PHOSPHORYLASE PHOSPHATASE: MOLECULAR CONFIGURATION AND ACTIVITY

L.R. KALALA, J. GORIS and W. MERLEVEDE

Afdeling Biochemie, Departement Humane Biologie, Fakulteit Geneeskunde, Katholieke Universiteit te Leuven, Belgium

Received 19 March 1973

1. Introduction

Phosphorylase phosphatase of bovine adrenal cortex [1], liver [2] and skeletal muscle [3], has been shown to exist in an active form and an inactive form; activation of the inactive form can be obtained by ATP in the presence of Mg ions. In preliminary observations with gel filtration [4, 5] it appeared that the active and inactive forms obtained from adrenal cortex as well as from liver had different molecular weights*.

In this communication we report that phosphorylase phosphatase from adrenal cortex can be separated by gel filtration into an active form (M_r = 195 000) and an inactive form (M_r = 88 000); in sucrose density gradient centrifugation two sedimentation coefficients (corresponding M_r 139 000 and 54 500) were observed for the active form, while the inactive form displayed only one sedimentation coefficient (M_r = 54 500). The enzyme from liver could be separated by gel filtration in three forms: two active forms (M_r = 215 000 and 77 000) and one inactive form (M_r = 138 000). In sucrose density gradient centrifugation one sedimentation coefficient (corresponding M_r = 59 000) was displayed by both an active and the inactive form of the enzyme while another active form was shown to have a M_r of about 155 000. After activation of both preparations by ATP-Mg the elution patterns were unchanged, but in gradients the enzyme was recovered under the active form.

* M_r is the abbreviation used for molecular weight.

2. Materials and methods

2.1. *Chemicals and enzymes*

Marker proteins (γ-globulin, bovine serum albumin, myoglobin) as well as DNP-L-alanine were purchased from Serva (Germany). The purified [6] β-lactoglobulin-A was a gift of L. Vanduffel. Blue Dextran 2000 was obtained from Pharmacia (Sweden).

The active liver phosphorylase was the same as used previously [2].

For the preparation of phosphorylase phosphatase from adrenal cortex slices were homogenized in 1 vol of 10 mM Tris-HCl (pH 7.4) and centrifuged during 10 min at 8 000 *g*. When not otherwise indicated, all steps were carried out at 4°. The extract was spun during 30 min at 40 000 *g*, and the supernatant incubated at 30° during 5 min in the presence of 0.5% glycogen. After cooling to 0°, glycogen was precipitated by adding 0.25 vol absolute ethanol at −20°, and centrifuging at −15° for 20 min at 8 000 *g*. The supernatant was dialysed against 10 mM Tris (pH 7.4) and brought to 30% saturation by adding solid $(NH_4)_2SO_4$ while adjusting to pH 7 with 1 mM NH_4OH. After standing for 15 min at 0° the mixture was centrifuged for 20 min at 8 000 *g*. The supernatant was brought to 60% saturation with solid $(NH_4)_2SO_4$ and precipitation was carried out in the same way. The precipitate was dissolved in a volume of 10 mM Tris (pH 7.4) equal to 0.10 of the weight of the initial material and dialysed against the same buffer. The preparation could be stored at −20° for months. The ratio of the inactive form of phosphorylase phosphatase over the active form was nearly the same as in the homogenate (about 1:1), the increase in specific activity about 20-fold. Neither phosphorylase, nor phosphorylase kinase could be detected in the purified preparation.

