REGULATION OF GLYCOGEN METABOLISM IN INSECT FLIGHT MUSCLE. ACTIVATION OF PHOSPHORYLASE *b* KINASE BY CALCIUM AND INORGANIC PHOSPHATE

Richard G.HANSFORD and Bertram SACKTOR

Gerontology Research Center, National Institute of Child Health and Human Development, National Institutes of Health, Baltimore, Maryland, USA

Received 17 February 1970

1. Introduction

Studies of the kinetic properties of flight muscle phosphorylases [1] and the concentrations of glycogen, ATP, AMP and P_i in the muscle [2, 3] indicated that the activity of phosphorylase *b* cannot account for the rate of glycogenolysis in the muscle during flight. It was suggested that a change of the enzyme to the phosphorylase *a* form must occur. This was supported by direct measurements of the two forms of phosphorylase in the thorax, during rest and during flight [1]. Because of the crucial nature of this conversion, a study of the phosphorylase *b* kinase (ATP: phosphorylase phosphotransferase, E.C.2.7.1.38) from this tissue was undertaken. That this activity controls the conversion process in other tissues was suggested by Krebs et al. [4, 5], Danforth et al. [6] and Duncan and Drummond [7]. In this paper evidence is presented showing that flight muscle phosphorylase *b* kinase is stimulated by low levels of Ca^{2+}, as is the enzyme from mammalian skeletal muscle [8, 9]. It was also found that the kinase is markedly stimulated by high levels of P_i. These properties suggest a mechanism by which phosphorylase *b* kinase activity and, hence, glycogenolysis is controlled.

2. Methods

A crude preparation of phosphorylase *b* kinase was routinely made each day because of the instability of the enzyme, as described below. Typically, flight muscle expressed from the thoraces of 50 blowflies (*Phormia regina*) was gently homogenized in 5 ml of 0.1 M KCl, 10 mM KP_i, pH 7.1, 4 mM tris EDTA, pH 7.1, using a Dounce homogenizer. The suspension was centrifuged at 10,000 *g* for 5 min to sediment mitochondria and myofibrils, and was then passed through glass wool to remove some lipid material and small pieces of cuticle. Glycogen was added to 10 mg/ml and the solution was centrifuged at 140,000 *g* for 60 min. This procedure cosedimented the phosphorylases. The supernatant was carefully removed and served as a partially purified phosphorylase *b* kinase. Activities of phosphorylases, phosphorylase *a*, phosphatase and ATPase were negligible; some adenylate kinase was present, however. When a further purification of the kinase was desired, the preparation was recentrifuged at 320,000 *g* for 90 min. This sedimented the phosphorylase *b* kinase. The barely visible pellet was washed with medium and the resulting solution was used for kinetic studies. It contained negligible adenylate kinase activity. The muscles from 200 flies were usually used in preparations of this sort. All manipulations were carried out at 0–4°.

Phosphorylase *b* was prepared from thoraces of blowflies as described previously [1]. Phosphorylase *b* kinase activity was measured by assaying the amount of phosphorylase *a* formed in a 5 min incubation at 23°. The compositon of the incubation medium varied with the experiment and is stated in each case in the relevant table or figure. At the end of this time a 10 or 20 μl sample was taken from the incubation and pipetted into 0.7 ml of phosphorylase *a* assay medium. Measurement of phosphorylase *a* activity relied upon the formation of glucose-1-P

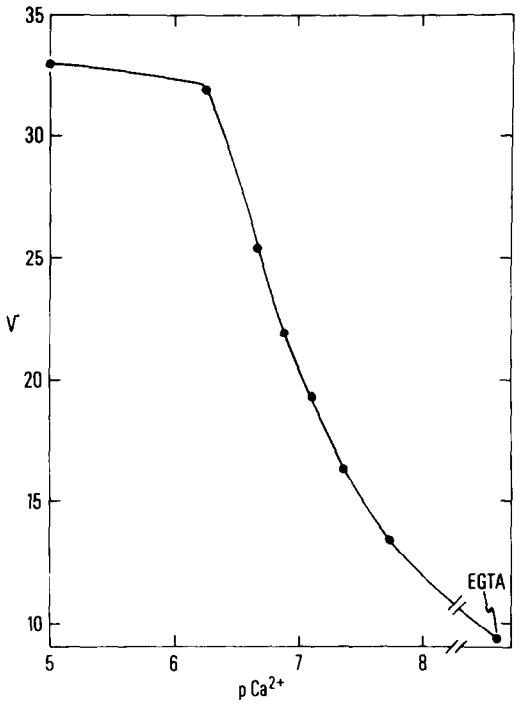

Fig. 1. The dependence of phosphorylase *b* kinase activity upon the Ca^{2+} level. The incubation medium comprised 0.1 M tris acetate, pH 7.1, 10 mM Mg acetate, 3 mM ATP and contained 1 Unit/ml of phosphorylase *b*. Each incubation contained 2 mM EGTA, and an amount of $CaCl_2$ sufficient to give the levels of Ca^{2+} indicated. The kinase was a partially purified preparation and was added in a volume of 10 µl. The velocity is quoted in arbitrary units. The point EGTA on the plot corresponds to no added Ca^{2+}.

