ATP-Mg-DEPENDENT PHOSPHORYLASE PHOSPHATASE IN MAMMALIAN TISSUES

Shiaw-Der YANG, Jackie R. VANDENHEEDE, Jozef GORIS and Wilfried MERLEVEDE

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

Received 27 November 1979
Revised version received 19 December 1979

1. Introduction

In [1] we described in dog liver a phosphorylase phosphatase which depends on the interaction of two protein fractions and ATP-Mg for activity. These two protein fractions (called F_A and F_C) can be separated by DEAE-cellulose chromatography, and recombination of both fractions in the presence of ATP-Mg results in an active phosphatase enzyme. We have now extended these observations to other tissues and animals: fresh high-speed supernatant fractions of both rat and rabbit tissues (liver, heart and skeletal muscle) were shown to contain a similar ATP-Mg requiring phosphatase system. It was observed that this phosphatase constitutes an important fraction of the total phosphorylase phosphatase activity present in these high-speed supernatant fractions.

2. Materials and methods

Crystalline phosphorylase *b* from rabbit skeletal muscle was purified following [2], its specific activity was ~60 U/mg protein. Phosphorylase *b* kinase was isolated from the same source according to [3,4]; its specific activity at pH 8.2 varied from 2 000–7 000 U/mg protein. [^{32}P]Phosphorylase *a* (2.5×10^6 counts . mm^{-1} . mg $protein^{-1}$) was prepared from phosphorylase *b* using [γ-^{32}P]ATP, cyclic AMP and phosphorylase *b* kinase as in [5].

ATP, cyclic AMP and dithiothreitol were obtained from Sigma Chemical Co. (USA) and DEAE–Sephacel from Pharmacia (Sweden). [γ-^{32}P]ATP was purchased from the Radiochemical Centre (England) and Hydroluma from Lumac (Switzerland).

ATP-Mg-dependent phosphatase (F_C) and its activating protein fraction F_A were obtained from dog liver as in [6].

Protein determinations were done as in [7] with bovine serum albumin as a standard, or estimated from A_{280} assuming an absorption index of 1.0 for 1 mg protein/ml solution.

Phosphorylase phosphatase activity was measured by the release of [^{32}P]phosphate from [^{32}P]phosphorylase *a*: the assay mixture (60 μl) contained 20 mM Tris–HCl (pH 7.4), 1 mM dithiothreitol, 5 mM caffeine, 1 mg/ml of bovine serum albumin and 1 mg/ml of [^{32}P]phosphorylase *a*. Caffeine was included in the phosphatase assay mixture to counteract the effect of AMP, that might be produced during the preincubation reaction. After a 10 min incubation at 30°C, the reaction was stopped by the addition of 250 μl ice cold 20% trichloroacetic acid and 250 μl 6 mg/ml of bovine serum albumin. After 15 min in ice water, the samples were centrifuged and the radioactivity of 0.45 ml portions of the supernatant fluid was measured in 2 ml scintillation fluid (Hydroluma). One unit of phosphatase releases 1 nmol [^{32}P]phosphate/min.

The ATP-Mg-dependent phosphatase was commonly measured after a 10 min preincubation with a saturating concentration of the activating protein fraction F_A from dog liver, 0.5 mM ATP, 2.5 mM $MgSO_4$, 20 mM Tris–HCl (pH 7.4), 1 mM dithiothreitol and 1.0 mg/ml of bovine serum albumin. The spontaneously active phosphatase activity was determined in a similar way, except that F_A and ATP-Mg were omitted from the preincubation mixture.

To measure F_A in the column eluates, the preincubation used was essentially as above, except that

Address correspondence to: Professor Dr W. Merlevede, Afdeling Biochemie, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium

the protein fractions were incubated with 2 U/ml of partially purified (inactive) dog liver ATP-Mg-dependent phosphatase [6]. The activity of F_A is measured by the activation of this phosphatase, and is expressed as the % of the maximal activation that can be produced.

