ELSEVIER

Biochimica et Biophysica Acta 1194 (1994) 35–43

BBA Biochimica et Biophysica Acta

Quantification and removal of glycogen phosphorylase and other enzymes associated with sarcoplasmic reticulum membrane preparations

Ana Cuenda, Fernando Henao, Manuel Nogues, Carlos Gutiérrez-Merino *

Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Extremadura, E-06080 Badajoz, Spain

Received 16 February 1994; revised 29 March 1994

Abstract

The enzymatic characterization of sarcoplasmic reticulum membrane fragments from rabbit skeletal muscle presented in this paper shows that glycogen phosphorylase, as well as other enzymes (e.g., creatine kinase, myokinase, phosphorylase kinase, glycosidase, AMP-deaminase, phosphoglucomutase) are associated with these membrane preparations. Amongst these enzymes, the highest activity associated with sarcoplasmic reticulum membranes is that of glycogen phosphorylase, which is mostly (at least 95%) in its *b* form (dephosphorylated form), since its activity in sarcoplasmic reticulum membranes is largely dependent upon AMP. A protocol is presented to quantify the amount of phosphorylase bound to sarcoplasmic reticulum membranes from fluorimetric measurements of the content of its coenzyme, pyridoxal 5'-phosphate. The content of phosphorylase ranged from 0.03 to 0.37 mg phosphorylase per mg of membrane protein, in sarcoplasmic reticulum membrane preparations made following several of the protocols most commonly used and also depending upon the length of the starvation period of the animal before killing. We also show that dilution of sarcoplasmic reticulum membranes to 0.1–0.2 mg protein per ml in a buffer containing 50 mM Tes-KOH (pH 7.4), 0.1 M KCl and 0.25 M sucrose removes at least 95% of glycogen phosphorylase from these membrane fragments, as well as other enzymes like myokinase and glycosidase. On these grounds, we suggest to introduce a final dilution step as indicated above in protocols of sarcoplasmic reticulum membrane preparations.

Key words: Sarcoplasmic reticulum; Glycogen phosphorylase; Phosphoglucomutase; Glycosidase; Myokinase; Creatine kinase

1. Introduction

Several laboratories have noticed that sarcoplasmic reticulum (SR) membranes contain enzymes not directly implicated in the control of cytosolic calcium concentration in the muscle cell. These can be split into two groups: (i) enzymes of glycogen metabolism, and (ii) other enzymes relevant to purine and hexose metabolism in skeletal muscle. The contaminants usually present in isolated SR membranes are enzymes like creatine kinase [1,2], myokinase [3,4], phosphorylase *b* kinase and glycogen synthase [5–7], protein kinases and phosphatases [8–11], and glycogen phosphorylase that is the most cited as a contaminant protein in SR membranes prepared following different protocols [4,12–14]. Glycogen phosphorylase plays a key role in the control of glycogen metabolism, and in the cell is partially bound to the glycogenolytic particles [15,16], which in turn associate with the sarcoplasmic reticulum membrane [16–18]. It has been shown from different laboratories that these enzymes are present in SR membranes prepared following different protocols. No particular effort has been made to quantify the amount of phosphorylase bound per mg of membrane protein in the preparations of fragmented SR, although a priori this question is of particular relevance to many studies with SR membranes.

In this paper we present further experimental evidence showing the presence of glycogen phosphorylase, as well as other enzymatic activities, in SR membrane fragments prepared following standard protocols. Fur-

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; IU, μmol product per min per mg protein; LDH, lactate dehydrogenase; phosphorylase, glycogen phosphorylase *b*; PK, pyruvate kinase; PLP, pyridoxal 5'-phosphate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gelelectrophoresis; SR, sarcoplasmic reticulum.

* Corresponding author. Fax: +34 24 271304.

0005-2736/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0005-2736(94)00119-A

thermore, we report here methods to quantify and remove the glycogen phosphorylase bound to SR preparations.

2. Materials and methods

Unless stated otherwise SR membranes and phosphorylase have been prepared from male rabbit (New Zealand white) skeletal muscle as indicated in Gutierrez-Merino et al. [19] and Gutierrez-Merino et al. [20], respectively. In addition, for comparative purposes SR membranes have been prepared as well following the protocols outlined in Warren et al. [21] and in Champeil et al. [22].

SDS PAGE has been performed following the method of Laemmli [23].

The protein concentration was determined using the method of Lowry et al. [24] with bovine serum albumin as standard.

The phosphorylase activity of SR membranes (30–50 μg protein/ml) has been measured in the direction of glycogen degradation as indicated in Cuenda et al. [14]. The ATPase activity was measured using the coupled enzyme system pyruvate kinase (PK)/lactate dehydrogenase (LDH) [21], as indicated elsewhere [19,25] with the following assay mixture: 100 mM Tes-KOH (pH 7.4), 100 mM KCl, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 7.5 IU PK, 18 IU LDH, 0.25 mM NADH, 0.42 mM phospho*enol*pyruvate, 2.5 mM ATP and 10 μg SR protein per ml. The Ca^{2+} independent ATPase activity was measured in the presence of 3.5 mM EGTA. This assay medium was used to determine other enzymatic activities assayed in SR membrane preparations: myokinase (in the presence of 1 mM AMP), creatine kinase (in the presence of 25 mM creatine) and hexokinase (in the presence of 2 mM glucose).

The phosphorylase *b* to phosphorylase *a* conversion by the endogenous phosphorylase kinase activity in SR membrane preparations was monitored assaying the phosphorylase activity in the presence of 2 mM ATP, 1 mM $CaCl_2$ plus 50 μM P^1P^5-di(adenosine-5')pentaphosphate (to inhibit the myokinase activity) and 5 IU PK and 2 mM phospho*enol*pyruvate (to regenerate ATP).