from glycogen and phosphate. The rate of glucose-1-P formation was measured using phosphoglucomutase and glucose-6-P dehydrogenase, and monitored spectrophotometrically by reduction of $NADP^+$. Details of the assay were described previously [1]. Two precautions are noted: (a) the activity of the kinase was adjusted so that not more than 10% of the phosphorylase *b* was converted in the course of the reaction; (b) controls were run with all components except the kinase present, as the phosphorylase *b* preparations always showed a slight (1–2% V_{max}) rate in the phosphorylase *a* assay medium. This procedure also insured that metabolites added to the incubation medium specifically to affect the kinase were not in fact exerting an effect on phosphorylase *a* or on one of the enzymes in the coupled assay mixture.

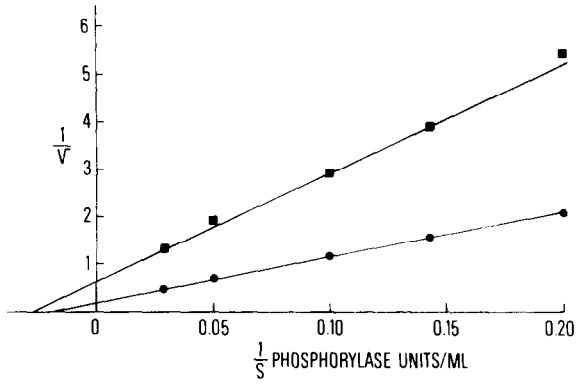

Fig. 2. The effect of Ca^{2+} on kinetics with respect to phosphorylase *b*. The incubation medium was as described for fig. 1, except that in those experiments marked with squares 2 mM EGTA was present and in those marked with circles a Ca^{2+} buffer containing 2 mM EGTA, and stabilizing 10^{-5} g ion/l Ca^{2+} was present. The K_m was estimated from the Ca^{2+} plot as 52 Units of phosphorylase *b*/ml. Velocity is in arbitrary units. The kinase was a partially purified preparation, and had stood for a few hours.

3. Results

3.1. *Stimulation by Ca^{2+}*

Studies with Ca^{2+}/EGTA buffers [10] showed that the activity of phosphorylase *b* kinase in the presence of 10^{-6} g ions/l Ca^{2+} is 3-times that in the presence of EGTA alone, at saturating ATP and limiting phosphorylase *b* concentrations (fig. 1). These results closely resemble those of Ozawa et al. [8] obtained with the enzyme from mammalian skeletal muscle.

The question as to whether Ca^{2+} changes the apparent K_m of the enzyme for phosphorylase *b* or causes a change in V_{max} has not been answered definitively. When the kinase is freshly prepared the apparent K_m for phosphorylase *b* is too high (above 100 Units/ml) to be estimated at neutral pH, and the kinetic effect of Ca^{2+} is not amenable to study. However, on storage overnight at 0° the kinase shows an enhanced affinity for phosphorylase whereas the stimulation by Ca^{2+} is essentially undiminished. When this aged enzyme is employed, Ca^{2+}

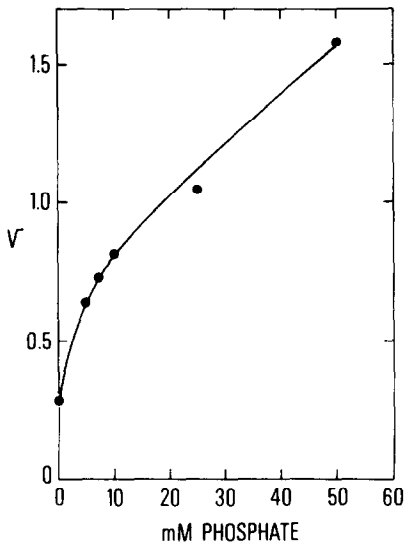

Fig. 3. The effect of phosphate concentration upon kinase activity. Each incubation contained 20 mM Mg acetate, 10 mM ATP and 1 Unit/ml of phosphorylase *b*. The different phosphate levels were achieved by adding varying volumes of two solutions, 0.1 M K phosphate pH 7.1. and 0.2 M tris acetate pH 7.1, such that the total volume added was always the same. [Ca^{2+}] was stabilized at 10^{-5} g ion/l.

