RESOLUTION OF THE ATP-Mg-DEPENDENT PHOSPHORYLASE PHOSPHATASE FROM LIVER INTO A TWO PROTEIN COMPONENT SYSTEM

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1. Introduction

The inactivation of a hormone-sensitive lipase from rat adipose tissue with ATP in the presence of Mg^{2+} has been described [1] and the resolution of an ATP-Mg-dependent system for the degradation of proteins in rabbit reticulocytes into two protein components reported [2]. An inhibition of phosphorylase phosphatase by ATP has been described in some enzyme preparations [3–7] whereas other preparations of phosphorylase phosphatase in bovine adrenal cortex [8,9], avian skeletal muscle [10] and dog liver [6] are activated by ATP-Mg. From these observations one may conclude that ATP-Mg could play an important role in the regulation of these enzyme systems. The ATP-Mg-dependent phosphorylase phosphatase present in liver cytosol constitutes an important fraction of the total phosphorylase phosphatase activity [6]; its activity is controlled by heat stable protein inhibitors, one of which is activated by cyclic AMP-dependent protein kinase [11]. We describe here that this phosphorylase phosphatase activity results from the interaction of at least two different thermolabile fractions which can be separated by DEAE-cellulose chromatography. Part of this work has been reported in [12].

2. Materials and methods

A partially purified preparation of ATP-Mg-dependent phosphorylase phosphatase (EC 3.1.3.17) was obtained from dog liver cytosol as in [6]. This preparation, 20 ml (± 30 mg protein/ml) was applied to a DEAE-cellulose column (2.5 × 20 cm), equilibrated with 10 mM Tris–HCl (pH 7.8) at 4°C. After washing with an additional 100 ml of the same buffer, phosphatase activity was eluted with a stepwise gradient using 100 ml 0.1 M, 100 ml 0.2 M and 100 ml 0.3 M NaCl in the same buffer.

Phosphorylase phosphatase was measured after preincubation at 30°C during 15 min in 240 μl total vol. containing 10 mM Tris–HCl (pH 7.4), 0.5 mM ATP, 2.5 mM $MgSO_4$. The phosphatase reaction was then initiated by the addition of an equal volume (240 μl) of a mixture containing 2 U/ml of liver phosphorylase *a* (EC 2.4.1.1) [9], 10 mM caffeine, 320 μg/ml of β-lactoglobulin and 20 mM Tris–HCl (pH 7.4). At different intervals up to 20 min a 100 μl aliquot was withdrawn for the assay of phosphorylase *a* [9]. Protein was determined by Lowry's method [13], β-lactoglobulin A was purified according to [14].

Rabbit muscle phosphorylase *b* kinase (EC 2.7.1.38) was a generous gift of Dr P. Cohen (Dundee University). ATP and cyclic AMP were obtained from Sigma Chemical Co (USA); blue dextran 200, Sephadex G-200 from Pharmacia (Sweden), DEAE-cellulose (DE52) from Whatman (England), hemoglobin, myoglobin and DNP-L-alanine from Serva (FRG).

3. Results and discussion

After DEAE-cellulose chromatography of the phosphorylase phosphatase preparation (fig.1) no phos-

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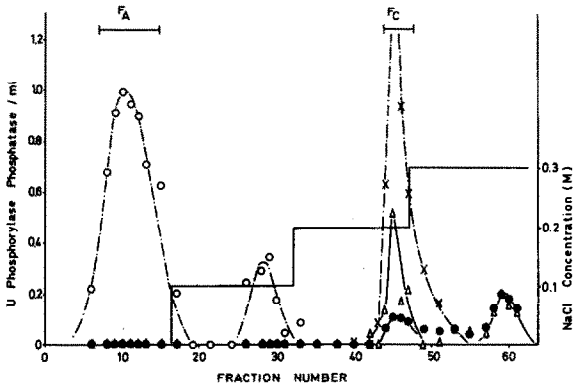

Fig.1. Separation of F_A and F_C on DEAE-cellulose. DEAE-cellulose chromatography of the liver phosphorylase phosphatase preparation was performed by a stepwise NaCl gradient as in section 2. Fractions (6.25 ml) were collected. Phosphorylase phosphatase present in 10 μl eluate was measured as in section 2 in the presence of Tris–HCl (pH 7.4): (●——●) as control; 0.5 mM ATP and 2.5 mM $MgSO_4$ (△——△); 0.5 mM ATP, 2.5 mM $MgSO_4$ and 5 μg F_A; (X–·–X); 0.5 mM ATP, 2.5 mM $MgSO_4$ and 20 μg F_C (○–·–○). Fractions A and C were pooled as indicated by the horizontal bars, concentrated by precipitation with 60% $(NH_4)_2SO_4$, resuspended in a small volume of Tris–HCl (pH 7.4) and dialyzed against the same buffer (final vol. ± 6 ml).