The AMP-deaminase activity of SR membrane preparations was determined from measurements of the AMP concentrations at different times as in Entman et al. [18], with the following assay mixture: 10 mM glucose 1-phosphate, 0.45 $g l^{-1}$ glycogen, 0.1 mM AMP, 1 mM EGTA, 150 mM KCl, 10 mM Tes-KOH (pH 7.4), with a SR membrane concentration ranging from 1 to 8 mg protein per ml. The concentration of AMP was determined using PK (7.5 IU), LDH (18 IU) and myokinase (5 IU) in a reaction medium containing 0.25 mM NADH, 2 mM phospho*enol*pyruvate, 2.5 mM ATP, 1 mM EGTA, 5 mM $MgCl_2$, 100 mM Tes-KOH (pH 7.2) at 25 and 37°C. The spectrophotometric decrease of absorption at 340 nm was calibrated by addition of known concentrations of AMP, and was corrected for the AMP contamination of ATP solutions ($< 1\%$ w/w).

The phosphoglucomutase and glycosidase activities of SR membrane preparations were measured using glucose-6-phosphate dehydrogenase as coupled enzyme with the following assay mixture: 10 mM Tes-KOH (pH 7.4), 100 mM KCl, 0.45 $g l^{-1}$ glycogen, 0.1 mM AMP, 0.5 mM $NADP^+$, 10 IU glucose-6-phosphate dehydrogenase and SR membranes (200–400 μg SR protein/ml), plus either 10 mM glucose 1-phosphate, 50 μM glucose 1,6-bisphosphate for phosphoglucomutase activity measurements, or 5 mM $MgCl_2$, 2.5 mM ATP, 10 IU hexokinase in the glycosidase activity assay medium.

The glycogen content of SR preparations has been measured following the phenol sulfuric method [26], using glycogen as standard.

The type of polysaccharide produced (α-amylose or amylopectin) under experimental conditions of phosphorylase activity, in the direction of glycogen synthesis [14], was revealed using the I_2/I^- method described by Krisman [27]. The ratio between the absorption at 500 and 700 nm (A_{500} and A_{700}, respectively) was taken as a branching index of glycogen synthesized by glycogen phosphorylase bound to SR membranes.

Pyridoxal 5'-phosphate (PLP) was removed from glycogen phosphorylase following the method of Strausbauch et al. [28] with minor modifications, and PLP was taken as a measurement of phosphorylase content of SR membranes. Briefly, to remove PLP from phosphorylase, the enzyme is treated with 0.6 M perchloric acid, and after 30 min incubation at 30°C under mild stirring the sample is centrifuged for 30 min at $15\,000 \times g$. The concentration of PLP in the supernatant has been determined using two methods:

(i) From measurements of the intensity of fluorescence (with excitation and emission wavelenghts of 335 and 410 nm, respectively): the samples were treated with sodium bisulfite (1 mg/ml) to enhance fluorescence intensity [29], and allowed to stand for 30 min to obtain a steady fluorescence reading.

(ii) From absorbance measurements at 410 nm of the PLP-phenylhydrazone derivative ($\epsilon = 3 \cdot 10^4$ $M^{-1} cm^{-1}$) following Strausbauch et al. [28]. Briefly, 1 vol. of sample was mixed with 1/20 vol. of 2% phenylhydrazine in 5 M N H_2SO_4, then allowed to stand for 30 min before absorbance reading.

Fluorescence measurements have been carried out at 25°C using a Hitachi/Perkin-Elmer model 650–40 spectrofluorimeter operating in ratio mode.

Rabbit polyclonal antibodies were raised against glycogen phosphorylase following the protocol indicated by Johnstone and Thorpe [30] and tested using

ELISA plates. Readings were taken with a Titertek Multiscan Plus.

2.1. Chemicals

Bovine serum albumin, AMP, ATP, EGTA, phospho*enol*pyruvate, protein molecular weight markers, β-mercaptoethanol, sodium dodecyl sulfate, glucose 1-phosphate, glucose 6-phosphate, Tris (TRIZMA base) and Tes were obtained from Sigma. Glycogen, NADH, $NADP^+$, calcimycin, P^1P^5-di(adenosine-5')pentaphosphate, PK, LDH, phosphoglucomutase and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim. All the other chemicals used in this study were obtained from Merck.

3. Results

3.1. Characterization of relevant enzymatic activities bound to SR membranes

Table 1 lists the most relevant enzyme activities measured in SR membrane preparations obtained by different protocols (see Materials and methods). These results show that the largest activity of non-transmembrane enzymes, associated with SR preparations is by far the glycogen phosphorylase activity.

Table 1
Enzyme activities [a] of preparations of fragmented sarcoplasmic reticulum membranes from rabbit skeletal muscle

		μmol product/ min per mg SR protein
Creatine kinase		0.045 ± 0.026
Myokinase		0.073 ± 0.036
Phosphorylase kinase		(+)
Glycosidase		$(1.33 \pm 0.05) \cdot 10^{-3}$
AMP-deaminase		$(9.05 \pm 5.00) \cdot 10^{-3}$
Phosphoglucomutase		$(0.86 \pm 0.33) \cdot 10^{-3}$
Glycogen phosphorylase [b]		0.30–0.75 [e]
		0.60–1.00 [f]
		0.75–2.00 [g]
Mg^{2+}-ATPase	Ca^{2+}-independent [c]	0.17 ± 0.08
	Ca^{2+}-dependent [d]	3.60 ± 0.40
Hexokinase		(−)

[a] The activities listed have been measured at 25°C and with the assay medium indicated in Materials and methods.
[b] Total glycogen phosphorylase activity measured in the presence of 1 mM AMP.
[c] The Ca^{2+}-independent Mg^{2+}-ATPase activity was measured in the presence of 4% (w/w) ionophore calcimycin (A23187), plus 3.5 mM EGTA.
[d] The Ca^{2+}-dependent Mg^{2+}-ATPase activity was measured in the presence of calcimycin, as indicated above, substracting the Ca^{2+}-independent Mg^{2+}-ATPase activity from total Ca^{2+},Mg^{2+}-ATPase activity.
[e] SR prepared as in [19].
[f] SR prepared as in [21].
[g] SR prepared as in [22].