To follow the time course of activation of the ATP-Mg-dependent phosphatase from rabbit liver, heart and muscle, a 2 min phosphatase assay was used. The activities were determined on the pooled column fractions after a time course preincubation with their respective activating protein fractions (pooled from the same column).

Adult male Wistar rats were decapitated and liver, heart and back muscle quickly removed and chilled in ice. Rabbits were killed by severing the neck, thoroughly bled and again the liver, heart and some back muscle were taken and put on ice. Heart and muscle were homogenized using a low speed overhead mixer (Novamix II) whereas the livers were homogenized in a motor-driven glass teflon Potter-Elvehjem homogenizer. The rabbit heart (7 g tissue), rabbit and rat liver (10 g tissue) and muscle tissues (30 g tissue) were homogenized individually (only tissue from one animal was used), whereas 20 rat hearts (30 g tissue) were homogenized together per experiment described. After homogenization in 3 vol. ice-cold buffer containing 20 mM Tris–HCl (pH 7.4), 1 mM dithiothreitol, 4 mM EDTA and 250 mM sucrose, a 10 min 8000 × *g* extract was made, and filtered through glass wool. The filtered extract was then centrifuged for 1 h at 100 000 × *g* and the resulting clear supernatant again filtered through glass wool. The rabbit heart, liver and rat liver high-speed supernatants were put entirely on DEAE–Sephacel columns (1.0 × 10 cm), whereas from the rat heart and the muscle tissues, only 1/3rd of the volume of the high-speed supernatants were used, corresponding in all cases to ~10 g tissue. The DEAE–Sephacel was equilibrated with 20 mM Tris–HCl (pH 7.4) and 1 mM DTT, and after a sample was applied, the column was washed with 50 ml of this buffer, before a 200 ml linear gradient going from 0–0.4 M NaCl in the same buffer was used to elute the proteins.

3. Results

Fig.1 shows the elution profiles on DEAE–Sephacel of the rat liver, heart and skeletal muscle preparations. The column was assayed in 3 different ways: for spontaneously active phosphorylase phosphatase, for ATP-Mg-dependent phosphatase and also for the presence of the phosphatase activating protein fraction F_A, as outlined in section 2.

As can be deduced for the 3 graphs, there was very little qualitative difference in the elution pattern of the F_A and phosphatase activities between the different rat tissues used. Rabbit liver, heart and muscle gave very similar results (not shown). In all cases, the F_A activities eluted in two peaks, analogous to the result obtained fror dog liver [1]. The ATP-Mg-dependent phosphatase eluted later in the gradient (with 0.2–0.25 M NaCl), together with some of the spontaneously active phosphorylase phosphatase. The peak activity fractions were pooled and assayed for spontaneously active phosphorylase phosphatase and for ATP-Mg-dependent phosphatase in the presence of ATP-Mg and saturating amounts of dog liver

