Biochimica et Biophysica Acta, 429 (1976) 809—816

BBA 67779

THIOPHOSPHATE-ACTIVATED PHOSPHORYLASE KINASE AS A PROBE IN THE REGULATION OF PHOSPHORYLASE PHOSPHATASE

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(Received October 10th, 1975)

Summary

Rabbit muscle nonactivated phosphorylase kinase (EC 2.7.1.38) is converted to thiophosphate-activated phosphorylase kinase by cyclic AMP dependent protein kinase, Mg^{2+} and ATP-γ-S /adenosine-5'-*O*-(s-thiotriphosphate)/. The formation of thiophosphate-activated phosphorylase kinase was also observed in the protein-glycogen complex from skeletal muscle. This new form of kinase is resistant to the action of phosphatase and behaves as a competitive inhibitor in the dephosphorylation of phosphorylase *a* by phosphorylase phosphatase (K_i = 0.04 mg per ml). The fact that the inhibitory effect of thiophosphate-activated phosphorylase kinase is 3 times higher than in the case of nonactivated kinase, may explain the transient inhibition of phosphorylase phosphatase in the protein-glycogen complex. The use of activated (phosphorylated) phosphorylase kinase supports this assumption since it causes a delay in the dephosphorylation of phosphorylase *a*, i.e. the conversion of phosphorylase *a* into *b* could start only after the dephosphorylation of activated phosphorylase kinase.

Introduction

The regulation of glycogen breakdown and synthesis depends on the successive phosphorylation and dephosphorylation of several enzymes. The activation of phosphorylase (EC 2.4.1.1) with synchronous inhibition of phosphorylase phosphatase (EC 3.1.3.17) was observed in the protein-glycogen complex isolated from rabbit skeletal muscle [1—4]. No clear explanation has been offered for the transient inhibition of phosphorylase phosphatase during the "flash activation" though Gratecos and Fischer [5] have postulated a complex formation between the enzymes of protein-glycogen complex.

This assumption was supported by frontal gel filtration of muscle extract and purified enzymes showing a strong association between phosphorylase, phosphorylase kinase and phosphatase [6]. The complex formation depends on

the protein concentration and the presence of Ca^{2+} [7]. The inhibition of phosphorylase phosphatase by nonactivated phosphorylase kinase may be considered as a result of the complex formation [8]. On the other hand, this effect of nonactivated kinase does not explain the transient inhibition of phosphatase since it may cause a permanent inhibition. Cohen and Antoniw [9] reported on the phosphorylation of nonactivated phosphorylase kinase in two subunits and demonstrated the phosphorylation of kinase in the protein-glycogen complex [10].

It would appear that phosphorylated (activated) kinase maintains phosphatase in its inactive state during the "flash activation". In the present communication this question is examined with the use of ATP-γ-S (adenosine-5′-*O*-(3-thiotriphosphate), Boehringer Mannheim) which is the thiophosphate analog of ATP [11—13]. It is demonstrated that thiophosphorylation of phosphorylase kinase may occur on the effect of ATP-γ-S in the protein-glycogen complex resulting in the formation of thiophosphate-activated phosphorylase kinase. This new form of phosphorylase kinase is resistant to the action of phosphatase [5], similar to thiophosphorylase *a* described by Gratecos and Fischer [5], therefore its inhibitory effect on phosphorylase phosphatase can be readily studied.

Methods

^{32}P-labeled phosphorylase *a* was prepared from three times crystallized rabbit skeletal muscle phosphorylase *b* using [γ-^{32}P] ATP (obtained from The Radiochemical Centre, Amersham) as described previously by Bot et al. [8]. AMP was removed by Norit-cellulose treatment [14] reaching a ratio of $A_{260} : A_{280} \leqslant 0.52$. Phosphorylase activity was assayed by the procedure of Illingworth and Cori [15]. Specific activity of phosphorylase *a* was 58 units · mg^{-1} in the presence of 16 mM glucose 1-phosphate and in the absence of AMP. The specific radioactivity was $1.95 \cdot 10^5$ cpm per mg protein.

Nonactivated phosphorylase kinase was prepared from rabbit skeletal muscle [16,17]. Its activity was measured according to the method of Cohen [17]. The specific activity of nonactivated phosphorylase kinase was 5.80 units · mg^{-1} at pH 8.2. The ratio of activity at pH 6.8 to 8.2 was 0.02. Therefore the phosphorylase kinase could be considered to be nonactivated (dephosphorylated). Activated (phosphorylated) phosphorylase kinase was prepared by the method of Cohen and Antoniw [9].

