highest ionic strength [8]. Here protein kinase activity is shown associated with isolated homogenous calsequestrin which can be differentiated from phosphorylase kinase.

2. Materials and methods

Phosphorylase *b* (80 U/mg) was prepared from rabbit skeletal muscle as in [10]. Rabbit skeletal muscle phosphorylase kinase was isolated according to [11] as modified [12]. The enzyme was assayed according to [13] or manually according to [4]; it had a spec. act. ~7000 nmol phosphate transferred . min^{-1} . mg^{-1} at pH 8.2. Calsequestrin was isolated from the protein–glycogen complex according to [8]. For the protein kinase activity of calsequestrin when assayed in the manual test [4] 9.2 mg/ml phosphorylase *b* was employed.

Immunization of sheep with rabbit muscle phosphorylase kinase was as in [14] and the antibodies were purified according to [2].

Calcium-dependent regulator protein was the heat-stable supernatant of purified rabbit skeletal muscle phosphorylase kinase [15]. Protein was determined by the method in [16] on a Technicon Autoanalyzer using bovine serum albumin as standard ($A_{280}^{1\%}$ = 4.42×10^4 $M^{-1}.cm^{-1}$). Polyacrylamide gel electrophoresis was carried out in the presence of dodecylsulphate as in [17].

3. Results and discussion

Calsequestrin which is purified in the penultimate step by DEAE-cellulose chromatography [8] is eluted in parallel with phosphorylase *b* to *a* converting activity (fig.1). All fractions show the same specific activity (20.66 ± 1.82 U/mg) of this protein kinase in the whole profile. Calsequestrin shows in gel filtration over Sephacryl S-300 an app. mol. wt 52 000 (second peak, fig.2A). It was obtained as a homogenous protein (fig.2B). Again protein kinase activity follows exactly the calsequestrin elution profile; the specific activity of 9.94 ± 1.30 U/mg is constant in the fractions of the second peak. The first peak contains also calsequestrin together with other proteins, mostly glycogen synthase; protein kinase activity of a lower specific activity (~2.10 ± 0.17 U/mg) is detected. In

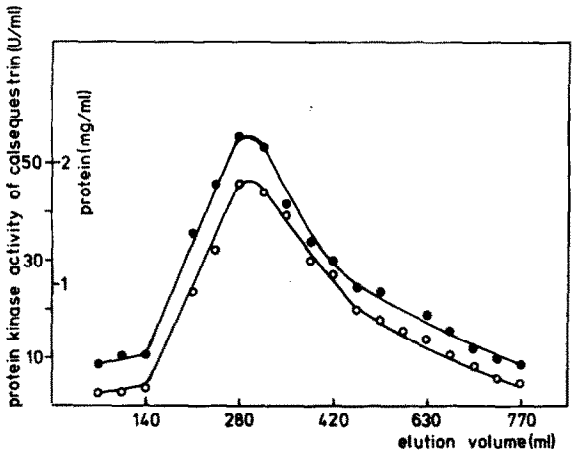

Fig.1. Chromatography of calsequestrin on DEAE-cellulose. Calsequestrin was eluted from DEAE-cellulose (Whatman DE-52, 5 × 20 cm) with 0.5 M Tris–HCl, 1 mM EDTA (pH 7.5) [12]. Protein (○——○) and protein kinase activity (pH 8.2) (●——●) were assayed automatically as in section 2. AMP formation was assayed and phosphorylase *b* activation by this nucleotide was taken in correction.

