Identification of a 68 kDa protein which copurifies with type-1 protein phosphatase as albumin

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Proteins of 60–70 kDa copurify with some preparations of type-1 or type-2 phosphatases. In our system chromatography on polylysine-Affi-Gel 10 separates a 68 kDa protein from rabbit muscle glycogen particle phosphorylase phosphatase. The separation affects neither the activity nor the size of the phosphatase. The 68 kDa protein, although pure by SDS gel electrophoresis criteria, still displays phosphatase activity of approx. 6–8 U/mg. However, rechromatography either on Bio-Gel A-0.5 m or on Blue Sepharose CL-6B followed by gel filtration shows that the activity is due to a contamination with phosphatases of type 1 and type 2, displaying a molecular mass of 35 kDa, which can be totally removed from the 68 kDa protein. The amino acid composition of the 68 kDa protein is identical to that of rabbit serum albumin, within the limits of variation of the method. Furthermore, the sequence of the 38 N-terminal amino acids is the same in the isolated 68 kDa protein and in rabbit serum albumin.

Protein phosphatase *Phosphorylase phosphatase* *Glycogen metabolism* *(Muscle)* *Albumin* *Amino acid composition* *N-terminal sequence analysis*

1. INTRODUCTION

In crude muscle extracts type-1 phosphatase exhibits a high molecular mass of 250 kDa [1]. However, purification yields either the catalytic subunit of 35–38 kDa solely (review [1,2]), or its complex with the modulator protein (inhibitor-2, 31 kDa) [3,4]. In some preparations a third component of 62–70 kDa copurifies with phosphatase [4,5]. 60–70 kDa components are also present in type-2 phosphatases [6,7] and in phosphatases purified from tissues other than skeletal muscle (review [6]). These proteins have been proposed as subunits of type-2 phosphatase complexes, displaying molecular masses between 250 and 107 kDa [6,7]. However, in none of these systems could a functional role for these 65–70 kDa proteins be demonstrated. Here we report that the 68 kDa protein that in our system copurifies with the glycogen particle phosphatase from rabbit skeletal muscle [8] is identical to albumin.

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Abbreviations: BSA, bovine serum albumin; RSA, rabbit serum albumin; PITC, phenylisothiocyanate; F_A, protein kinase that activates type-1 phosphatase; DTT, dithiothreitol

2. MATERIALS AND METHODS

Polylysine (average 25 kDa), BSA, RSA, ovalbumin and carbonic anhydrase were purchased from Sigma. Brij 35 was from Pierce, Affi-Gel 10 and Bio-Gel A-0.5 m from Bio-Rad and Blue Sepharose CL-6B from Pharmacia. PITC was obtained from Beckman. The solvents for the amino acid analysis were of the highest purity available, Sequanal grade when possible. All the reagents for sequence analysis were obtained from Applied Biosystems. Isolated catalytic subunit of type-1 phosphatase (phosphorylase phosphatase) was purified from glycogen particles of rabbit