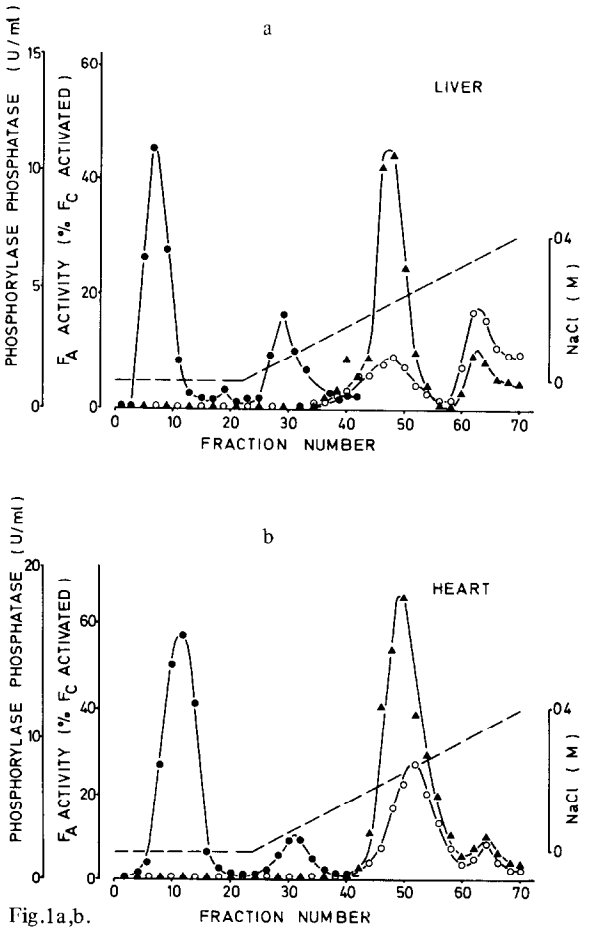

Fig.1a,b.

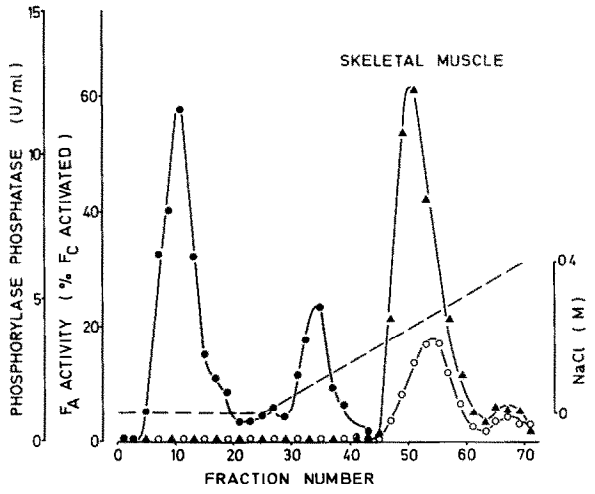

Fig.1. Elution profile of the ATP-Mg-dependent phosphorylase phosphatase and the phosphatase activating protein fraction F_A from rat tissues on DEAE–Sephacel chromatography High speed supernatant fractions from rat liver, heart and skeletal muscle were applied to a DEAE–Sephacel column, and fractionated with a linear NaCl gradient (– – –). The eluate was assayed for spontaneously active phosphorylase phosphatase (○), ATP-Mg-dependent phosphatase (▲) and for F_A (●), as in section 2. The activity of F_A is expressed as % of the maximal activation of the phosphatase.

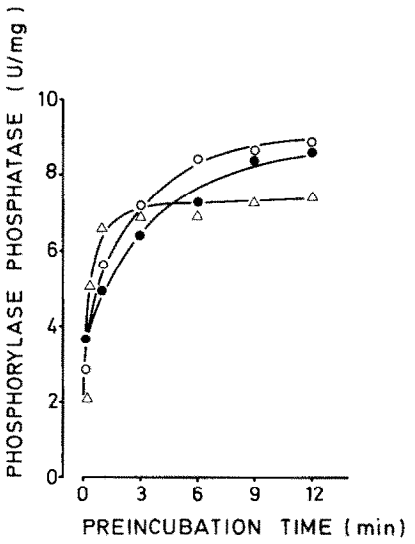

Fig.2. Time-dependent activation of the ATP-Mg-dependent phosphatase from rabbit liver, heart and skeletal muscle by the activating protein fraction F_A of the corresponding tissues. Activations of the ATP-Mg-dependent phosphatase from rabbit liver (△), heart (○) and muscle (●) were measured as in section 2.

F_A. Results for all the tissues examined are shown in table 1.

The time dependency of the F_A-mediated activation of the ATP-Mg-dependent phosphatase was investigated for rabbit liver, heart and skeletal muscle preparations. The pooled ATP-Mg-dependent phosphatase fractions from the DEAE–Sephacel columns were preincubated with aliquots of their correspondingly pooled F_A fractions. The first peak of F_A activities were used here, since its activity was usually much greater than the second F_A activity peak. Fig.2 shows the time-dependent activation for the three rabbit tissues used. The rabbit heart and muscle enzyme were activated at a comparable rate, whereas the activation of the liver enzyme was much more rapid. Similar amounts of protein were used for the ATP-Mg-dependent phosphatase and F_A in the activation reactions for all these tissues.

