Regulation of Muscle Phosphorylase *b* Kinase Activity by Inorganic Phosphate and Calcium Ions

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The regulation of the activity of blowfly flight-muscle phosphorylase *b* kinase by P_i and Ca^{2+} was studied, and the actions of these effectors on the kinases from insect flight and rabbit leg muscles were compared. Preincubation of blowfly kinase with P_i increased activity severalfold. The effect was concentration-dependent, with an apparent K_m of about 20 mM, and time-dependent, requiring at least 10 min for maximal activation. Neither ATP nor cyclic AMP was needed, suggesting that a protein kinase may not be involved. Maximal activation of the insect kinase required Mg^{2+} in addition to P_i . The apparent K_m for Mg^{2+} was 3 mM. Rabbit leg-muscle phosphorylase *b* kinase was slightly inhibited, rather than stimulated, by P_i , and was strongly inhibited by K^+ , Na^+ and Li^+ . At physiological concentrations, Ca^{2+} activated the phosphorylase *b* kinases from both blowfly flight and rabbit leg muscles. However, the responses to Ca^{2+} of the enzymes from the two tissues were different. The mammalian kinase had virtually no activity in the absence of Ca^{2+} , and showed a large increase in activity over a narrow range of Ca^{2+} concentrations. Flight-muscle kinase had appreciable activity in the absence of Ca^{2+} , and had a smaller increase over a wide range of Ca^{2+} concentration. The concentrations of Ca^{2+} required for half-activation were 0.1 and $1 \mu M$ for the blowfly and rabbit enzymes respectively. The pH-activity profiles of the non-activated, phosphate- and Ca^{2+} -activated kinase revealed considerable enhancement of activity with little, if any, increase in the ratio of activities at pH 6.8 to those at 8.2. These results are discussed in relation to the mechanism coupling contraction to glycogenolysis and to the biochemical distinction between asynchronous and synchronous types of muscle.

Glycolytic flux and respiratory exchange in blowfly flight muscle are increased approximately 100-fold on initiation of flight (Sacktor, 1970). This remarkable control of metabolism, to support the muscle contracting at rates of several hundred times per second, is dependent exclusively on the utilization of carbohydrate, principally glycogen. Previous studies on the kinetic properties of flight-muscle phosphorylases (Childress & Sacktor, 1970) and the concentrations of glycogen, ATP, AMP and P_i in the muscle (Sacktor & Wormser-Shavit, 1966; Sacktor & Hurlbut, 1966) have indicated that the activity of phosphorylase *b* cannot account for the rate of glycogenolysis in the working muscle. It was suggested that, to satisfy the rate of glycogenolysis during flight, phosphorylase *b* must be converted into phosphorylase *a*. This was supported by direct measurements of the two forms of phosphorylase in the muscle, during rest and during

flight (Childress & Sacktor, 1970). Thus a study of the mechanisms by which phosphorylase *b* kinase itself becomes activated is of significance. That this enzyme controls the conversion process in other tissues was demonstrated by Krebs *et al.* (1959, 1964), Danforth *et al.* (1962) and Drummond & Duncan (1966). It was shown previously that blowfly flight-muscle phosphorylase *b* kinase is stimulated by physiological concentrations of Ca^{2+} (Hansford & Sacktor, 1970), as is the enzyme from mammalian skeletal muscle (Ozawa *et al.*, 1967; Heilmeyer *et al.*, 1970; Brostrom *et al.*, 1971). It was also found that the insect kinase is markedly activated by high concentrations of P_i (Hansford & Sacktor, 1970). In the present paper the regulation of blowfly flight-muscle phosphorylase *b* kinase by P_i and Ca^{2+} is described further. In addition, the actions of these effectors on the enzymes from insect flight muscle and rabbit leg muscle are compared. A preliminary account of portions of this study has been reported (Sacktor *et al.*, 1971).

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Experimental Procedures

Crude preparations of phosphorylase *b* kinase from flight muscles of the blowfly, *Phormia regina*, were made each day by the procedure described previously (Hansford & Sacktor, 1970), with the exception that the homogenization medium was 0.1M-KCl-20mM-imidazole-4mM-Tris-EDTA, adjusted to pH 7.1 with Tris. Phosphorylase *b* kinase from rabbit leg muscle was prepared in a similar manner, but the homogenization medium contained 5mM-imidazole and 1mM-Tris-EDTA, adjusted to pH 7.1 with Tris. Phosphorylase *b* was prepared from thoraces of blowflies, as described previously (Childress & Sacktor, 1970). In experiments with rabbit muscle kinase, crystalline rabbit muscle phosphorylase *b* was used. This was prepared by the method of Fischer & Krebs (1958) or was kindly supplied by Dr. E. Bueding. No apparent differences were observed in the behaviour of blowfly and rabbit muscle kinases tested with the phosphorylase *b* from either of the two species.

