AUTOCATALYTIC PHOSPHORYLATION OF CALSEQUESTRIN

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1. Introduction

Isolated skeletal muscle sarcoplasmic reticulum from rabbit [1] and I-strain mice [2] contains Ca^{2+}-dependent membrane-bound protein kinase activity. This kinase seems to be similar but not identical to phosphorylase kinase and presumably is involved in the regulation of the Ca^{2+}-transport ATPase activity [3]. We have demonstrated that calsequestrin isolated from rabbit skeletal muscle sarcoplasmic reticulum is associated with Ca^{2+}-independent protein kinase activity [4] which however can be stimulated by calcium-saturated calmodulin or troponin C [5].

In addition to the Ca^{2+}-dependent protein kinase activity a cAMP-stimulated protein kinase seems also to be present in these vesicles and may play an important role in the Ca^{2+}-dependent regulation of membrane-bound protein phosphatase activity [6].

Here, we show that isolated calsequestrin can undergo autocatalytic phosphorylation during its incubation with [γ-^{32}P]ATP/Mg^{2+}. This autophosphorylation can be stimulated ~10-fold (from ~0.04 to ~0.4 mol/mol protein) by preincubation of the isolated calsequestrin with protein phosphatase.

2. Material and methods

Phosphorylase *b* was prepared from rabbit skeletal muscle as in [7]. Rabbit skeletal muscle phosphorylase kinase was isolated according to [8] and as modified in [9]. The enzyme activity was measured according to [10] or manually as in [2]. The enzyme had spec. act. ~9800 nmol phosphate transferred . min^{-1} . mg^{-1} at pH 8.2. Homogeneous calsequestrin was isolated from the protein glycogen complex as in [11] as modified in [4]. Its kinase activity measured with phosphorylase *b* as substrate was 13 U/mg at pH 8.2, the pH 6.8/8.2 activity ratio 0.45. Protein phosphatase from rabbit skeletal muscle was prepared according to [12] and as in [6]. The catalytic subunit of the cyclic AMP-dependent protein kinase (470 nmol . min^{-1} . mg^{-1}) was prepared according to [13]. [γ-^{32}P]ATP was prepared according to [14]. [^{32}P]Phosphate was obtained from Amersham Buchler, Braunschweig. Protein was determined by the Lowry method [15] on a Technicon Autoanalyzer using bovine serum albumin as standards ($\epsilon_{280} = 4.42 \cdot 10^4$ M^{-1} . cm^{-1}). Protein bound radioactivity was determined as in [16]. SDS–Polyacrylamide gel electrophoresis (5%) was carried out as in [17]. Densitometry was measured at 540 nm. For the estimation of the radioactivity distribution the gels were cut into 1 mm slices with a Gilson gelfractionator. The radioactivity of the slices was counted in a Packard liquid scintillation counter. Endogeneous phosphate of calsequestrin was determined as in [18].

3. Results and discussion

Calsequestrin incorporates a very low amount of phosphate, maximally 0.04 mol/mol protein, in a time-dependent reaction if incubated with [γ-^{32}P]ATP and Mg^{2+}. This reaction is neither influenced by addition of ~10 μM Ca^{2+}, nor 0.2 mM EGTA, nor phosphorylase kinase (not shown) nor the catalytic subunit of the cAMP-dependent protein kinase (fig.1). However, this self-phosphorylation of calsequestrin can be stimulated ~10-fold (from ~0.04 ~0.4 mol phosphate/mol protein) by simultaneous addition of protein phosphatase. Again, neither the rate of this reaction nor the total amount of incorporated phosphate is influenced by addition of cAMP-dependent protein kinase (fig.1). The observed enhancement of the autocatalytic phosphorylation in the presence of