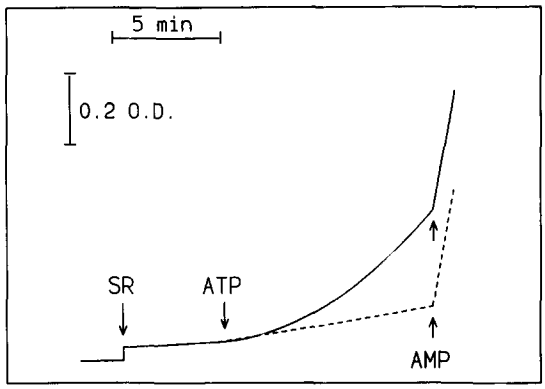

Fig. 1. Calcium dependence of glycogen phosphorylase *a* production in SR membranes. The reaction was carried out at 30°C in a medium containing 100 μg SR protein per ml, 20 mM Tes (pH 7.0), 10 mM magnesium acetate, 15 mM KH_2PO_4, 1 mM β-mercaptoethanol, 50 μM glucose 1, 6 bisphosphate, 2. 5 mM phospho*enol*pyruvate, 2.5 $g l^{-1}$ glycogen, 50 μM P^1P^5-di(adenosine-5')pentaphosphate, 1 $\mu g ml^{-1}$ calcimycin, 0.5 mM $NADP^+$, 7.5 IU PK, 5 IU phosphoglucomutase, 5 IU glucose-6-phosphate dehydrogenase, in the presence of either 50 μM $CaCl_2$ (continuous line) or 5 mM EGTA, no calcium added (dotted line). The onset of glucose 1-phosphate production by phosphorylase *b* to *a* conversion and phosphorylase *b* activity was initiated by addition of 2 mM ATP and 1 mM AMP, respectively, at the times indicated by arrows in the figure.

Fig. 1 shows the phosphorylase *a* production in SR membranes. Immediately after addition of SR the phosphorylase activity is negligible in the absence of AMP. Addition of ATP to the assay medium produces the activation of glycogen phosphorylase, due to phosphorylase *b* to phosphorylase *a* conversion by the kinase activity associated with SR membranes [5–7]. In the presence of EGTA (dotted line) phosphorylase *a* production is negligible. Therefore, the presence of Ca^{2+} in the assay medium is required for phosphorylase *a* production under these experimental conditions. These results show that the phosphorylase kinase activity present in SR membranes is calcium dependent (see Fig. 1), as expected since no cAMP was added and glycogen phosphorylase *b* kinase is activated by either Ca^{2+} or cAMP (31,32), and that phosphorylase bound to SR membranes is mostly (> 95%) in its dephosphorylated *b* form.

To monitor the glycogen branching activity in SR membranes we have analysed the spectral characteristics of the complex of glycogen with I_2/I^- by following the ratio A_{500}/A_{700}, in the presence of 1 mg SR protein per ml. As a result we have found no significant change – after 60 min incubation – in the ratio A_{500}/A_{700} (data not shown). Therefore, the glycogen branching activity of SR membrane preparation appears to be negligible.

Glycogen has been shown to be involved in the stabilization of the association of phosphorylase with

the SR-glycogenolytic complex [15,16]. We have characterized the content of glycogen of preparations of SR membranes ($n = 12$), and we have found that on average it amounts to 32 ± 10 mg glycogen per g of SR membrane protein. The content of glycogen is reduced (approx. 2-fold) in SR membranes prepared from skeletal muscle of rabbits which were left 48 h starvation before sacrifice, and in these membrane preparations the amount of phosphorylase is moderately decreased as well (see below).

3.2. Characterization and quantification of phosphorylase bound to SR membranes

Earlier we have noticed glycogen phosphorylase activity in sarcoplasmic reticulum membrane preparations [12,14]. As indicated in Materials and methods we have prepared SR membranes from back and hind leg skeletal muscles using several protocols employed by research groups active in this field [21,22]. We have found that in all the preparations of SR membranes glycogen phosphorylase activity is detected, with activities ranging from 0.3 to 2 μmol glucose 1-phosphate released per min per mg of membrane protein in the presence of 1 mM AMP (Table 1), data obtained with a pool of more than 28 preparations following different protocols. Following the protocol of MacLennan slightly modified as indicated in [19] and killing the rabbits after a 48 h starvation period, the range of total phosphorylase activity associated to SR membranes ($n = 20$) is narrowed from 0.30 to 0.75 μmol glucose 1-phosphate per min per mg of membrane protein, similar to those reported elsewhere [12]. The presence of glycogen phosphorylase in SR membranes prepared as indicated above was confirmed by gel electrophoresis (Fig. 2A) and further assessed using rabbit polyclonal antibodies (not shown). As it can be seen in SDS-PAGE the Ca^{2+}-ATPase and phosphorylase *b* appear as bands of M_r 110000 and 98000, respectively. Fig. 2B shows that glycogen phosphorylase activity is associated with SR membranes. In the absence of AMP the phosphorylase activity observed is due to the *a* form and is negligible. The addition of 1 mM AMP, a non-covalent allosteric activator of the *b* form [33–35], produces a fast increase of the phosphorylase activity which is inhibited by 5 mM caffeine, an inhibitor of phosphorylase *b* [16,36]. In conclusion, the phosphorylase activity associated with SR membranes is mostly in the *b*