North-Holland Publishing Company – Amsterdam

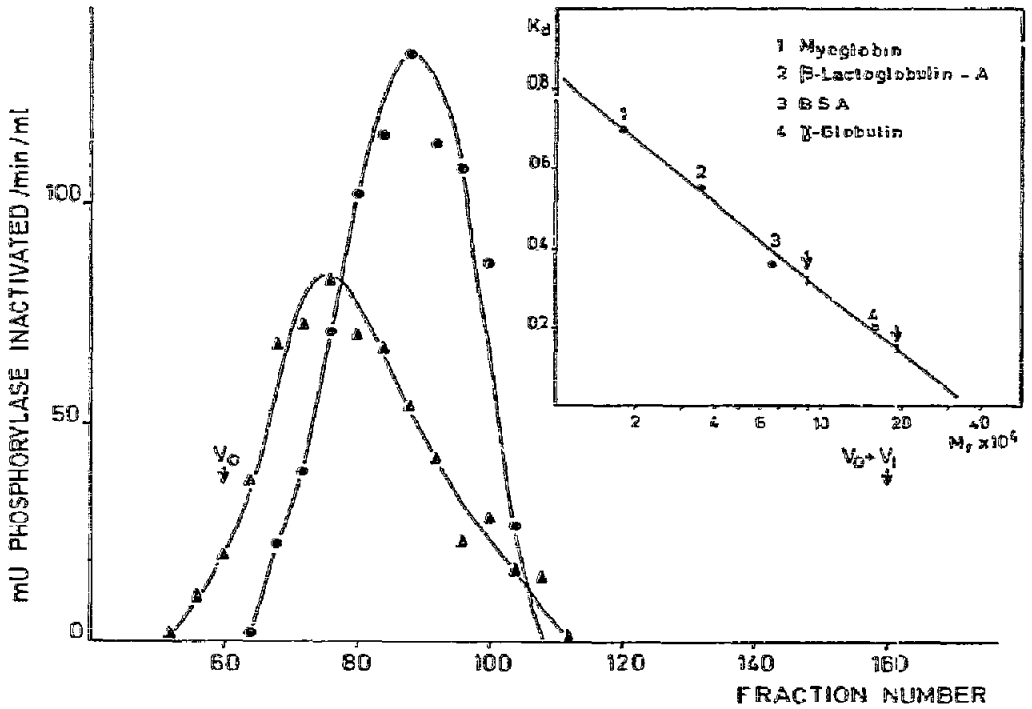

Fig. 1. Elution profile of adrenal cortex phosphorylase phosphatase on Sephadex G-200, assayed after preincubation with (●—●—●) and without (▲—▲—▲) ATP-Mg.

Full activation of the enzyme preparation was obtained within 15 to 20 min by incubation in the presence of 4 mM ATP and 5 mM Mg ions at 30°. An activation to the same extent was observed with high Mg^{2+} concentrations, the K_m for this activation being about 60 mM Mg^{2+}. With other bivalent metal ions activation of the phosphatase could also be obtained [7].

Phosphorylase phosphatase was isolated from dog liver perfused with 0.15 M NaCl [8].

2.2. *Methods*

Phosphorylase phosphatase activity was measured by a method previously described [8], the concentration of caffeine in the assay mixture being 5 mM (adrenal cortex) or 0.5 mM (liver). Phosphatase activity is expressed as mU of phosphorylase inactivated per min and per ml. Activation of the inactive phosphatase in the elution and centrifugation fractions was obtained by preincubation for 15 min at 30° in the presence of 4 mM ATP and 5 mM $MgSO_4$ (adrenal cortex) or 0.4 mM ATP and 0.5 mM $MgSO_4$ (liver).

Columns (2.5 X 100 cm) of Sephadex G-200, equilibrated with 10 mM Tris (pH 7.4) were eluted by upward flow at 4° using the same buffer. Constant flow rates were maintained between 12 and 18 ml/hr by means of a peristaltic pump. The ultraviolet absorption of the column eluate was monitored at 280 nm. The columns were calibrated with the following proteins: γ-globulin, M_r = 160 000; bovine serum albumin (BSA), M_r = 67 000; β-lactoglobulin-A, M_r = 36 000 and myoglobin, M_r = 17 800. V_o and V_i were determined with Blue Dextran and DNP-L-alanine. The slight retention of DNP-L-alanine when compared to sucrose was of no significance in the calculations of M_r. After calibration of the column each filtration run was done in the presence of Blue Dextran (4 mg) and DNP-L-alanine (2 mg). From the K_d values, a linear plot was calculated by the method of least squares and used to estimate the molecular weights [9].