Table 1
Phosphorylase phosphatase activities of the pooled DEAE–Sephacel column fractions from different mammalian tissues measured in the absence or presence of ATP-Mg and dog liver F_A

Tissues	Phosphatase activity	
	No additions U/mg protein	In the presence of F_A, ATP and Mg (U/mg protein)
Rat		
Liver	0.62	9.90
Heart	14.0	25.80
Muscle	1.70	10.20
Rabbit		
Liver	3.15	6.70
Heart	3.33	8.80
Muscle	4.10	8.13

4. Discussion

Liver, heart and skeletal muscle extracts obtained from rats and rabbits were shown to contain an ATP-Mg-dependent phosphatase enzyme and an activating protein fraction F_A similar to [1,6] in dog liver and bovine adrenal cortex [8]. The inactive phosphatase of each tissue could be activated in an ATP-Mg-dependent way by either dog liver F_A or by the activating factor separated from the corresponding tissue

extract. The activating protein fraction (F_A) of each tissue could be assayed in the column eluates by the activation of the inactive dog liver ATP-Mg-dependent phosphatase. These results suggest that this ATP-Mg-dependent phosphatase system is present in most tissues. Two protein fractions are involved, and both are present in and interchangeable between the tissues used, which emphasizes even more the importance of this phosphatase enzyme. The activated ATP-Mg-dependent phosphatase was shown to be very sensitive to the heat-stable phosphatase inhibitors [9], and recent results obtained with the dog liver enzyme system have shown that the phosphatase activating factor could not be separated from a cyclic AMP- and Ca-independent synthase kinase present in the same F_A preparation [6]. The ATP-Mg-dependent phosphatase is not easily demonstrated in crude extracts of most tissues for two major reasons. The first is the presence of the spontaneously active phosphorylase phosphatase which is inhibited by free ATP (K_i 7 μM) [10]. Thus incubation of crude extracts with ATP-Mg produces a simultaneous activation of the ATP-Mg-dependent phosphatase and an inhibition of the spontaneously active enzyme, leading to little net increase (or even a decrease) in phosphorylase phosphatase activity. The second reason is the presence of heat-stable protein phosphatase inhibitors which inhibit the ATP-Mg-dependent enzyme more potently than the spontaneously active enzyme [9].

Acknowledgements

The authors are grateful to R. Verbiest for expert technical assistance. This work was supported by the Fonds voor Wetenschappelijk Geneeskundig Onderzoek. S.-D.Y. is a recipient of a KUL scholarship. J. R. V. is Bevoegdverklaard Navorser van het NFWO.

References

[1] Goris, J., Defreyn, G. and Merlevede, W. (1979) FEBS Lett. 99, 279–282.
[2] Fischer, E. H. and Krebs, E. G. (1958) J. Biol. Chem. 231, 65–71.
[3] Hayakawa, T., Perkins, J. P., Walsh, D. A. and Krebs, E. G. (1973) Biochemistry 12, 567–573.
[4] De Lange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A. and Krebs, E. G. (1968) J. Biol. Chem. 243, 2200–2208.
[5] Krebs, E. G., Kent, A. B. and Fischer, E. H. (1958) J. Biol. Chem. 231, 73–83.
[6] Goris, J., Doperé, F., Vandenheede, J. R. and Merlevede, W. (1980) submitted.
[7] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[8] Merlevede, W., Defreyn, G., Goris, J., Kalala, L. R. and Roosemont, J. (1976) Arch. Int. Physiol. Biochim. 84, 359–378.
[9] Goris, J., Defreyn, G., Vandenheede, J. R. and Merlevede, W. (1979) Eur. J. Biochem. 91, 457–464.
[10] Goris, J., Defreyn, G. and Merlevede, W. (1977) Biochimie 59, 171–178.