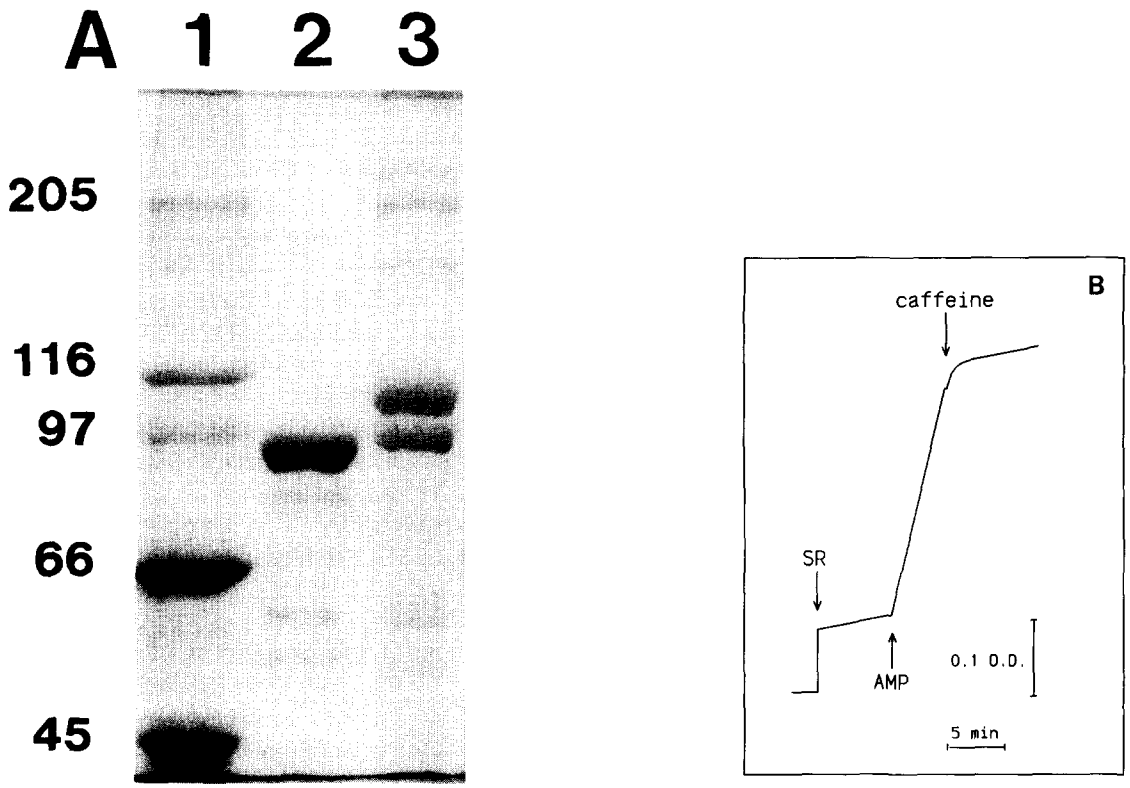

Fig. 2. (A) SDS-PAGE of SR membranes. Lane 1, molecular weight markers: myosin (205000), β-galactosidase (116000), glycogen phosphorylase *b* (97400), bovine serum albumin (66000) and ovalbumin (45000). Lane 2, 3 μg of purified glycogen phosphorylase *b*. Lane 3, 7 μg of SR membrane proteins, with a contamination of 0.15 ± 0.02 mg phosphorylase per mg of total SR protein. (B) Glycogen phosphorylase activity in SR membranes. The assay mixture contained: 50 mM imidazol (pH 6.9), 10 mM magnesium acetate, 12 mM KH_2PO_4, 0.63 mM $NADP^+$, 5 IU phosphoglucomutase, 5 IU glucose-6-phosphate dehydrogenase plus 35–50 μg SR protein per ml. Temperature 30°C. The addition of 1 mM AMP initiates the onset of rapid glucose 1-phosphate production due to phosphorylase *b* activity, since it is inhibited by addition of 5 mM caffeine at the time indicated by an arrow.

form (> 95%), because it requires AMP to be expressed and is inhibited by caffeine.

Activity measurements does not allow to correctly quantify the amount of phosphorylase bound to the SR membranes, because there is ample experimental evidence showing that this enzymatic activity is modulated by a large variety of effectors [34,37], and even by its association with this membrane [14]. In addition, direct estimation of the phosphorylase/Ca^{2+}, Mg^{2+}-ATPase from readings of the optical density of SR SDS-PAGE stained with Coomassie blue is uncertain, because of the much higher staining of phosphorylase than of ATPase (see, Figs. 2 and 6 of this paper) and also because of partial overlapping of bands of these proteins in the gels.

Therefore, we looked for a simple and reliable alternative approach to measure the phosphorylase content of this membrane, and to this end we have measured their content of PLP following the protocols described in the Materials and methods. PLP is the prosthetic group of glycogen phosphorylase, which is required for the activity of this enzyme and is covalently bound to the protein. Native glycogen phosphorylase contains 1 mol of PLP per enzyme monomer. This prosthetic group can be removed from phosphorylase [28]. Using solutions of known concentrations of purified glycogen phosphorylase and solutions of known concentrations of PLP (prepared by weighing), we have assessed that PLP is completely removed from the enzyme using the treatment indicated in the Materials and methods (Fig. 3). Fig. 4 shows the fluorescence emission spectra (excitation wavelength 335 nm) of bound and free PLP. It can be observed that the fluorescence of PLP bound to the native enzyme has a band centered at 520 nm, while the emission band of free PLP is about 410 nm,

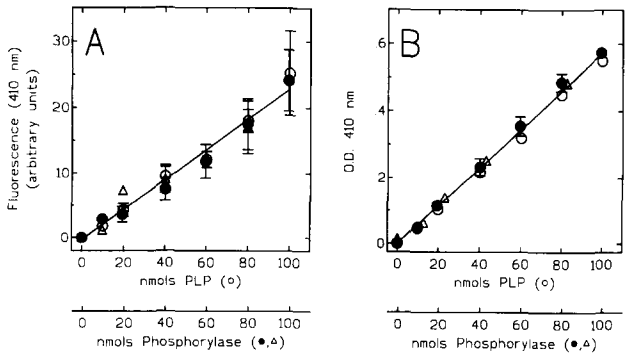

Fig. 3. Calibration lines of solutions of increasing PLP concentrations, prepared by weighing (○) or from resolution of purified glycogen phosphorylase *b* in the absence (●) and in the presence (△) of 5 mg SR protein per ml. (A) From measurements of the intensity of fluorescence (λ_{exc} 335 nm and λ_{em} 410 nm). (B) From absorbance measurements at 410 nm of the PLP-phenylhydrazone derivative prepared as indicated in Materials and methods.