Centrifugation in a sucrose density gradient [10] was carried out at 4° with the 9792 swinging bucket rotor in an Omega II Martin Christ ultracentrifuge. The sample (adrenal cortex: 0.1 ml containing ±3 mg of protein; liver: 0.2 ml containing ±2 mg of protein)

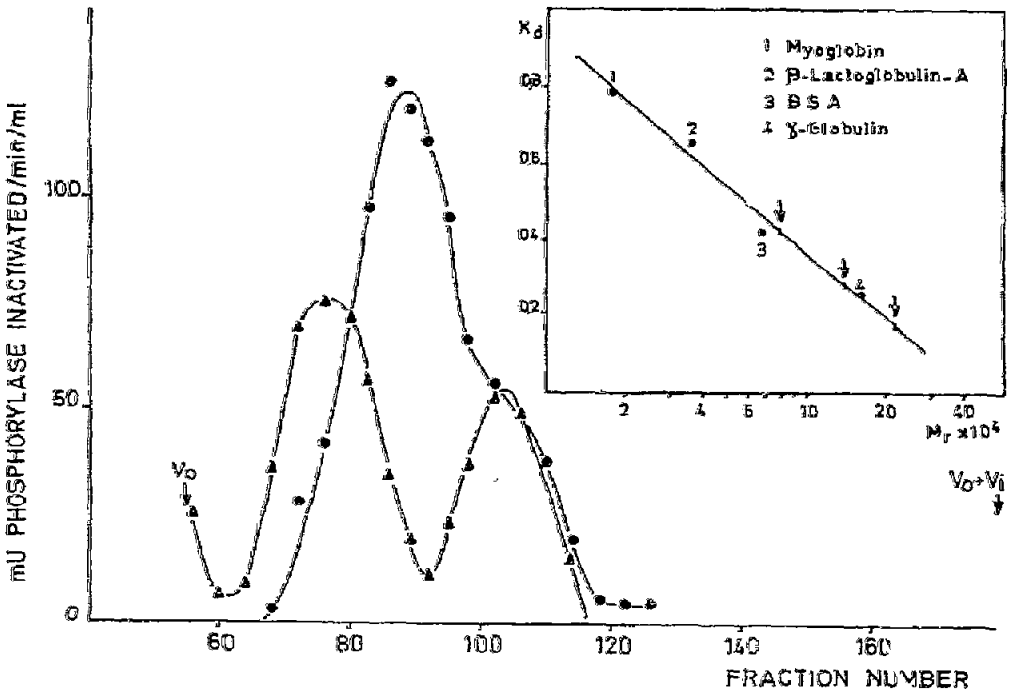

Fig. 2. Elution profile of liver phosphorylase phosphatase on Sephadex G-200, assayed after preincubation with (●—●—●) and without (▲—▲—▲) ATP-Mg.

was layered on a linear 5 to 20% (w/v) sucrose gradient (5 ml) in 10 mM Tris pH 7.4. For the calculation of $s_{20,w}$ either hemoglobin: $s_{20,w} = 4.6$ (adrenal cortex), or β-lactoglobulin-A: $s_{20,w} = 3$ (liver), were used as reference proteins.

3. Results and discussion

When the phosphorylase phosphatase preparation from adrenal cortex was eluted from a Sephadex G-200 column (fig. 1) two different fractions were obtained: one active form displaying a M_r of 195 000 and a second with a M_r of 88 000 which became active only after incubation with ATP-Mg. The trailing of the first active form (fig. 1) and the irregular appearance of a small active peak coincident with the major inactive one (not shown), suggest the existence of another active form with a M_r of about 88 000. A similar elution pattern was observed with the fully activated enzyme preparation.

With the liver enzyme three different forms were eluted from a Sephadex G-200 column (fig. 2): two active forms with molecular weights of about 215 000 and 77 000, and one inactive form with a M_r of about 138 000. As with the enzyme from adrenal cortex a similar elution pattern was observed with an enzyme preparation previously activated with ATP-Mg.