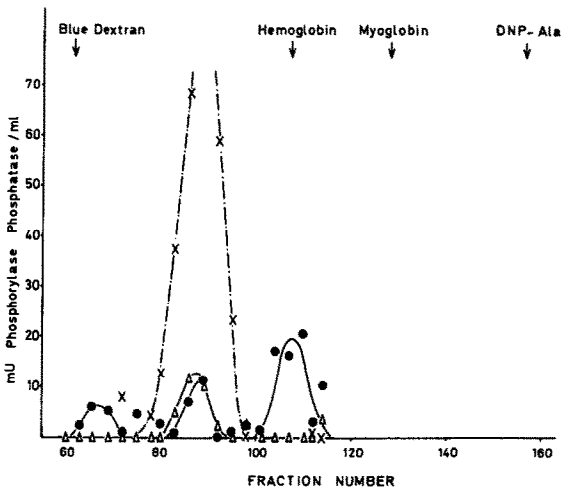

Fig.2. Elution profile of F_C on Sephadex G-200. F_C (6 ml, 120 mg protein) isolated by DEAE-cellulose chromatography (see fig.1) was applied to a column (2.5 × 95 cm) of Sephadex G-200 equilibrated in and eluted with 10 mM Tris–HCl (pH 7.4). Fractions (3 ml) were collected. Phosphorylase phosphatase present in 200 μl eluate was measured as in section 2 in the presence of Tris–HCl buffer (●——●) as control; 0.5 mM ATP and 2.5 mM $MgSO_4$ (△——△); 0.5 mM ATP, 2.5 mM $MgSO_4$ and 25 μg F_A (X–·–X).

phatase activity could be detected in the first wash (F_A) nor in the 0.1 M NaCl eluate. Some spontaneously active phosphatase was found in the 0.2 M and 0.3 M eluate. The addition of F_A or F_C to the individual fractions of the 0.2 M eluate or the first wash and 0.1 M eluate, respectively, yielded much additional phosphatase activity, which was completely dependent on ATP and Mg^{2+}. When the 0.2 M NaCl fractions (F_C) were pooled, concentrated by 60% $(NH_4)_2SO_4$ precipitation, dialyzed and chromatographed on a Sephadex G-200 column (fig.2) insignificant amounts of ATP-Mg-dependent phosphatase activity could be detected but a peak of phosphatase activity was obtained when the enzyme assay was performed in the presence of F_A, ATP and $MgSO_4$. In addition most of the spontaneously active form present in the 0.2 M NaCl eluate emerged separated from the F_C peak. For large scale preparations an additional DEAE-cellulose chromatography was included after the Sephadex G-200 filtration to eliminate detectable contamination with F_A. It was checked that the phosphorylase phosphatase activity observed by combining F_A and F_C was not due to proteolytic degradation of phosphorylase by the reactivation of the phosphorylase *b* to *a* using phosphorylase *b* kinase (fig.3).

The present results indicate that the interaction of at least two protein factors in the presence of ATP-Mg results in phosphorylase phosphatase activity. Several characteristics of the reconstituted phosphatase activity point to the identity of the enzyme with the ATP-Mg-dependent phosphatase present in liver: the K_a for ATP (0.3 μM) was the same in both cases [6]; the ATP-Mg activation was also time dependent and reversible upon Sephadex G-25 filtration; the inhibitory effect of protein inhibitors 1, 2α and 2β on the reconstituted enzyme was identical (not shown) to the effect described on the ATP-Mg-dependent phosphorylase phosphatase [11]. The reconstituted enzyme as well as F_A and F_C was inactivated by urea, ethanol and trypsin, was thermolabile and non dialysable.

The effect of ATP-Mg on phosphorylase phosphatase is readily reversible and it was supposed [15] that