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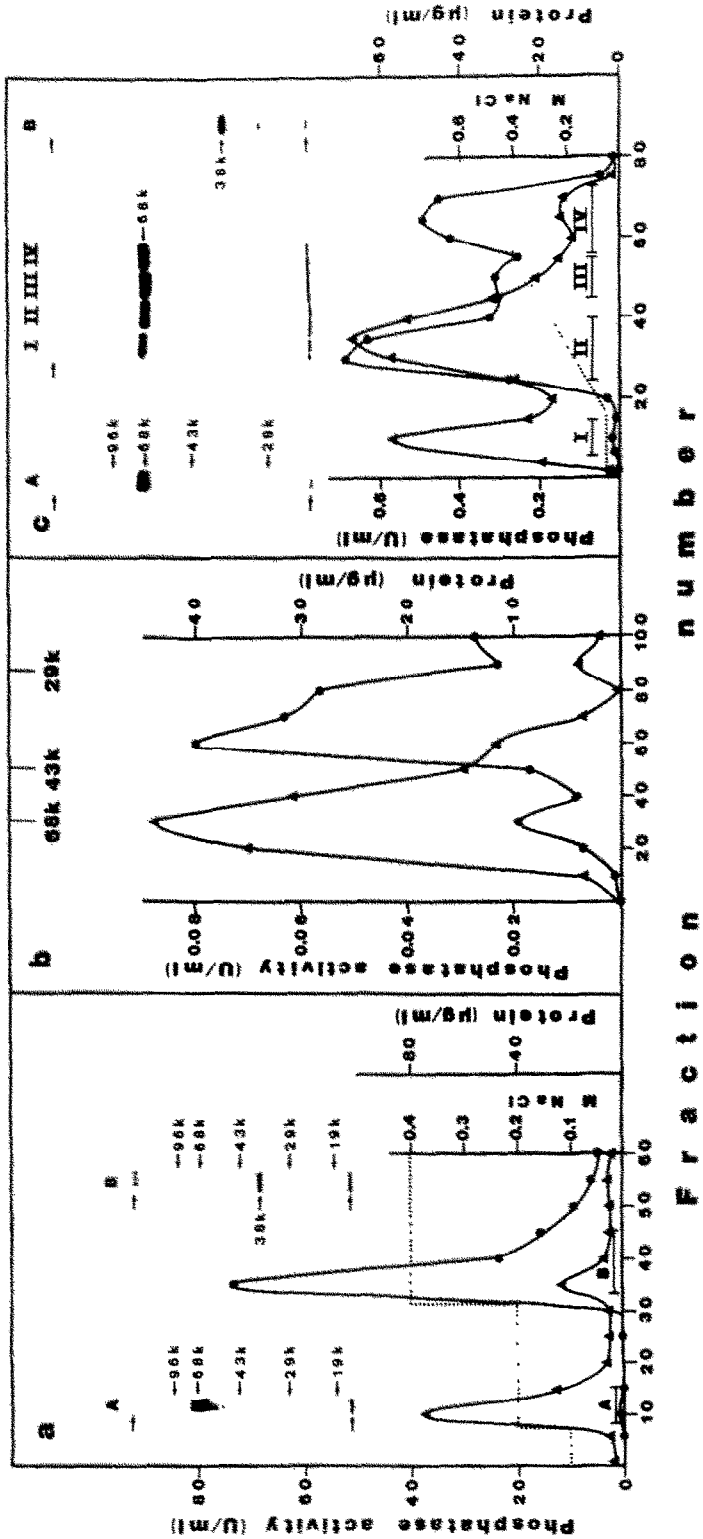

Fig. 1. (a) Separation of the 68 kDa protein from the catalytic subunit of phosphorylase phosphatase purified from glycogen particles of rabbit skeletal muscle [8]. 550 units (675 μg protein) of phosphatase were applied to a 2 ml polylysine-Affi-Gel 10 column [4] equilibrated in 20 mM imidazole, 0.2 mM EDTA, 5% glycerol, 0.01% Brij 35, 1 mM DTT, pH 7.5 (buffer A), containing 0.1 M NaCl, at a flow rate of 5 ml/h. 7 vols of the same buffer containing 0.2 M NaCl and 9 vols of buffer containing 0.4 M NaCl were successively applied and 0.6 ml fractions were collected. (●) Spontaneously active phosphorylase phosphatase, (▲) protein, (– – –) M NaCl. (Inset) Silver-stained SDS-polyacrylamide gel (10%) containing 2 μg protein from pool A or B per lane. Markers (in kDa): phosphorylase (96), BSA (68), ovalbumin (43), carbonic anhydrase (29), soybean trypsin inhibitor (19). (b) Gel filtration of the 68 kDa protein obtained from the polylysine column. 180 μg protein (1.4 phosphatase units) were applied to a 1 × 46 cm Bio-Gel A-0.5 m column, equilibrated in buffer A containing 0.1 M NaCl, at a flow rate of 3.9 ml/h. No protein eluted up to 17.2 ml, when collection of 0.1 ml fractions started. Symbols and markers as in a. (c) Chromatography of the 68 kDa protein obtained from the polylysine column on Blue Sepharose CL-6B. 630 μg protein (5 phosphatase units) were applied to a 1.4 ml column equilibrated in buffer A containing 0.05 M NaCl, at a flow rate of 4 ml/h. Following washing with 2 vols of the same buffer, the protein was eluted with a linear gradient of 7 vols of buffer at 0.05 M NaCl and 7 vols of buffer at 0.6 M NaCl. The fraction size was 0.28 ml. The protein or activity peaks were pooled as indicated. The inset shows a silver-stained SDS-polyacrylamide gel (9%) containing 2–2.5 μg protein per lane. (A) 68 kDa protein, (I–IV) pooled column fractions, (B) catalytic subunit of type-1 phosphatase isolated from the glycogen particles [8]. Symbols and markers as in a.