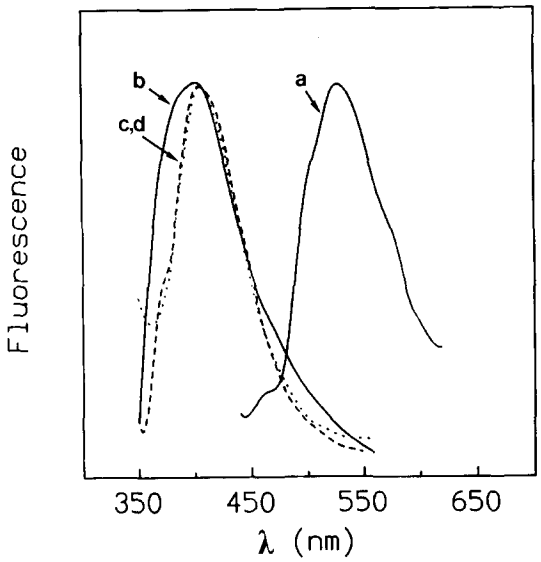

Fig. 4. Emission spectra of PLP (λ_{exc} 335 nm). The spectra shown correspond to PLP bound to purified glycogen phosphorylase (a) and commercial free PLP (b, ———) in 10 mM Tris-acetate (pH 7.4); and free PLP removed as indicated in Materials and methods from purified glycogen phosphorylase (c, - - -) and from SR membrane protein (d, · · ·) in 10 mM Tris-acetate (pH 7.4) plus 0.6 M perchloric acid.

and therefore it confirms that PLP has been efficiently removed from glycogen phosphorylase. Since the calibration line obtained with solutions of known concentrations of purified glycogen phosphorylase *b* is unaffected by the presence of a relatively high concentration of SR membranes (5 mg protein per ml), with low content of glycogen phosphorylase (see also Fig. 3), it follows that no other component of the SR membranes interferes with this assay. The concentration of PLP per mg of SR protein has been calculated from the slope of regression lines fitted to data like those shown in the Fig. 5, and interpolation in the calibration lines shown in Fig. 3.

The results obtained indicate that there is a relatively large variation of the content of phosphorylase in membrane preparations made following different experimental protocols, i.e., from 0.03 to 0.37 mg phosphorylase per mg of membrane protein. In particular, following the protocol currently employed in this laboratory [19], e.g., basically that of MacLennan [38], the content of glycogen phosphorylase ranged from 0.03 to 0.1 mg phosphorylase per mg membrane protein. The lowest content in phosphorylase were systematically found in SR membranes prepared from rabbits killed after 48 h starvation. From these results and activity measurements we obtain specific phosphorylase activities of 12 ± 6 IU ($n = 20$). This specific activity is clearly lower than that reported for soluble and purified enzyme (25–30 IU [33], and Cuenda, A. and

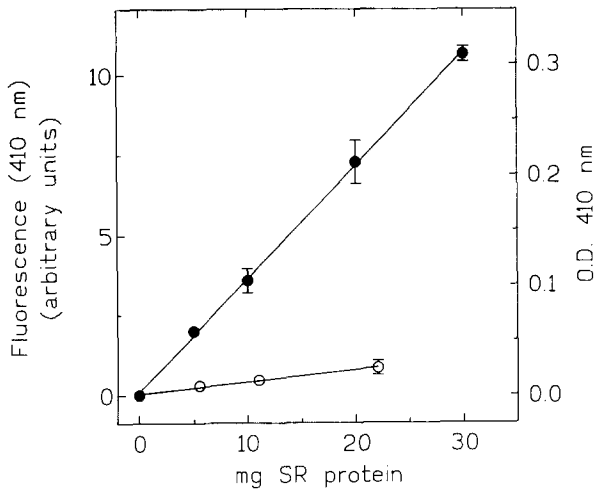

Fig. 5. Dependence upon membrane concentration of the fluorescence intensity (λ_{exc} 335 nm and λ_{em} 410 nm) and absorbance at 410 nm of samples treated with 0.6 M perchloric acid to remove PLP from glycogen phosphorylase copurifying with SR membranes: (•) before and (○) after 100-fold dilution in 50 mM Tes-KOH (pH 7.4), 0.1 M KCl and 0.25 M sucrose followed by centrifugation for 30 min at 78000 × *g*. Other experimental details as indicated in Materials and methods.

Gutierrez-Merino, C. unpublished results), and is consistent with the low activity of phosphorylase in the SR-glycogenolytic complex [18].

3.3. Glycogen phosphorylase removal from SR membranes

The association of phosphorylase with SR membranes is largely reversed (90–95%) by simple dilution into a buffer containing 50 mM Tes-KOH (pH 7.4), 100 mM KCl, 0.25 M sucrose, and then centrifugation for 30 min at an average 78000 × *g*, as shown by (i) the large decrease (≥ 95%) of PLP content of SR membranes (Fig. 5), (ii) the loss of more than 90% of initial phosphorylase activity in the membranes pelleted after dilution, (iii) the presence of glycogen phosphorylase activity in the supernatant (Fig. 6A) and (iv) by the gel electrophoretic pattern of the supernatant (Fig. 6B). Owing to its methodological relevance it is to be noted that no Ca^{2+}-dependent ATPase activity was found in the supernatants after the centrifugation step, and within experimental errors all the initial SR Ca^{2+}-ATPase activity (measured in the presence of 4% (w/w) calcymicin) was recovered in the pellet. Therefore, SR membranes were well precipitated by the centrifugation step. Moreover, phosphorylase was indeed activated upon dissociation from the SR membrane, reaching specific activities in the supernatant of 20–27 IU, values that are identical within experimental errors to those determined under the same experimental condi-