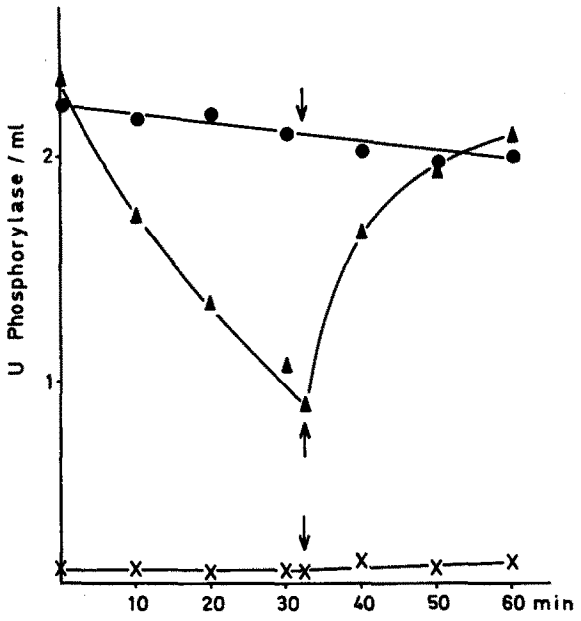

Fig.3. Proof of phosphorylase phosphatase activity by reactivation of phosphorylase *b*. 20 μl F_A and 20 μl F_C (obtained after a second DEAE-cellulose column) were preincubated with 2 mM ATP, 10 mM Mg^{2+}, 1 mM dithiothreitol and 10 mM Tris–HCl (pH 7.4) in 60 μl total vol. for 10 min at 30°C. After a 80-fold dilution in 10 mM Tris–HCl (pH 7.4) and 1 mM dithiothreitol 2 U liver phosphorylase *a* were inactivated by 250 μl diluted preincubation mixture in the presence of 5 mM caffeine and 160 μg β-lactoglobulin in 500 μl total vol. After 32 min, reactivation was carried out on 200 μl of this mixture by 4 U/ml of phosphorylase *b* kinase in the presence of 2 mM ATP, 2 mM $MgSO_4$ and 50 mM NaF in 400 μl total vol. Phosphorylase *a* was assayed on 25 μl samples during inactivation and 50 μl samples during reactivation (▲——▲). Controls of phosphorylase activity in the absence of F_A and F_C (●——●) and of a mixture of F_A and F_C (×——×) were also measured in the same conditions.

the activation of the ATP-Mg-dependent phosphorylase phosphatase could result from a ligand–enzyme interaction followed by a time-dependent conformational change. Since ATP-Mg is involved a kinase type of action is possible. The easy reversibility of the activation could then be explained by the action of an endogenous (autocatalytic?) phosphatase. The activation occurs in the presence of protein kinase inhibitor [11] or 1 mM EGTA (not shown) therefore the involvement of cyclic AMP-dependent protein kinase, or a Ca^{2+}-dependent kinase seems unlikely. However, recent results have shown that at this stage it is not that simple to explain the mechanism of this phosphatase activation: it seems to be the result of a protein–protein interaction between one or more 'regulatory' and 'catalytic' subunits, but the exact role of ATP-Mg in this activation is still unclear.

There is some resemblance between the ATP-Mg-dependent phosphatase and an ATP-Mg-dependent proteolytic system from reticulocytes which has also been resolved into two components by DEAE-cellulose chromatography [2]. Here ATP-Mg-dependent proteolytic activity involves a polypeptide with a mol. wt ~9000. This factor is characterised by a remarkable stability at high temperatures, a feature common to several regulatory proteins such as the protein inhibitors of phosphorylase phosphatase described in liver [11] and other tissues and the protein deinhibitor of the same enzyme demonstrated in liver [16].

The inactivation of a hormone-sensitive lipase from adipose tissue which requires ATP and Mg^{2+} [1] also bears some analogy. There ascorbic acid in the μM range plays an important role in the ATP-Mg-dependent regulation; this cannot be completely excluded in our experiments although addition of ascorbic acid has no effect on the activity of the ATP-Mg-dependent phosphorylase phosphatase.

The role of ATP-Mg in the regulation of these enzymes is undoubtedly important and further experiments are conducted in this laboratory to elucidate the exact nature of the interaction of the protein factors and the role of ATP-Mg in the activation of phosphorylase phosphatase.

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References

[1] Tsai, S.-C., Fales, H. M. and Vaughan, M. (1973) J. Biol. Chem. 248, 5278–5281.

[2] Ciehanower, A., Hod, Y. and Hershko, A. (1978) Biochem. Biophys. Res. Commun. 81, 1100–1105.
[3] Kato, K., Kobayashi, M. and Sato, S. (1975) J. Biochem. 77, 811–815.
[4] Hsiao, K.-J., Sandberg, A. L. and Li, H.-C. (1978) J. Biol. Chem. 253, 6901–6907.
[5] Khandelwal, R. L. (1977) Biochim. Biophys. Acta 485, 379–390.
[6] Goris, J., Defreyn, G. and Merlevede, M. (1977) Biochimie 59, 171–178.
[7] Gratecos, D., Detweiler, T. and Fischer, E. H. (1973) in: Metabolic Interconversion of Enzymes (Fischer, E. H. et al. eds) pp. 43–52, Springer-Verlag, Berlin.
[8] Merlevede, W. and Riley, G. A. (1966) J. Biol. Chem. 241, 3517–3524.
[9] Kalala, L. R., Goris, J. and Merlevede, W. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 575–581.
[10] Chelala, C. A. and Torres, H. N. (1970) Biochim. Biophys. Acta 198, 504–513.
[11] Goris, J., Defreyn, G., Vandenheede, J. R. and Merlevede, W. (1978) Eur. J. Biochem. 91, 457–464.
[12] Defreyn, G., Goris, J. and Merlevede, W. (1976) Arch. Int. Physiol. Biochim. 84, 595–596.
[13] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[14] Aschaffenburg, R. and Drewry, J. (1957) Biochem. J. 65, 273–277.
[15] Goris, J. and Merlevede, W. (1974) FEBS Lett. 48, 184–187.
[16] Defreyn, G., Goris, J. and Merlevede, W. (1977) FEBS Lett. 79, 125–128.