In a sucrose density gradient both the active and inactive forms of phosphorylase phosphatase from adrenal cortex sedimented as a main peak with $s_{20,w} = 4.15$ corresponding to a M_r of 54 500; in about half of the experiments active phosphatase also sedimented with a $s_{20,w}$ of about 7.7 corresponding to a M_r of 139 000 (fig. 3a). By full activation of the preparation the inactive phosphatase was converted to, and recovered as an active form with the same molecular weight (fig. 3b).

Similar results were obtained with liver phosphorylase phosphatase. Both the active and inactive forms sedimented with a $s_{20,w}$ value of 4.35, corresponding to a M_r of 59 000. A second active phosphatase displayed a mean $s_{20,w}$ value of 8.3 (corresponding M_r of 155 000), but with considerable variability (fig. 4a and b). After full activation the enzyme remained active in the gradient and displayed very similar sedimentation coefficients (fig. 4b).

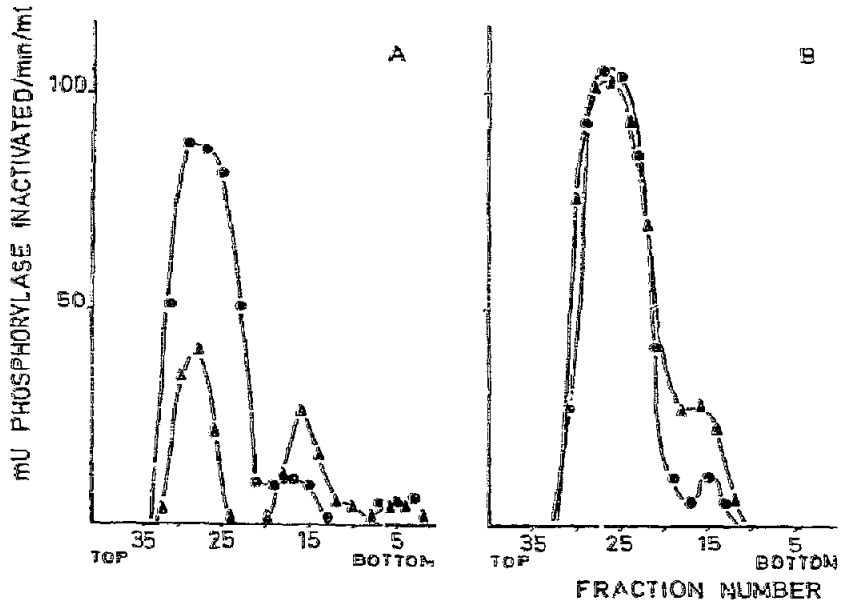

Fig. 3. Sedimentation profile of adrenal cortex phosphorylase phosphatase in a sucrose gradient before (A) and after (B) activation by ATP-Mg, and assayed after preincubation with (●—●—●) and without (▲—▲—▲) ATP-Mg.

The active forms obtained in both types of experiments (figs. 1 to 4) appeared to be inhibited by ATP-Mg. This inhibition was clearly evident in the absence of inactive phosphatase.

From these data it can be concluded that in adrenal cortex as well as in liver two active and one inactive form of phosphorylase phosphatase can be identified. The discrepancies between the M_r values