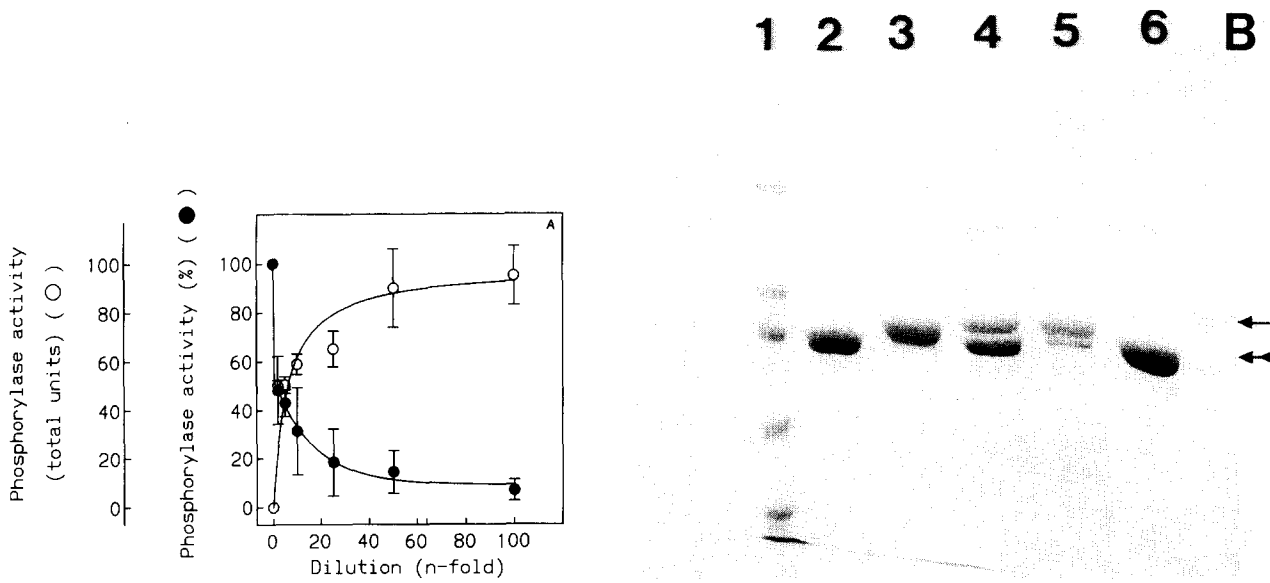

Fig. 6. Dissociation of glycogen phosphorylase from SR membranes by dilution. (A) Dependence of the phosphorylase activity of SR membranes upon dilution in 50 mM Tes-KOH (pH 7.4), 0.1 M KCl and 0.25 M sucrose (100-fold dilution correspond to a final SR protein concentration of 0.2 mg per ml). The phosphorylase activity of pellet (•) and of supernatant (○) after dilution and centrifugation for 30 min at 78000 × *g*, was measured in the direction of glycogen phosphorolysis with the assay medium indicated in Fig. 2. The phosphorylase activity of the pellet is given as percentage of total activity of SR membranes before the dilution step. Total units in the supernatant are given in μmol product per min per 18 mg SR protein. (B) SDS-PAGE of purified glycogen phosphoryalse (3 μg) prepared as indicated in Materials and methods (lane 2); 3 μg of ATPase purified following the protocol of MacLennan (1970) (lane 3); 6 μg of SR membranes prepared as indicated in Materials and methods (lane 4); and 6 μg of pellet proteins (lane 5) and 6 μg of supernatant proteins (lane 6) from SR membranes diluted 100-fold in the buffer indicated in the Panel A and then centrifuged for 30 min at 78000 × *g*. Lane 1, major molecular weight markers: myosin (205000), β-galactosidase (116000), glycogen phosphorylase *b* (97400), bovine serum albumin (66000) and ovalbumin (45000). The arrow and double arrow at the right indicate the bands of ATPase and phosphorylase, respectively. The results shown in Fig. 6B have been obtained using a SR membrane preparation containing approx. 0.27 mg phosphorylase per mg of total SR protein.

tions with purified glycogen phosphorylase (data not shown). Wanson and Drochmans [16] showed that the polysaccharide of glycogen particles plays a major role in maintaining the association of SR membranes with these particles. It is to be noted, however, that about 5% of initial phosphorylase remained bound to the SR membrane even after a combined treatment with α-amylase (0.5%, w/v) and dilution of the SR membrane to a final concentration lower than 0.2 mg/ml. This phosphorylase activity remaining bound to the SR membranes (approx. 3–5% of total activity of untreated SR membranes) was not eliminated even by additional treatment with solutions of high (non-physiological) ionic strength, 1 M KCl.

In addition to glycogen phosphorylase, myokinase and glycosidase are also more than 90% dissociated by dilution to 0.1–0.2 mg SR protein per ml in the buffer indicated above. Nevertheless, it is to be noted that as inferred from the PLP content (in nmol PLP per mg protein) more than 95% of the protein of the supernatant is glycogen phosphorylase (data not shown). In both cases, dissociated myokinase and glycosidase are fully active, as shown by activity measurements of the supernatant. Fig. 6B also shows that the band of approximate M_r 145 000–155 000 of SR membranes is largely reduced after dilution, and consistently it is readily seen in the SDS-PAGE of the supernatant. It is worth-noting here that McIntosh et al. [39] have noticed that treatment of SR membrane with 1 μg/ml α-amylase removes from these membranes a protein of approximate M_r 140 000. Since the presence of muscle contratile proteins contamination in SR membrane preparations was already noticed by MacLennan [38], it is likely that this band is mostly reflecting the presence of residual traces of these proteins. However, neither phosphorylase *b* kinase nor AMP deaminase are significantly dissociated from SR membranes by dilution to 0.1–0.2 mg protein per ml.