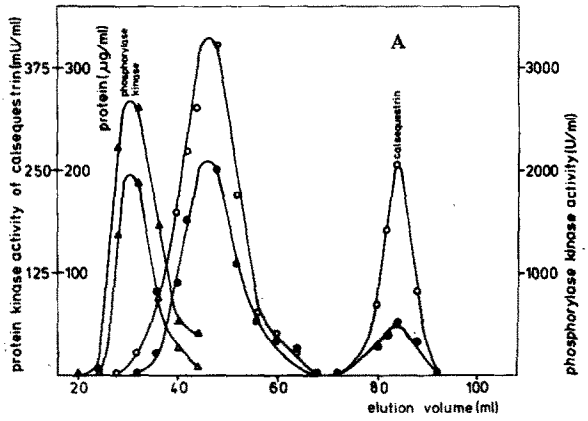

Fig.2A. Gel filtration of calsequestrin over Sephacryl S-300. An aliquot of the concentrated pool of the fractions from the DEAE-cellulose column (4.5 mg = 3.5 ml) was applied to a Sephacryl S-300 superfine column (98 × 1.3 cm) which was equilibrated in 50 mM sodium-glycerolphosphate, 2 mM EDTA (pH 7.0). Protein (●——●) and the phosphorylase *b* to *a* concerting activity of calsequestrin (○——○) were assayed at pH 8.2 in the manual test as in section 2. Separately, purified rabbit skeletal muscle phosphorylase kinase (3.8 mg = 2.5 ml) was applied to the same column. Its activity (△——△) was determined at pH 8.2 and protein (▲——▲) as in section 2. AMP formation was assayed and phosphorylase *b* activation by this nucleotide was taken in correction.

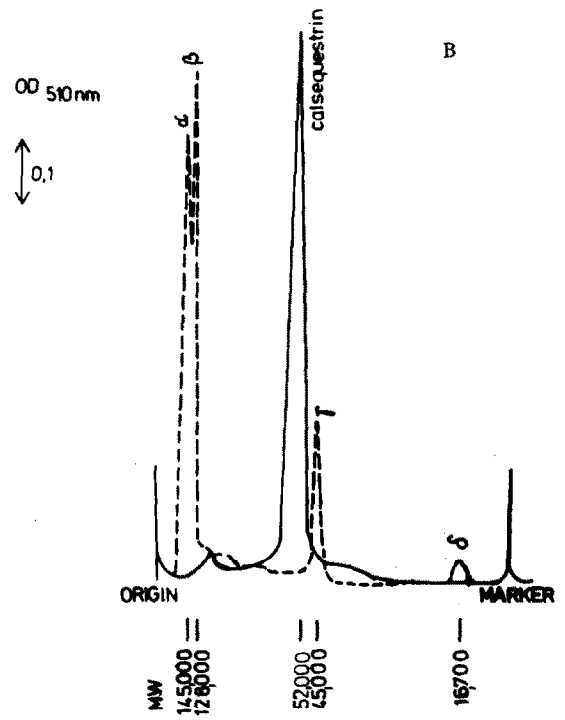

Fig.2B. Homogenous calsequestrin, 6.5 μg (concentrated pool of the second peak of fig.2A) (——) and phosphorylase kinase, 15 μg (– – –) were applied to 6.6% polyacrylamide gels and separated by electrophoresis as in section 2.

both peaks the specific activity is lower than in the ion-exchange chromatography step. A decrease of the protein kinase activity during preparation is always observed which indicates a high lability of the isolated enzyme.

Calsequestrin isolated in homogenous form according to [7] by the desoxycholate solubilization procedure yields a product which has essentially the same protein kinase activity as that isolated above.

The *b* to *a* converting activity of calsequestrin shows a lower molecular weight than phosphorylase kinase; the latter enzyme appears under the same gel filtration conditions near the breakthrough (mol. wt 1.25×10^6, fig.2A). The protein kinase activity which is isolated as homogenous calsequestrin is not inhibited but rather slightly activated by concentrations of antibodies against phosphorylase kinase (fig.3) which completely inhibit an equivalent amount of purified phosphorylase kinase activity. On this basis and the appar-

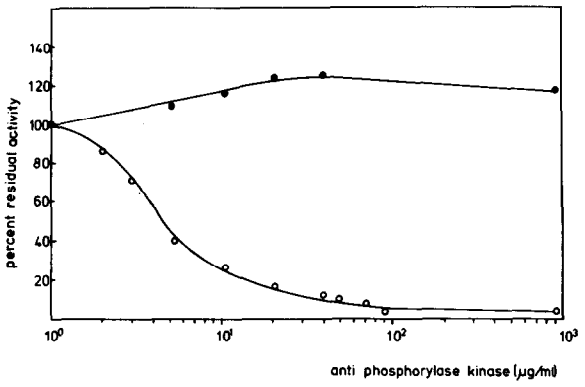

Fig.3. Immunotitration of purified rabbit skeletal muscle phosphorylase kinase (o——o) and the kinase activity of calsequestrin (•——•). In 1 ml 10 mM sodium-glycerolphosphate, 1 mM DTE (pH 7.0) were present 2 U either phosphorylase kinase or homogenous calsequestrin kinase activity (100%) and antibodies against phosphorylase kinase as indicated. After incubation for 10 min at 0°C, kinase activities were assayed automatically [13].