affects V_{max}, in a system in which ATP is nearly saturating and phosphorylase *b* concentration is varied (fig. 2). The precision of these data, and of other plots not shown, is not sufficient to rule out unequivocally an effect on K_m as well. Ca^{2+} does not seem to change the apparent K_m for ATP. Indeed, such an effect could not explain the Ca^{2+} stimulation since the level of ATP normally used is several times its apparent K_m value. This value for flight muscle phosphorylase *b* kinase has been estimated variously as 0.05–0.15 mM, at pH 7.1, and in the presence of a fixed level of Mg^{2+} (10 mM) and low levels of phosphorylase *b* (0.90–3.0 Units/ml). Within this range of phosphorylase *b* concentration, no systematic variation in the apparent K_m for ATP with phosphorylase *b* level has been found.

3.2. *Stimulation by phosphate*

The freshly prepared kinase is stimulated several-fold by 50 mM KP_i, at non-saturating phosphorylase *b* and saturating ATP levels (fig. 3). The effect is not one of ionic strength, as the ionic strength of the incubations without P_i was matched. Nor is it an effect of pH. The latter was checked carefully, as a shift towards alkalinity does activate this enzyme, though not as markedly as with the skeletal muscle enzyme [5]. KCl does not replace KP_i. The effect of P_i on the kinetics of the kinase with respect to phosphorylase *b* concentration is not susceptible to experiment, as the enzyme which shows an effect of P_i (see below) also has an apparent K_m for phosphorylase *b* that is too high to measure.

Table 1
Activation of phosphorylase *b* kinase by phosphate and calcium.

Incubation medium	Activity
EGTA, $-P_i$	0.07
EGTA, $+P_i$	0.28
EGTA, $+Ca^{2+}$, $-P_i$	0.20
EGTA, $+Ca^{2+}$, $+P_i$	1.80

The incubation medium contained: phosphorylase *b*, 1 Unit/ml; ATP, 4.8 mM; Mg acetate, 9.5 mM; EGTA, 1 mM; and, as indicated, $CaCl_2$, 1 mM; KP_i, pH 7.1, 50 mM; or tris acetate, pH 7.1, 100 mM.

The stimulation in kinase activity by P_i is found in the presence or absence of Ca^{2+} (table 1). Moreover, the activation by P_i can be coupled to the 3-fold enhancement by Ca^{2+}, resulting in an overall increase in enzymic rate of 20-times or more.

3.3. *Selective loss of phosphate and calcium control*

The control of kinase activity by P_i is extremely labile (table 2). Freezing at $-20°$ or aging overnight either at $0°$ or at room temperature causes a transition of the enzyme from a P_i-controlled state to an activated (decontrolled) state that is about 4-times more active than is the enzyme in the absence of P_i, but is no longer responsive to P_i. The stimulation in enzymic rate by Ca^{2+}, however, still persists after such treatments. The decontrolled enzyme has a higher affinity (lowered apparent K_m) for phosphorylase *b*. A brief exposure of the enzyme to pH 4.5 at $0°$ also results in an irreversible change to the aged form, control by P_i is lost but that by Ca^{2+} is maintained. A less severe acid treatment, pH 5.4, induces an intermediate response; the kinase is activated 2-fold by the acid, and the addition of P_i effects a further 2-fold stimulation.

Table 2
Loss of phosphate and calcium control of phosphorylase *b* kinase activity.

Treatment	Phosphorylase *a* formed		
	Tris, EGTA	Tris, Ca^{2+}	Phosphate Ca^{2+}
None	0.32	0.99	4.82
Frozen, -20°	1.34	4.06	4.39
Acidified, pH 4.5, 2 min	2.68	5.00	5.55
Acidified, pH 5.4, 2 min	0.65	2.24	4.14
Heated, 40°, 27 min	0.40	0.39	0.71

Activity is expressed as phosphorylase units $\times 10^2$. The incubation medium contained 1 Unit/ml of phosphorylase *b*, 3 mM of ATP, 10 mM of Mg acetate in a total volume of 0.1 ml. As designated, the buffer was 0.1 M tris acetate, pH 7.1, or 0.05 M KP_i, pH 7.1. As indicated, Ca^{2+} was added, at an EGTA concentration of 4 mM, resulting in a Ca^{2+} buffer of 10^{-5}g ion/l. In those treatments marked EGTA, the concentration of EGTA was 1 mM. The partially purified kinase was used in all experiments. Acidification of the kinase was with acetic acid and the pH was readjusted with $NaHCO_3$. The procedure was performed at 0°.