4. Discussion

The results presented in this paper confirm previous observations (see the Introduction) that preparations of SR membrane are contaminated with glycogen phosphorylase, and with other enzymes like phosphorylase kinase, myokinase, creatine kinase, glycosidase, AMP-deaminase and phosphoglucomutase. To the best of our knowledge the presence of AMP-deaminase and phosphoglucomutase activities in SR membrane preparations is first described in this paper. Glycogen phosphorylase bound to SR membranes is mostly (at least it is 95%) in its *b* form (dephosphorylated form), since the activity of this enzyme in SR membranes is dependent upon AMP (Fig. 2). The content of phosphorylase varies from 0.03 to 0.37 mg phosphorylase per mg of membrane protein in SR preparations made following different experimental protocols, and we also show that upon simple dilution of SR membrane in a buffer solution containing 50 mM Tes-KOH, 0.1 M KCl, 0.25 M sucrose, 95% of glycogen phosphorylase as well as others enzymes like myokinase and glycosidase can be eliminated from SR vesicles. Several factors can account for the large variation observed in the content of glycogen phosphorylase associated with SR membranes prepared following different protocols. From the results reported in this paper it follows that the different dilutions during the preparation of SR membranes in different protocols is one of the most obvious cause of variations in the content of phosphorylase. However, as illustrated by the results obtained using MacLennan's protocols with minor modifications [19], the starvation period and any treatment producing glycogen depletion in skeletal muscle before killing (like the adrenergic shock) also tend to moderately decrease (2–4-fold) the content of phosphorylase of SR membrane preparations.

Regarding the basic kinetic mechanism of the Ca^{2+}, Mg^{2+}-ATPase catalysis, and the bioenergetics of the Ca^{2+} pump, careful measurements of the rate of phosphoenzyme formation and decomposition, and of phosphoenzyme levels are of utmost importance [40]. Using SR preparations with a high 'contamination' of glycogen phosphorylase (approx. 0.25 mg phosphorylase per mg total SR protein) we have obtained values of 2.5–2.7 nmol phosphoenzyme per mg of total SR protein, under assay conditions used in these type of studies, e.g., 100 mM Mes-Tris (pH 6.0), 20 mM $MgCl_2$, 0.25 mM EGTA, 0.35 mM $CaCl_2$ and 25 μM ATP (see [41]). After treatment of SR membranes to remove glycogen phosphorylase as indicated above, we have obtained values of 4.0 ± 0.25 nmol phosphoenzyme per mg total SR protein. Values of nmol phosphoenzyme per mg of SR protein between 2.5 and 4.0 are currently reported in the literature (see, e.g., [42–44]). By itself, the highest steady state level of phosphoenzyme attained with SR membranes depleted of glycogen phosphorylase excludes a significant contribution of glycogen phosphorylase *a* to the steady state level of phosphoenzyme in SR membrane studies under these experimental conditions. Because dephosphorylation of glycogen phosphorylase *a* is only partly achieved in handlings involving acid precipitation with 5% perchloric acid and filtration through glass fiber filters (results not shown), this merely confirms that the activity of phosphorylase kinase of SR membrane preparations is low, as indicated by the data of Fig. 1.

In addition, the presence of phosphorylase should also produce artifactual results both in differential scanning calorimetry, because the thermal denaturation of glycogen phosphorylase partially overlaps with that of the Ca^{2+},Mg^{2+}-ATPase [45], and in proteolytic

studies of the Ca^{2+}-ATPase in SR membranes. Furthermore, SR membranes to be used to raise monoclonal and/or polyclonal antibodies should be treated to largely remove glycogen phosphorylase to avoid production of antibodies against the latter enzyme. On the other hand, the presence of myokinase might also produce artifactual results in SR membrane preparations to be used in functional studies aiming to rationalize modulation of the ATPase by ADP, or the operation of the Ca^{2+} pump in the reverse mode (e.g., in the direction of ATP synthesis coupled to Ca^{2+} gradient dissipation).

On these grounds, to avoid some of the problems described above, we suggest to introduce a dilution step to 0.1–0.2 mg protein per ml in the protocols of SR membrane preparations, followed by centrifugation at an average $78000 \times g$ in a buffer containing 0.25 M sucrose to prevent partial denaturation of SR membrane proteins by hydrostatic pressure, for example of Ca^{2+}, Mg^{2+}-ATPase [46]. This treatment is better than treatment with α-amylase because of two major reasons: (i) commercial α-amylase is contaminated with glycosidase activity and several major components of SR membrane are glycoproteins [47,48], and (ii) since the activity of glycogen phosphorylase is inhibited by α-amylase, probably by competition for the substrate glycogen (not shown, Nogues, M., unpublished results), after this treatment the phosphorylase that remains associated with SR membranes cannot be accurately measured without further sample handlings.

Acknowledgments

The help of Dr. A.M. Mata in the preparation of antibodies against glycogen phosphorylase is deeply appreciated. Dr. P. Champeil's critical reading and suggestions are gratefully acknowledged. This work has been funded by Grant Nos. PB87-1020 and PB91-0311 of the Spanish DGICYT.A. Cuenda and M. Nogues were the recipient of a Pre-Doctoral Fellowship of the Junta de Extremadura.