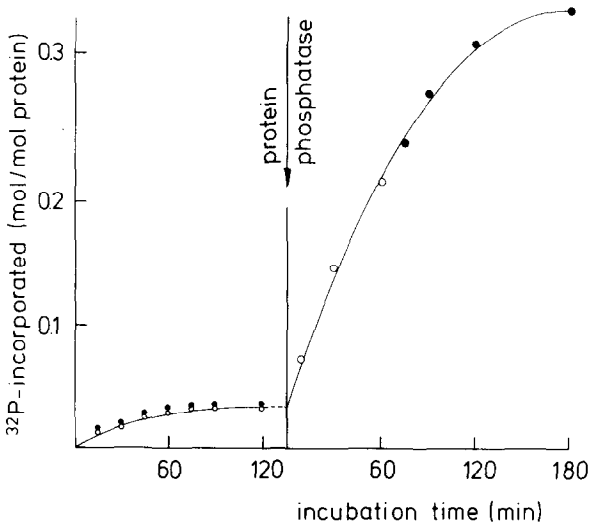

Fig.1. Phosphorylation of calsequestrin: 2 mg/ml homogeneous calsequestrin was incubated in the presence of 20 mM Tris, 20 mM sodium-glycerolphosphate, 1 mM DTE, 1 mM [γ-^{32}P]ATP, 10 mM Mg^{2+}, 0.2 mM EGTA with (○) and without (●) the catalytic subunit of the cyclic AMP-dependent protein kinase (45 μg/mg calsequestrin) at pH 7.5 (30°C). During the incubation aliquots were removed for determination of ^{32}P incorporation from [γ-^{32}P]ATP into the calsequestrin according to [16]. After 120 min to 100 μl reaction mixture without cAMP-dependent protein kinase, 270 μg phosphoprotein phosphatase was given and further incubated. After 60 min, 10 μg catalytic subunit of the cyclic AMP-dependent protein kinase was given to 60 μl reaction mixture and further incubated (●). During the whole incubation, samples were removed and the protein-bound radioactivity was determined as above.

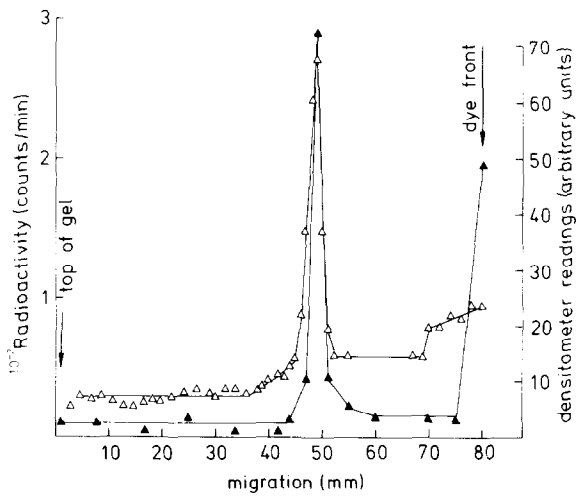

Fig.2. Two aliquots autophosphorylated calsequestrin (10 μg) were subjected to SDS electrophoresis. The first gel was scanned after staining and destaining with Coomassie brilliant blue (▲) and the second was used for the determination of the radioactivity in 1 mm slices as in section 2 (△).

protein phosphatase may indicate that the isolated calsequestrin contains endogeneously bound phosphate. Upon incubation of calsequestrin with protein phosphatase it presumably is removed, at least partially, and the dephosphorylated calsequestrin is re-phosphorylated in the autocatalytic process. Following trichloroacetic acid precipitation (3 times) and digestion of the protein with sulphuric acid, an amount of 0.61 mol phosphate/mol protein was determined. Therefore, the presence of endogeneous phosphate in calsequestrin is compatible with the enhancement of the autocatalytic incorporation of phosphate into calsequestrin upon pre-dephosphorylation. However, contamination of the protein with other trichloroacetic acid-precipitable phosphorous-containing materials cannot be excluded [19].

SDS–polyacrylamide gel electrophoresis reveals that the phosphate incorporated during the autocatalytic reaction is present exclusively in calsequestrin (fig.2).

To exclude an influence of the added protein phosphatase on the autocatalytic phosphorylation of calsequestrin the latter protein was preincubated with the protein phosphatase which should result in dephosphorylation. Then the protein phosphatase was removed by gel filtration (not shown). The dephosphorylated calsequestrin incorporates again autocatalytically 0.26 mol phosphate/mol protein (fig.3). The initial velocity of the self-phosphorylation before preincubation with protein phosphatase was 3.7 pmol . min^{-1} . mg^{-1} whereas the phosphatase pretreatment increases it 10-fold (37 pmol . min^{-1} . mg^{-1}). It should be noted that the initial velocity of phosphorylation of an exogeneous substrate phosphorylase *b* (13 nmol . min^{-1} . mg^{-1}) is ~350-fold faster than the phosphatase stimulated autophosphorylation rate of calsequestrin. Similar relationship can be observed with rabbit skeletal muscle phosphorylase kinase, too: it phosphorylates phosphorylase *b* at a 300-fold higher rate than autocatalytically its own subunits α and β [20].