skeletal muscle [8]. Phosphorylase *b* was purified according to [9]. Phosphorylase phosphatase was assayed by the release of P_i from ^{32}P-labeled phosphorylase *a* [4]. In some experiments preactivations of phosphatase were performed [4,10]. One unit of phosphatase activity is defined as the amount of enzyme that releases 1 nmol P_i per min. Polyacrylamide gel electrophoresis was performed in the presence of SDS [11,12] and the slab gels were silver-stained [13,14]. Protein was determined by the method of Bradford [15] using BSA as standard. Amino acid analysis was performed by reverse-phase HPLC using precolumn derivatization with PITC [16,17]. PTC-amino acids were separated on a Spherisorb ODS II 3 μm column (4.6 × 125 mm) and detected with a Kontron Uvikon 722 LC spectrophotometer at 260 nm. The sequence analysis was performed on an Applied Biosystems model 470 A sequencer. The resultant PTH derivatives were identified and quantified at 269 nm using an isocratic HPLC method [18].

3. RESULTS AND DISCUSSION

The 68 kDa protein is enriched in parallel with the glycogen particle phosphatase (type-1 phosphatase) during 100000 × *g* sedimentation, digestion with α-amylase, DEAE-Sepharose chromatography and acetone precipitation [8]. Most of the catalytic subunit of the phosphatase (38 kDa) can be separated from the 68 kDa protein during chromatography on polylysine-Affi-Gel 10 (fig.1a). SDS gel electrophoresis shows that the 68 kDa protein is eluted from the polylysine column in apparently pure form (fig.1a, inset, lane A). However, phosphatase assay shows that the 68 kDa protein still contains 6–8 U/mg protein. This is a specific activity at least 1000-fold lower than that expected for purified catalytic subunit, and consequently correponds to an amount of protein well below the detection limit on silver-stained SDS gel. This activity can be partially inhibited by the phosphatase inhibitor-2, but cannot be further increased following any of the treatments known to activate inactive phosphatase (i.e. Mn^{2+}, trypsin, trypsin and Mn^{2+}, kinase F_A, as described in [10].

The activity in the 68 kDa protein might be either due to some contamination with 38 kDa catalytic subunit or the 68 kDa protein itself might have some enzyme activity. The latter was suggested for a 70 kDa protein that is obtained together with the sarcoplasmic phosphorylase phosphatase [19]. To distinguish between these two possibilities the 68 kDa protein obtained from the polylysine purification step was additionally subjected to two types of chromatography. The first, gel filtration on Bio-Gel A-0.5 m (fig.1b), shows that 90% of this residual activity elutes at ≈38 kDa and is well separated from the 68 kDa protein, which then contains only a trace amount of activity (0.44 U/mg at most). With the second approach, chromatography on Blue Sepharose CL-6B (fig.1c), 4 protein peaks are obtained, all of which contain the 68 kDa protein (fig.1c, inset, lanes I–IV). The first peak has practically no enzymatic activity. Peak II contains a type-2 phosphatase, since it is not inhibited by inhibitor-2 and treatment with trypsin decreases the activity to 30–40% [4]. Peaks III and IV contain type-1 phosphatase, since they are both totally inhibited by inhibitor-2 and are slightly activated (120–140%) by treatment with trypsin (cf. [8]). In none of these peaks is a protein band in the range 35–38 kDa detectable using silver-stained SDS gel electropherograms (the catalytic subunit of type-1 phosphatase is shown in fig.1c, inset, lane B, for comparison). However, upon rechromatography of peaks II and IV on Bio-Gel A-0.5 m, in both cases the activity elutes at 35 kDa and no activity is detectable at the 68 kDa position (not shown). These results clearly indicate that the activity present in the 68 kDa protein is due to contamination by low-molecular-mass phosphatase catalytic subunits rather than the 68 kDa protein itself having enzyme activity.