References

[1] Levitsky, D.O., Levchenko, T.S., Saks, V.A., Sharov, V.G. and Smirnov, V.D. (1978) Membr. Biochem. 2, 81–96.
[2] Rossi, A.M., Eppenberger, H.M., Volpe, P., Cotrufo, R. and Wallimann, T. (1990) J. Biol. Chem. 264, 5258–5266.
[3] Ronzani, N., Migala, A. and Hasselbach, W. (1979) Eur. J. Biochem. 101, 593–606.
[4] Pickart, C.M. and Jenks, W.P. (1982) J. Biol. Chem. 257, 5319–5322.
[5] Jennissen, H.P., Hörl, W.H., Gröschel-Stewart, U., Velick, S.F. and Heilmeyer, L.M.G. Jr. (1975) in Metabolic Interconversion of Enzymes (Shaltiel, S., ed.), p. 19, Springer-Verlag, Berlin.
[6] Varsanyi, M. and Heilmeyer, L.M.G. Jr. (1981) FEBS Lett. 131, 223–228.
[7] Thieleczek, R., Behle, G., Messer, A., Varsanyi, M., Heilmeyer, L.M.G. Jr. and Dreckhahn, D. (1987) Eur. J. Cell Biol. 44, 333–340.
[8] Hörl, W.H. and Heilmeyer, L.M.G. Jr. (1978) Biochemistry 17, 766–772.
[9] Hörl, W.H., Jennisen, H.P. and Heilmeyer, L.M.G. Jr. (1978) Biochemistry 17, 759–766.
[10] Campbell, K.P. and Shamoo, A.E. (1980) J. Membr. Biol. 56, 241–248.
[11] Chu, A., Sumbilla, C., Inesi, G., Jay, S.D. and Campbell, K.P. (1990) Biochemistry 29, 5899–5905.
[12] Gutierrez-Merino, C. (1983) Trends Biochem. Sci. 8, 8.
[13] Ross, D.C. and McIntosh, D.B. (1987) J. Biol. Chem. 262, 2042–2049.
[14] Cuenda, A., Centeno, F. and Gutierrez-Merino, C. (1991) FEBS Lett. 283, 273–276.
[15] Meyer, F., Heilmeyer, L.M.G. Jr., Haschke, R.H. and Fischer, E.H. (1970) J. Biol. Chem. 245, 6642–6648.
[16] Wanson, J.C. and Drochmans, P. (1972) J. Cell Biol. 54, 206–224.
[17] Entman, M.L., Kaniike, K., Goldstein, M.A., Nelson, T.E., Bornet, E.P., Futch, T.W. and Schwartz, A. (1976) J. Biol. Chem. 251, 3140–3146.
[18] Entman, M.L., Keslensky, S.S., Chu, A. and Van Winkle, W.B. (1980) J. Biol. Chem. 255, 6245–6252.
[19] Gutierrez-Merino, C., Molina, A., Escudero, B., Diez, A. and Laynez, J. (1989) Biochemistry 28, 3398–3406.
[20] Gutierrez-Merino, C., Garcia-Blanco, F., Pocovi, M., Menendez, M. and Laynez, J. (1980) J. Biochem. 87, 1483–1490.
[21] Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Proc. Natl. Acad. Sci. USA 78, 795–799.
[22] Champeil, P., Büschlen-Boucly, S., Bastide, F. and Gary-Bobo, C.M. (1978) J. Biol. Chem. 253, 1179–1186.
[23] Laemmli, U.K. (1970) Nature 277, 680–685.
[24] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[25] Cuenda, A., Henao, F. and Gutierrez-Merino, C. (1990) Eur. J. Biochem. 194, 663–670.
[26] Dubois, M., Giles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350–356.
[27] Krisman, C.R. (1962) Anal. Biochem. 4, 17–23.
[28] Strausbauch, P.H., Kent, A.B., Hedrick, J.L. and Fischer, E.H. (1967) Methods Enzymol. 11, 671–675.
[29] Coburn, S.P. and Mahuren, J.D. (1983) Anal. Biochem. 129, 310–317.
[30] Johnstone, A. and Thorpe, R. (1987) Immunochemistry in practice, 2nd Edn., pp. 30–34, Blackwell Scientific,Oxford.
[31] Graves, D.J., Fischer, E.H. and Krebs, E.G. (1960) J. Biol. Chem. 235, 805–809.
[32] Fletterick, R.J. and Madsen, N.B. (1980) Annu. Rev. Biochem. 49, 31–61.
[33] Graves, D.J. and Wang, J.H. (1972) Enzymes, 3rd Edn, Vol. 7, pp. 435–482.
[34] Fletterick, R.J. and Madsen, N.B. (1977) Trends Biochem. Sci. 2, 145–148.
[35] Barford, D., Hu, S.H. and Johnson, L.N. (1991) J. Mol. Biol. 218, 233–260.
[36] Vereb, G., Fodor, A. and Bot, G. (1987) Biochim. Biophys. Acta 915, 19–27.
[37] Johnson, L.N., Jenkins, J.A., Wilson, K.S., Stura, E.A. and Zanotti, G. (1980) J. Mol. Biol. 134, 639–653.
[38] MacLennan, D.H. (1970) J. Biol. Chem. 245, 4508–4518.
[39] McIntosh, D.B., Ross, D.C., Champeil, P. and Guillain, F. (1991) Proc. Natl. Acad. Sci. USA 88, 6437–6441.
[40] de Meis, L. and Vianna, A.L. (1979) Annu. Rev. Biochem. 48, 275–292.

[41] Champeil, P., le Maire, M., Andersen, J.P., Guillain, F., Gingold, M., Lund, S. and Moller, J.V. (1986) J. Biol. Chem. 261, 16372–16384.
[42] Ikemoto, N., Garcia, A.M., Kurobe, Y. and Scott, T.L. (1981) J. Biol. Chem. 256, 8593–8601.
[43] Lund, S. and Moller, J.V. (1988) J. Biol. Chem. 263, 1654–1664.
[44] Michelangeli, F., Grimes, E.A., East, J.M. and Lee, A.G. (1991) Biochemistry 30, 342–351.
[45] Centeno, F., Fernandez-Salguero, P. and Gutierrez-Merino, C. (1992) J. Bioenerg. Biomembr. 24, 625–634.
[46] Champeil, P., Büschlen, S. and Guillain, F. (1981) Biochemistry 20, 1520–1524.
[47] Michalak, M. (1985) in Enzymes of Biological Membranes (Martonosi, A.N., ed.), Vol. 3, pp. 115–153, Plenum Press, New York.
[48] Campbell, K.P., MacLennan, D.H. and Jorgensen, A.O. (1983) J. Biol. Chem. 258, 11267–11273.