In contrast to the stability of the Ca^{2+}-activation towards acid or aging, the Ca^{2+} effect is obliterated by moderate heat treatment. A relatively modest increase in activity is obtained with the additon of P_i, although the full potential of the enzyme is never attained.

4. Discussion

The present results demonstrating the control of flight muscle phosphorylase *b* kinase by P_i are quite distinct from any activation of the enzyme previously described. It is possible that it is an intrinsic property of freshly prepared phosphorylase *b* kinase from other tissues, but this would have been overlooked because of the acid treatment and storage at freezing temperatures usually used in preparations of the mammalian enzyme [5]. The activation by P_i also differs fundamentally from the activation (decontrol) seen after the enzyme has been frozen, aged or treated mildly with acid. Although the resulting enzymic rates are comparable, the kinase activated by P_i has an immeasurably high K_m for phosphorylase *b* at a neutral pH, whereas the decontrolled kinase has a considerably lower K_m for phosphorylase *b* at this pH. The "decontrolled" form of the kinase resembles in some respects the "activated" form from mammalian skeletal muscle as described by Krebs et al. [5]. However, the Ca^{2+}-dependent proteolysis [11] should not have occurred here, as EDTA was always present in these experiments.

The activation of phosphorylase *b* kinase by both P_i and Ca^{2+} provides additional support for the view that in muscle the conversion of phosphorylase *b* to *a* represents an essential component in the mechanism coupling contraction to glycogenolysis [12]. On initiation of flight, the P_i concentration in flight muscle is elevated instantaneously [3] and, presumably, Ca^{2+} is released from the sarcoplasmic reticulum (cf. [13]). It is suggested that it is the availability of both activators, enhancing kinase activity about 20-fold, that is largely responsible for the rapid conversion of phosphorylase *b* to *a* [1] and, hence, breakdown of glycogen [2] that is concomitant with the intitiation of flight. It is probably more than coincidental that other key enzymes in flight muscle that are also facilitated during the rest-to-flight transition, namely: phosphofructokinase, and the mitochondrial α-glycerophosphate, isocitrate and proline dehydrogenases [2], are activated variously by either Ca^{2+} or P_i [2, 3, 14–17].

References

[1] C.C.Childress and B.Sacktor, submitted for publication.
[2] B.Sacktor and E.Wormser-Shavit, J. Biol. Chem. 241 (1966) 624.
[3] B.Sacktor and E.C.Hurlbut, J. Biol. Chem. 241 (1966) 632.
[4] E.G.Krebs, D.J.Graves and E.H.Fischer, J. Biol. Chem. 234 (1959) 2867.
[5] E.G.Krebs, D.S.Love, G.E.Bratvold, K.A.Trayser, W.L.Meyer and E.H.Fischer, Biochemistry 3 (1964) 1022.
[6] W.H.Danforth, E.Helmreich and E.F.Cori, Proc. Natl. Acad. Sci. U.S. 48 (1962) 1191.
[7] G.I.Drummond and L.Duncan, J. Biol. Chem. 241 (1966) 5893.
[8] E.Ozawa, K.Hosoi and S.Ebashi, J. Biochem. (Tokyo) 61 (1967) 531.

[9] E.G.Krebs, R.B.Huston and F.L.Hunkeler, Advan. Enzyme Regulation 7 (1969) 245.
[10] H.Portzehl, P.C.Caldwell and J.C.Ruegg, Biochim. Biophys. Acta 79 (1964) 581.
[11] R.B.Huston and E.G.Krebs, Biochemistry 7 (1968) 2116.
[12] C.F.Cori, Enzymes: Units of Biological Structure and Function, ed. O.H.Gaebler (Academic Press, New York, 1956) p. 573.
[13] B.Sacktor, Advan. Insect Physiology 7, in press.
[14] R.G.Hansford and J.B.Chappell, Biochem. Biophys. Res. Commun. 27 (1967) 686.
[15] R.G.Hansford and J.B.Chappell, Biochem. Biophys. Res. Commun. 30 (1968) 643.
[16] R.G.Hansford and B.Sacktor, J. Biol. Chem. 245 (1970) 991.
[17] R.W.Estabrook and B.Sacktor, J. Biol. Chem. 233 (1958) 1014.