Some reports indicate that 60–70 kDa proteins represent subunits of type-2 phosphatases [6,7] and this has also been proposed for cytosolic type-1 phosphatase [5]. In our case, as well as in that of the sarcoplasmic phosphatase purified according to [4], removal of the 68 kDa protein from the phosphatase changes neither the characteristics nor the size of the enzyme. Moreover, there is no indication of an interaction when the isolated catalytic subunit [8] or its complex with inhibitor-2 is remixed with the 68 kDa protein, as assessed by gel filtration (not shown). Thus, in our system the 68 kDa protein does not behave as a phosphatase subunit but rather as a contaminant. This was

Table 1

Amino acid composition

Amino acid	Residues per mol	
	Rabbit serum albumin	68 kDa protein
Asx	39.3	47.5
Glx	67.7	73.3
Ser	29.6	28.3
Gly	23.6	33.6
Thr	32.6	25.5
Ala	53.9	59.6
His	28.6	20.2
Pro	39.1	34.1
Arg	29.1	30.7
Tyr	29.4	26.2
Val	39.7	39.7
Met	2.5	5.0
Ile	19.9	18.8
Leu	69.5	63.4
Phe	29.8	29.0
Lys	77.0	65.3
Cys	n.d.	n.d.
Trp	n.d.	n.d.

Results represent the mean values of duplicate analyses of 2 different hydrolysates (1 h at 150°C)

already proposed in the case of the sarcoplasmic phosphatase, suggesting also that the 70 kDa contaminant might, for instance, be rabbit albumin [4]. Indeed, the 68 kDa protein isolated here displays on 6% polyacrylamide SDS gels the same molecular mass as RSA, which is ≈1 kDa higher than that of BSA under these same conditions.

To identify further this 68 kDa protein we performed amino acid analysis. The compositions of RSA and 68 kDa protein are indeed identical within the limits of variation of the method (table 1). Further proof was obtained by N-terminal sequence analysis of the two proteins. The sequence of the first 38 amino acid residues of the 68 kDa protein and of RSA is identical, as shown in fig.2. Furthermore, the extrapolated initial yield of the PTH-amino acids represents, in the case of the 68 kDa protein, over 90% of what could be calculated from the amount of protein applied, showing that the protein being sequenced corresponds to practically all of the protein present in the sample. This comparison demonstrates that the isolated 68 kDa protein is RSA. Albumin has been shown previously to be synthesized by the muscle itself [20], which rules out the possibility that it is present due to contamination of the tissue with blood.

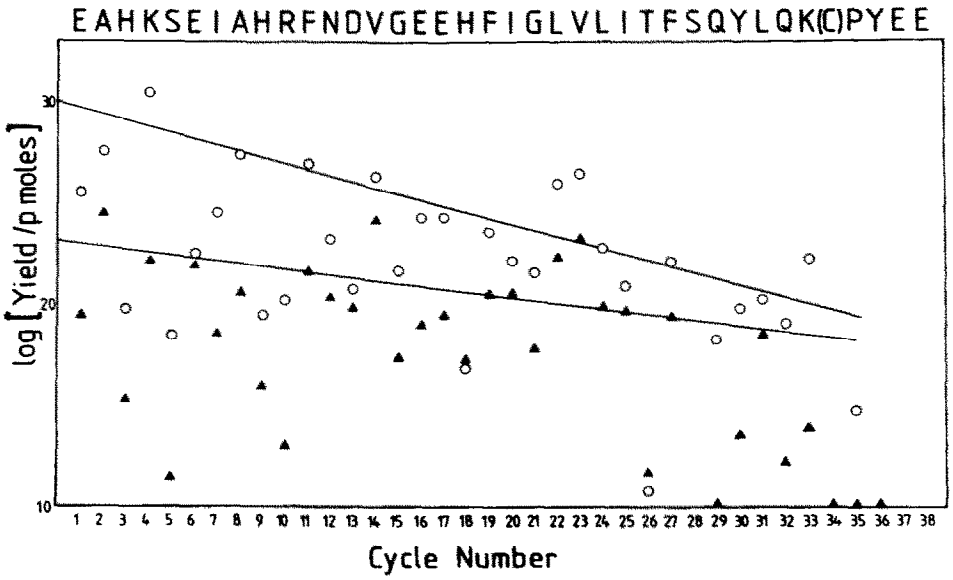

Fig.2. N-terminal sequence analysis of the 68 kDa protein and of RSA. 200 pmol of 68 kDa protein (▲) or 2000 pmol RSA (○) were applied to the automatic sequencer. The amounts of protein applied were calculated from amino acid determinations. The extrapolated initial yield was 90% in the case of the 68 kDa protein and 50% in the case of RSA.

At the present stage we do not know whether the copurification of albumin and phosphatase catalytic subunit is due to unspecific binding or whether albumin serves as carrier in the cell as it does in blood.

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