Phosphorylase phosphatase was prepared from rabbit skeletal muscle as described by Bot et al. [8]. This preparate can catalyze the dephosphorylation of phosphorylase kinase and phosphorylase *a*. Phosphorylase phosphatase assay: ^{32}P-labeled phosphorylase *a* was incubated with phosphatase in 0.04 M Tris/0.01 M mercaptoethanol/0.002 M EDTA (pH 6.8) buffer. The concentration of phosphorylase phosphatase was chosen so as not to induce more than 50% dephosphorylation of phosphorylase *a* in 10 min. Aliquots (0.1 ml) were removed from the incubation mixture and precipitated with trichloroacetic acid. The precipitate was dissolved in 0.1 M NaOH, reprecipitated and washed once more with trichloroacetic acid, and finally redissolved in 0.25 M NaOH. The radioactivity was measured by counting an aliquot using the Cerenkov-effect of ^{32}P with a Packard Tri-Carb scintillation spectrometer [18,19]. Similar conditions

were used in the assay of the dephosphorylation of phosphorylated phosphorylase kinase. The activity of remaining phosphorylated kinase was assayed at pH 6.8.

Protein was determined by the biuret-procedure [20] or by measuring the absorbance at 280 nm using an absorbance index of phosphorylase $A_{280}^{1\%} = 12.5$ [21] and of phosphorylase kinase $A_{280}^{1\%} = 12.4$ [17].

The protein-glycogen complex of rabbit skeletal muscle was prepared according to the method of Meyer et al. [1]. The "flash activation" of phosphorylase and phosphorylase kinase was studied at 30°C in an incubation medium containing: 0.4 ml protein-glycogen complex, 12 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.002 mM cyclic AMP and 3 mM ATP or ATP-γ-S (pH 7.0, final volume 0.6 ml). The ATP initiated the reaction. Aliquots were removed, diluted and assayed for phosphorylase *a* and phosphorylase kinase activity at pH 6.8. The enzymic activities were expressed in per cent of activity of total phosphorylase (measured in the presence of 1 mM AMP) and total phosphorylase kinase (assayed at pH 8.2).

Results

Preparation and properties of thiophosphate-activated phosphorylase kinase

Protein-glycogen complex from rabbit skeletal muscle which contains the most enzymes associated with glycogen metabolism has been described by Meyer et al. [1]. In this complex phosphorylase is in the *b* form and, phosphorylase kinase in the nonactivated form; "flash activation" triggers the formation of phosphorylase *a* while phosphorylase kinase remains in the nonactivated form [2,3]. Recently, Yeaman and Cohen [10] demonstrated that the activation of phosphorylase kinase occurred simultaneously with the formation of phosphorylase *a*. As can be seen in Fig. 1A the activation of phosphorylase and phosphorylase kinase occurs simultaneously after adding ATP, Mg^{2+} and Ca^{2+}.

Fig. 1B shows the formation of thiophosphate-activated kinase and thiophos-

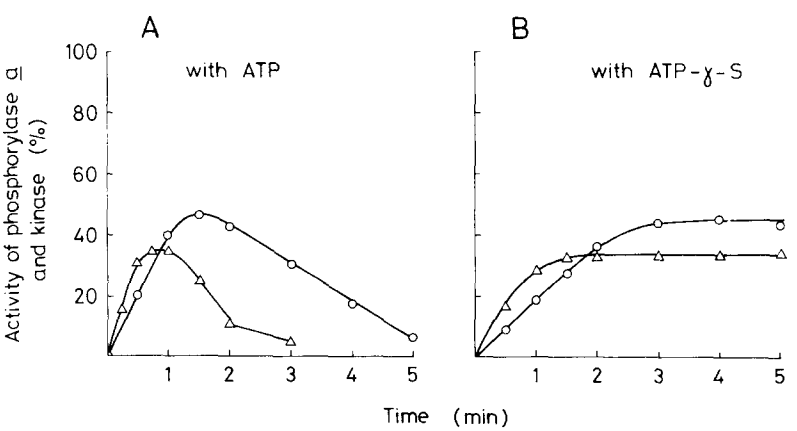

Fig. 1. Activation of phosphorylase and phosphorylase kinase in protein-glycogen complex. Conditions of activation process are described in Methods. Activity of activated phosphorylase kinase at pH 6.8 (△———△) and phosphorylase *a* (○———○): upon addition of ATP, Mg^{2+} and Ca^{2+} (A); ATP-γ-S, Mg^{2+} and Ca^{2+} (B).