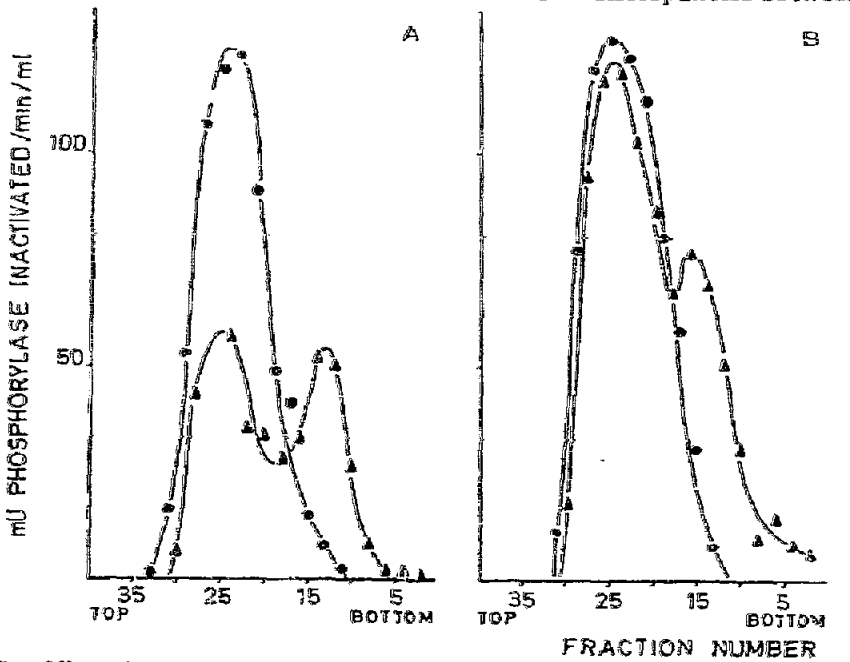

Fig. 4. Sedimentation profile of liver phosphorylase phosphatase in a sucrose gradient before (A) and after (B) activation by ATP-Mg, and assayed after preincubation with (●—●—●) and without (▲—▲—▲) ATP-Mg.

obtained in sucrose density gradient centrifugation and gel filtration, are probably related to the different separation principles, and similar observations have been reported by other authors [11, 12]. Full activation of the enzyme preparation did not cause a change in the elution pattern with gel filtration, while after a sucrose gradient the enzyme was recovered under the active form. Therefore it appears that only during gel filtration could the activated phosphatase be separated from an unknown stabilizing factor.

The existence of one active phosphatase with a high M_r deserves some comments. It could result from the aggregation of active enzyme of lower M_r; indeed, when the 4.15 S active enzyme (figs. 3–4) is chromatographed on Sephadex G-200, the same pattern as that of figs. 1 and 2 emerges. Since this persists even when the column is eluted with 10% sucrose, it is puzzling that this aggregation is much less apparent in a sucrose density gradient. This high M_r could also have resulted from an association with either phosphorylase or glycogen. However, no phosphorylase was measured in the samples applied to the column, even in the presence of 2 mM AMP which confers a definite activity to phosphorylase *b* from liver and adrenal cortex [1]. Treatment of the sample with α-amylase did not change the elution pattern. Since gel filtration of the 40 000 *g* supernatant of an untreated homogenate produced an elution profile similar to that of the more purified preparation, it seems that no artefact is responsible for the high M_r species of the active form.

Acknowledgements

The authors are grateful to Mrs. M. Buckinx-Veeckmans and Mrs. R. Cabergs-Verbiest for expert technical assistance. This work was supported by a grant of the "Fonds voor Wetenschappelijk Geneeskundig Ongerzoek", and by a fellowship (L.R.K.) awarded by the "Office de la Cooperation au Développement".

References

[1] W. Merlevede and G.A. Riley, J. Biol. Chem. 241 (1966) 3517.
[2] W. Merlevede, J. Goris and C. De Brandt, European J. Biochem. 11 (1969) 499.
[3] C.A. Chelala and H.N. Torres, Biochim. Biophys. Acta 178 (1969) 423.
[4] W. Stalmans and W. Merlevede, Arch. Int. Physiol. Biochim. 75 (1967) 369.
[5] J. Goris and W. Merlevede, Arch. Int. Physiol. Biochim. 80 (1972) 967.
[6] R. Aschaffenburg and J. Drewry, Biochem. J. 65 (1957) 273.
[7] J.L. Roosemont and W. Merlevede, Arch. Int. Physiol. Biochim. 80 (1972) 981.
[8] J. Goris, L. Pinnenborg-Vercruysse and W. Merlevede, Biochim. Biophys. Acta 268 (1972) 158.
[9] B.J. Gelotte, J. Chromatogr. 3 (1960) 330.
[10] R.G. Martin and B.N. Ames, J. Biol. Chem. 236 (1961) 1372.
[11] E.M. Reimann, D.A. Walsh and E.G. Krebs, J. Biol. Chem. 246 (1971) 1986.
[12] S.A. Schwartz and D.R. Helinski, J. Biol. Chem. 246 (1971) 6318.