About 50% of the autocatalytically-incorporated radioactive phosphate into calsequestrin can be rapidly removed by incubation with protein phosphatase (fig.3); the hydrolysis of the remaining phosphate occurs slowly. It might be interpreted that calseques-

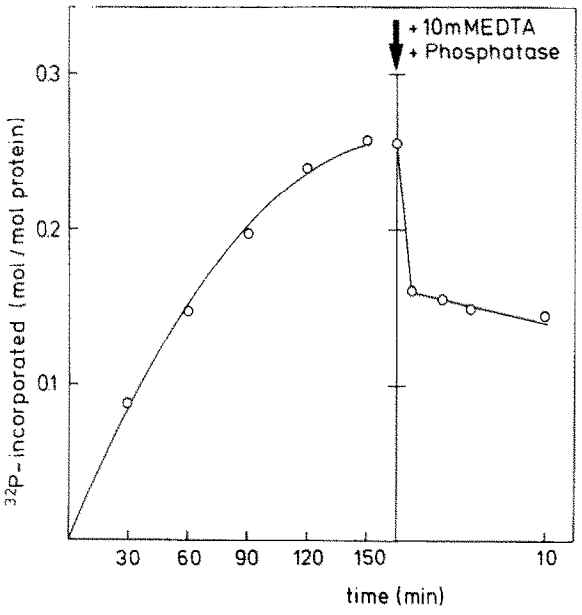

Fig.3. Calsequestrin (2.3 mg) was dialized against 20 mM Tris, 1 mM DTE (pH 7.5). After intensive dialysis for 6 h phosphoprotein phosphatase (= 2 mg) was added to the calsequestrin and incubated for 30 min at 30°C. To separate phosphoprotein phosphatase the incubation mixture was gelfiltered over a Sephacryl S 300 column (1.5 × 80 cm) equilibrated with 20 mM Tris, 1 mM DTE (pH 7.5). Calsequestrin was pooled according to gel electrophoresis according to [17] and concentrated on Amicon PM 10 filter. Thereafter 230 μg/ml dephosphorylated calsequestrin was autophosphorylated as in the legend of fig.1. After addition of 10 mM EDTA for binding Mg^{2+} in the incubation mixture and 300 μg phosphoprotein phosphatase/50 μg calsequestrin the dephosphorylation of calsequestrin was followed according to [16].

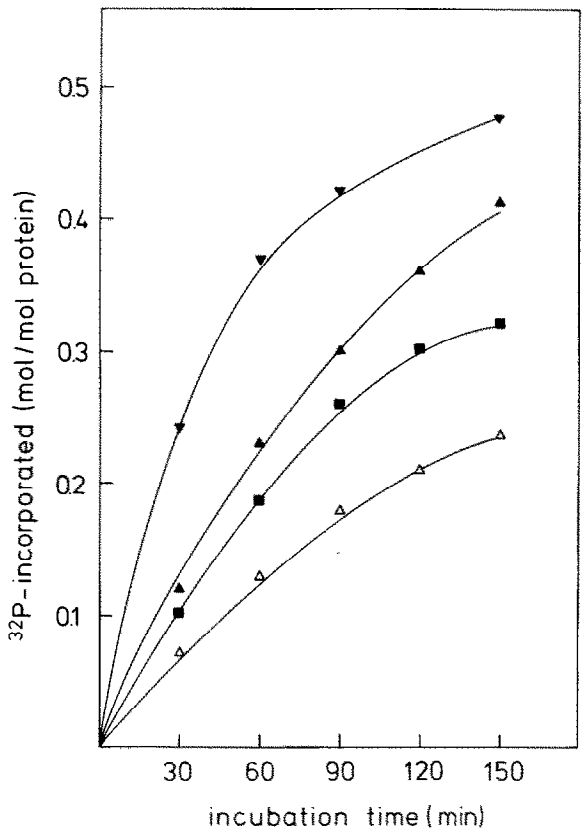

Fig.4. Autocatalytic phosphorylation of the dephosphorylated calsequestrin at various pH values was carried out as in the legend of fig.1: pH 6.6 (△), 7.2 (■), 8.9 (▲), 9.4 (▼).

trin is phosphorylated at different sites which are dephosphorylated with different velocities by the protein phosphatase. However such different sites have not yet been shown.

The rate of the calsequestrin autophosphorylation reaction increases with increasing pH (fig.4). This is in agreement with the acidic nature of calsequestrin [21]. Autophosphorylation has also been observed in other protein kinase preparations. Cyclic AMP-dependent protein kinase from bovine heart can catalyze self-phosphorylation of the regulatory [22] and catalytic [23] subunits. The phosphorylase kinase autocatalytic phosphorylation was extensively studied [24] and shown that the partial activities of the phosphorylase kinase (a_1, a_4) are activated through the autocatalytic process [25]. In [26], during a phosphoprotein formation study in sarcoplasmic reticular subfractions, phosphorylation of a 57 000 M_r polypeptide in the low density vesicles was observed, but it was not clear whether that polypeptide is calsequestrin. Assuming that it is calsequestrin a phosphorylation of ~0.001 mol/mol protein can be calculated from these data.

We cannot assign yet any physiological role to the autophosphorylation of calsequestrin. It might however occur in the intact membranes since the protein is isolated in phosphorylated form.

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