phorylase *a* in protein-glycogen complex upon addition of ATP-γ-S, Mg^{2+} and Ca^{2+}. Both thiophosphorylated enzymes remain in the activated form because phosphorylase phosphatase cannot split off the thiophosphate groups [5]. On the basis of this observation, purified nonactivated phosphorylase kinase was incubated with cyclic AMP dependent protein kinase, ATP-γ-S and Mg^{2+} (Fig. 2) to prepare and investigate the properties of thiophosphate-activated phosphorylase kinase.

It can be seen that phosphorylase kinase is activated by incorporation of thiophosphate group, however, this process is markedly slower than the usual phosphorylation by ATP and Mg^{2+}. In the presence of ATP-γ-S and Mg^{2+} the activity ratio of pH 6.8 : 8.2 reached its maximal value 0.3—0.33 only after longer incubation, but the specific activities of phosphorylated and thiophosphorylated kinase were identical. The activation process was stopped by addition of 10 volumes of 0.004 M EDTA and thiophosphate-activated kinase was precipitated by 0.35 volume saturated $(NH_4)_2SO_4$ and immediately centrifuged for 20 min at 10 000 × *g*, 4°C. The precipitate was dissolved in 0.04 M Tris/0.002 M EDTA (pH 6.8) buffer dialysed overnight against the same buffer and further purified by chromatography on Sepharose 4B (column size 1.5 × 60 cm, elution with 0.04 M Tris/0.01 M mercaptoethanol/0.002 M EDTA buffer, pH 6.8). The peak fractions containing kinase were collected and concentrated through a membrane filter (Amicon UM20). The purification steps of thiophosphate-activated kinase are listed in Table I.

Table II summarizes some properties of thiophosphate-activated kinase in comparison with that of activated (phosphorylated) kinase. Glucose 6-phosphate inhibits the activity of activated kinase and AMP almost entirely abolishes the effect of glucose 6-phosphate [22,23]. Glucose 6-phosphate causes a similar decrease in the activity of thiophosphate-activated kinase and AMP counteracts

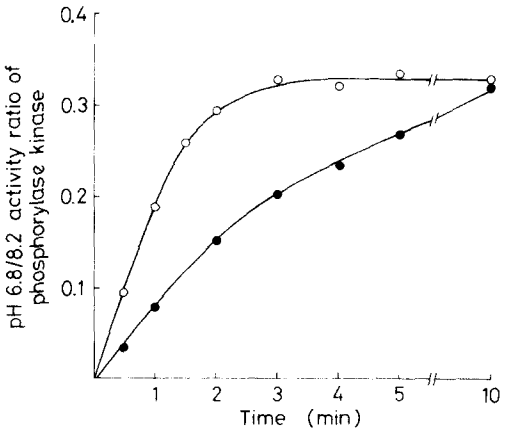

Fig. 2. Phosphorylation and thiophosphorylation of nonactivated phosphorylase kinase. 20 mg purified nonactivated phosphorylase kinase was incubated with 4 mM magnesium-acetate, 0.01 mM cyclic AMP, cyclic AMP-dependent protein kinase and 1 mM ATP (o———o) or ATP-γ-S (•———•) at 20°C, pH 6.8. Aliquots were removed, diluted and assayed for phosphorylase kinase activity at pH 6.8 and 8.2. The conversion of nonactivated phosphorylase kinase to activated form was estimated from the activity ratio of pH 6.8 : 8.2.

TABLE I

PURIFICATION STEPS OF THIOPHOSPHATE-ACTIVATED PHOSPHORYLASE KINASE

Step	Volume (ml)	Total protein (mg)	Spec. activity at pH 6.8 (units/mg)	Yield (%)
Kinase activation mixture (after EDTA addition)	12.0	86.0	1.62	100
$(NH_4)_2SO_4$ precipitate (after dialysis)	4.0	27.4	3.83	75.3
Sepharose 4B eluate	23.0	10.9	5.72	44.8

the inhibition of kinase caused by glucose 6-phosphate. It is known that both nonactivated and activated phosphorylase kinase have an absolute Ca^{2+} requirement for their enzymatic function [24,25,26]. According to the data of Table II thiophosphate-activated kinase has no activity in the presence of EGTA, i.e. in the absence of Ca^{2+}. It was also demonstrated (Fig. 2) that the pH 6.8 : 8.2 activity ratio of thiophosphate-activated kinase does not differ from that of activated kinase.