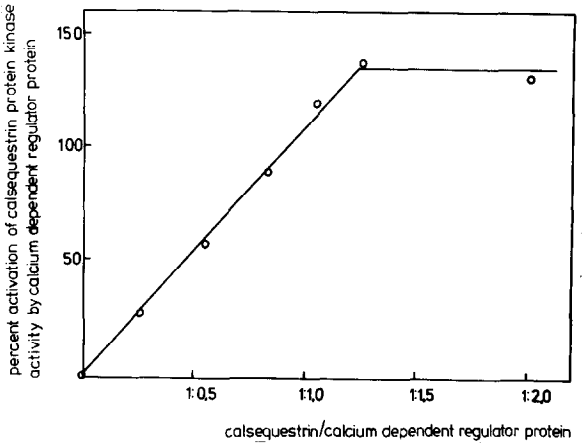

Fig.4. Activation of calsequestrin protein kinase activity by Ca^{2+} containing Ca^{2+}-dependent regulator protein. To 2.67 µmol calsequestrin in 1 ml total vol. increasing amounts of Ca^{2+}-dependent regulator protein in the ratio to calsequestrin as indicated were added. Activity was assayed in the manual test (see section 2).

ent molecular weight it is concluded that the protein kinase activity which is isolated as calsequestrin is different from phosphorylase kinase.

The expression of the protein kinase activity isolated as calsequestrin is not dependent on the presence of Ca^{2+} and shows a pH 6.8/8.2 activity ratio from 0.5–0.8 depending on the preparation. This also differentiates this kinase from phosphorylase kinase which expresses most of its activity only in the presence of Ca^{2+} either in its native or covalently modified forms [18]. The low specific activity of the calsequestrin kinase may be due to the unphysiological substrate phosphorylase *b*; the physiological substrate might be a membrane component.

The protein kinase activity of calsequestrin can be further enhanced ~2 fold by the Ca^{2+} containing Ca^{2+}-dependent regulator protein (fig.4). Activation is only observed in the presence of $\sim 10^{-5}$ M but not at $\sim 10^{-9}$ M Ca^{2+}. The extent varies according to the preparation from 50–150%, the maximal activation is obtained at ratio of ~1 mol calsequestrin to 1.3 mol Ca^{2+}-dependent regulator protein (fig.4). It suggests the formation of a calsequestrin–Ca^{2+}-dependent regulator protein complex. This Ca^{2+}-dependent regulator protein activation is similar to that of glycogen synthase kinase and of myosin light chain kinase, however both of these protein kinases do not accept phosphorylase *b* as substrate [19–23].

Phosphorylase kinase accepts 5′-GTP, 5′-UTP, 5′-ITP and 5′-CTP with decreasing efficiency as phosphate donors; the protein kinase activity of calsequestrin yields the same order of nucleotides; however, GTP is accepted with lower and UTP with higher efficiency (not shown). $MgCl_2$ and ATP at physiological concentrations were reported [7] to strongly inhibit the binding of Ca^{2+} to calsequestrin. This behaviour might be explainable on the basis of its protein kinase properties.

It has been estimated that calsequestrin comprises ~7% of the total sarcoplasmic reticular protein [7]. The protein kinase activity of the sarcoplasmic reticulum which can not be inhibited by antibodies against phosphorylase kinase and which is also present in the sarcoplasmic reticulum of I strain mice has spec. act. 1.0–1.5 U/mg. The specific activity of the isolated calsequestrin is high enough to explain the above-mentioned protein kinase activity of the membranes.

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