All of these results show the essential identity of both forms of activated phosphorylase kinase formed either by phosphorylation or thiophosphorylation. However, thiophosphate-activated kinase is resistant to phosphatase (Fig. 1B) and, therefore, it may behave as an inhibitor in the phosphorylase phosphatase reaction.

Inhibition of phosphorylase phosphatase by thiophosphate-activated phosphorylase kinase

Bot et al. [8] reported on the inhibition of nonactivated phosphorylase kinase in the phosphatase reaction. The effect of thiophosphate-activated phosphorylase kinase on the substrate saturation of phosphorylase phosphatase is shown in Fig. 3.

It is seen that thiophosphate-activated kinase inhibits the phosphatase reac-

TABLE II

PROPERTIES OF THIOPHOSPHATE-ACTIVATED PHOSPHORYLASE KINASE

The measurment of phosphorylase kinase activity was carried out in the following reaction mixture: 4 mg per ml phosphorylase *b*, 3 mM ATP, 10 mM Mg-acetate, 1.2 μg per ml phosphorylase kinase (either activated or thiophosphate-activated) and effectors at the indicated concentrations in 0.03 M glycero-phosphate/0.02 M Tris/0.01 M mercaptoethanol buffer (pH 6.8). Initial velocity was measured by phosphorylase *a* formation at 30°C. Phosphorylase kinase activity in the absence of effectors is taken as 100%.

Effector	Activity of	
	Activated kinase	Thiophosphate-activated kinase
None	100	100
5 mM glucose 6-phosphate	42	47
5 mM glucose 6-phosphate + 1 mM AMP	95	90
2.7 mM EGTA	0	0

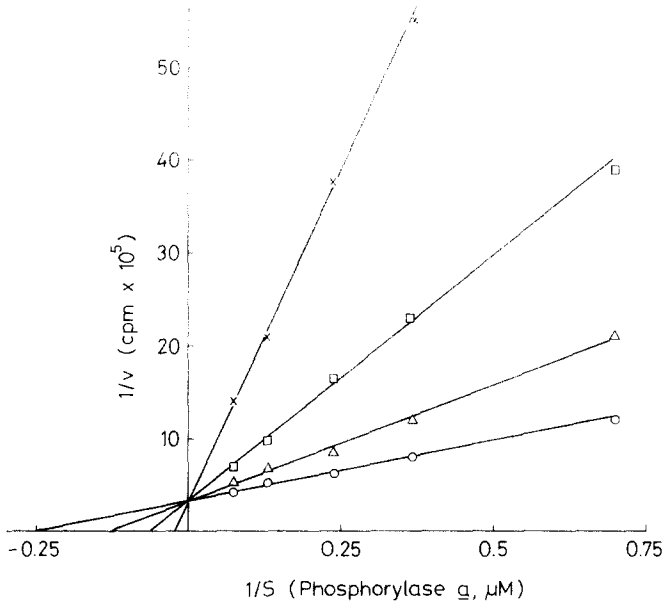

Fig. 3. Effect of thiophosphate-activated phosphorylase kinase on the phosphorylase phosphatase reaction. Lineweaver-Burk plot with respect to ^{32}P-labeled phosphorylase *a* in the absence (○———○) and in the presence of 0.05 (△———△), 0.1 (□———□) and 0.2 (X———X) mg per ml thiophosphate-activated kinase. The rate of phosphatase reaction was measured as described in Methods.

tion and the inhibition is enhanced by increasing the kinase concentration. Kinase does not influence the V of phosphatase reaction; the inhibition is competitive in nature. The K_M of phosphorylase phosphatase for phosphorylase *a* increases about 13 fold (from 4.0 to 52 μM) at the highest kinase concentration applied.

The inhibitor constant (K_i) of thiophosphate-activated kinase was determined according to Dixon and found to be 0.04 mg per ml kinase (for details of Dixon see plot, Bot et al. [8]). Both nonactivated and thiophosphate-activated kinases are competitive inhibitors of phosphatase. However the thiophosphate-activated kinase is about 3 fold more effective as an inhibitor than the nonactivated kinase. (K_i of nonactivated kinase is 0.11 mg per ml [8] in comparison to 0.04 mg per ml for the thiophosphate-activated kinase).

Sequential dephosphorylation of activated (phosphorylated) kinase and phosphorylase a

It may be supposed, on the basis of these observations, that not only thiophosphate-activated kinase but also phosphorylated kinase inhibits the dephosphorylation of phosphorylase *a* by phosphorylase phosphatase. Since both phosphorylated kinase and phosphorylase *a* are considered substrates of phosphatase, it is of interest to examine their competitive effects.

Fig. 4A shows the effects of phosphatase on the dephosphorylation of kinase and phosphorylase *a*. It can be seen that the presence of phosphorylase *a* does not influence the conversion of activated (phosphorylated) kinase into the nonactivated one. Whereas the presence of nonactivated and activated kinase con-

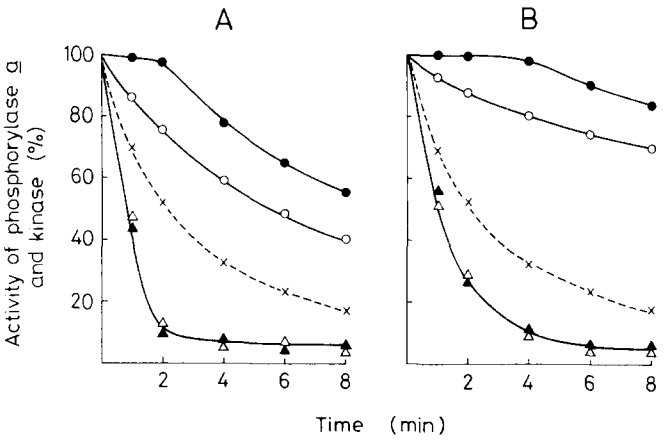

Fig. 4. Sequential dephosphorylation of activated phosphorylase kinase and phosphorylase *a*. Conditions of phosphorylase phosphatase reaction are described in Methods. The reaction mixture contained 1.3 mg phosphorylase *a*, 0.4 mg (A) and 0.8 mg (B) per ml kinase. Phosphorylase kinase activity in the absence (△———△) and in the presence (▲———▲) of phosphorylase *a*. Phosphorylase *a* activity in the presence of nonactivated (○———○) and activated/phosphorylated/kinase (●———●), in the absence of kinase (X- - - - -X).

siderably moderates the dephosphorylation of phosphorylase *a*, activated kinase does cause a delay. It is known that nonactivated kinase inhibits the conversion of phosphorylase *a* to *b* [8]. Therefore the moderation could be attributed to the presence or formation of the nonactivated form. The delay observed in the conversion of phosphorylase *a* into *b* is related to the presence of phosphorylated kinase. The inactivation of phosphorylase *a* could start only after the dephosphorylation of kinase. Increasing the amount of phosphorylated kinase (Fig. 4B) increases the period of delay. These results demonstrate the sequential order in the dephosphorylation of phosphorylated kinase and phosphorylase *a*.

Discussion

In light of experimental data presented here the events taking place in the protein-glycogen complex during "flash activation" might be explained as follows. At the start of glycogen mobilization occurs the phosphorylation of phosphorylase kinase and the conversion of phosphorylase *b* to *a* (see Fig. 1). These two processes could be considered as a trigger of glycogen breakdown.

It is to be remarked that the formation of hybrid phosphorylase containing phosphorylated and non-phosphorylated subunits was observed during "flash activation" [2,5]. Bot et al. [27] demonstrated that the extent of phosphorylation, i.e. the formation of hybrid, is controlled by the relative amount of phosphorylase kinase and phosphorylase. These results were also supported by in vivo investigations [28] proving the significance of hybrid phosphorylase in the glycogen metabolism.

The transient inhibition of phosphorylase phosphatase [4] observed in synchrony with the activation of phosphorylase kinase and phosphorylase could be attributed to the formation of activated kinase. The fact, that activated kinase (formed either by phosphorylation or thiophosphorylation) is a stronger inhibi-

tor of phosphatase than the nonactivated one, explains the decrease in the activity of phosphorylase phosphatase. The complex formation between phosphorylase kinase and phosphatase [6,7] supposes that the transient inhibition is directly related to the phosphorylation of kinase. The next step, the dephosphorylation of kinase results in the liberation of phosphatase from the inhibition caused by phosphorylated kinase hereby allowing the inactivation of phosphorylase *a* into *b* form. Accordingly, the phosphorylation of kinase, besides its important role in the activation of phosphorylase, serves as an inhibitor of phosphorylase phosphatase.

Since thiophosphate derivates are resistant to the action of phosphorylase phosphatase they should be helpful in the study of phosphorylation or even dephosphorylation processes. The use of ATP-γ-S makes the detection of transient phosphorylation easier and thiophosphate analogs might serve as a sensitive probe in the investigations of enzyme mechanism.

Acknowledgment

The authors are greatly indebted to Mrs. Anikó Gergely for her excellent technical assistance.

References

1 Meyer, F., Heilmeyer, L.M.G., Haschke, R.H. and Fischer, E.H. (1970) J. Biol. Chem. 245, 6642–6648
2 Heilmeyer, L.M.G., Meyer, F., Haschke, R.H. and Fischer, E.H. (1970) J. Biol. Chem. 245, 6649–6656
3 Haschke, R.H., Heilmeyer, L.M.G., Meyer, F. and Fischer, E.H. (1970) J. Biol. Chem. 245, 6657–6663
4 Haschke, R.H., Grätz, K.W. and Heilmeyer, L.M.G. (1972) J. Biol. Chem. 247, 5351–5356
5 Gratecos, D. and Fischer, E.H. (1974) Biochem. Biophys. Res. Commun. 58, 960–967
6 Gergely, P., Vereb, Gy. and Bot, Gy. (1974) Acta Biochim. Biophys. Acad. Sci. Hung. 9, 223–226
7 Gergely, P., Vereb, Gy. and Bot, Gy. (1975) Acta Biochim. Biophys. Acad. Sci. Hung. 10, 153–158
8 Bot, Gy., Varsányi, M. and Gergely, P. (1975) FEBS Lett. 50, 351–354
9 Cohen, P. and Antoniw, F.J. (1973) FEBS Lett. 34, 43–47
10 Yeaman, J. and Cohen, P. (1975) Eur. J. Biochem. 51, 93–104
11 Eckstein, F. (1966) J. Am. Chem. Soc. 88, 4292–4294
12 Goody, R.S. and Eckstein, F. (1971) J. Am. Chem. Soc. 93, 6252–6257
13 Goody, R.S., Eckstein, F. and Schirmer, R.H. (1972) Biochim. Biophys. Acta 276, 155–161
14 Fischer, E.H. and Krebs, E.G. (1958) J. Biol. Chem. 231, 65–71
15 Illingworth, B. and Cori, G.T. (1953) in Biochemical Preparations (Snell, E.E., ed.), Vol. 3, pp. 1–9, John Wiley and Sons, Inc., New York
16 DeLange, R.J., Kemp, R.G., Riley, W.D., Cooper, R.A. and Krebs, E.G. (1968) J. Biol. Chem. 243, 2200–2208
17 Cohen, P. (1973) Eur. J. Biochem. 34, 1–14
18 Clausen, T. (1968) Anal. Biochem. 22, 70–73
19 Haviland, R.T. and Bieber, L.L. (1970) Anal. Biochem. 33, 323–334
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–273
21 Sevilla, C.L. and Fischer, E.H. (1969) Biochemistry 8, 3315–3327
22 Bot, Gy., Kovács, E.F. and Pólyik, E.N. (1970) Acta Biochim. Biophys. Acad. Sci. Hung. 5, 9–18
23 Tu, J.-I. and Graves, D.J. (1973) Biochem. Biophys. Res. Commun. 53, 59–65
24 Ozawa, E., Hosoi, K. and Ebashi, S. (1967) J. Biochem. Tokyo 61, 531–533
25 Krebs, E.G., Huston, R.B. and Hunkeler, F.L. (1968) in Advances in Enzyme Regulation (Weber, G., ed.), Vol. 6, pp. 245–255, Pergamon Press, Oxford
26 Brostrom, C.O., Hunkeler, F.L. and Krebs, E.G. (1971) J. Biol. Chem. 246, 1961–1967
27 Bot, Gy., Kovács, E.F. and Gergely, P. (1974) Biochim. Biophys. Acta 370, 70–77
28 Gergely, P., Bot, Gy. and Kovács, E.F. (1974) Biochim. Biophys. Acta 370, 78–84