Phosphorylase *b* kinase activity was measured by assaying the amount of phosphorylase *a* formed in a 5min incubation at 25°C. The composition of the incubation medium was 100mM-Tris-acetate, pH 7.1, 10mM-magnesium acetate, 5mM-ATP, 1 unit of phosphorylase *b*/ml, and a preincubated sample of phosphorylase *b* kinase. At the end of the incubation a 10 or 20 μ l sample was taken and added to 0.62ml of phosphorylase *a* assay medium. Measurement of phosphorylase *a* activity was dependent on the formation of glucose 1-phosphate from glycogen and P_i . The rate of glucose 1-phosphate formation was measured by using phosphoglucomutase and glucose 6-phosphate dehydrogenase, and monitored spectrophotometrically by reduction of NADP⁺. Details of the assay were described previously (Childress & Sacktor, 1970). Controls were run with all components except the kinase present, as the phosphorylase *b* preparations always showed a slight rate in the phosphorylase *a* assay medium (Hansford & Sacktor, 1970). Also, 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. The composition of the preincubation medium varied with the experiment and is stated in each case in the relevant Table or Figure.

Buffers of Ca²⁺-EGTA [ethanedioxybis(ethylamine)tetra-acetate] at various pH values were prepared by the method of Portzehl *et al.* (1964). Protein was determined as described by Layne (1957).

Results

Hansford & Sacktor (1970) noted that, when blowfly flight-muscle phosphorylase *b* kinase was incubated with phosphorylase *b*, ATP and Mg²⁺, there

Table 1. Activation of phosphorylase *b* kinase by P_i

Phosphorylase *b* kinase activity was measured by assaying the phosphorylase *a* formed in a 5 min incubation at 25°C of kinase with phosphorylase *b* in an incubation mixture described in the text. The complete preincubation mixture contained 50mM-potassium phosphate, pH 7.1, 10mM-magnesium acetate, 5mM-ATP and 2mM-EGTA-Ca²⁺ buffer to yield a Ca²⁺ concentration of 10 μ M. The preincubation period was 10 min at 25°C. In the incubations without 50mM-phosphate, 100mM-Tris-acetate, pH 7.1, was substituted.

[P_i] (mM)		Activity (units/mg of protein)
Preincubation	Incubation	
0	0	0.66
0	5	0.62
0	50	2.11
50	5	2.34
50	50	2.11

was a severalfold increase in the phosphorylase *a* formed if high concentrations of P_i were present. The problem, whether P_i activated the kinase independently of the presence of phosphorylase *b*, or whether the kinase, phosphorylase *b* and P_i were required to be incubated together for P_i to facilitate their interaction, was not resolved. An experiment designed to answer this question is described in Table 1. Kinase was preincubated with P_i in the absence of phosphorylase *b*, and after 10 min a sample of the preincubated enzyme was incubated with phosphorylase *b*. Preincubation of the kinase with 50mM- P_i activated the kinase. The enzyme preincubated in P_i had an activity comparable with that preincubated in the absence of P_i , but subsequently incubated in the presence of 50mM- P_i and phosphorylase *b*. The data in Table 1 also demonstrate that low concentrations of P_i (5mM) transferred from the preincubation to the incubation media together with the kinase were without effect. Addition of 50mM- P_i to an incubation medium containing kinase previously activated by P_i did not further enhance the activity of the enzyme.

The effect of the concentration of P_i in the preincubation medium on the activation of the kinase is shown in Fig. 1. Maximal activity required a concentration of P_i as high as 50mM. The response to P_i was sigmoidal, suggesting that P_i activated allosterically. Transformation of the data into a Hill plot yielded a coefficient (*n*) of 2.2 ± 0.2 and an apparent K_m ($s_{0.5}$) of 21 mM.

The activation of the kinase in a preincubation mixture containing 50mM- P_i was not instantaneous. As illustrated in Fig. 2, maximal activation required at least 10 min under these experimental conditions. Activity remained essentially constant from 10 to 30 min.

Fig. 1. Effect of the concentration of P_i in the preincubation medium on the activity of blowfly flight-muscle phosphorylase *b* kinase

Phosphorylase *b* kinase activity was measured by assaying the phosphorylase *a* formed in a 5 min incubation at 25°C of kinase with phosphorylase *b* in an incubation mixture described in the text. The preincubation mixture contained the indicated potassium phosphate buffer, pH 7.1, 10mM-magnesium acetate, 5mM-ATP and 0.5mM-EGTA- Ca^{2+} buffer to yield a free Ca^{2+} concentration of 10 μ M. Osmolarity was maintained with Tris-acetate, pH 7.1. The preincubation period was 15 min at 25°C.

Fig. 2. Time-course of activation of blowfly flight-muscle phosphorylase *b* kinase by P_i

Phosphorylase *b* kinase activity was measured by assaying the phosphorylase *a* formed in a 5 min incubation at 25°C of kinase with phosphorylase *b* in an incubation mixture described in the text. The preincubation mixture contained 50mM-potassium phosphate, pH 7.1, 10mM-magnesium acetate, 5mM-ATP and 0.5mM-EGTA- Ca^{2+} buffer to yield a free Ca^{2+} concentration of 10 μ M.

In the experiments described above, the preincubation reaction media contained all constituents of the incubation mixture, including ATP, Mg^{2+} and Ca^{2+} , with the exception of phosphorylase *b*. When these other components were omitted from the preincubation medium (Table 2) we found that ATP was not

Table 2. Requirements for the activation of phosphorylase *b* kinase by P_i

Phosphorylase *b* kinase activity was measured by assaying the phosphorylase *a* formed in a 5 min incubation, at 25°C, of kinase with phosphorylase *b* in an incubation mixture described in the text. The complete preincubation mixture contained 50mM-potassium phosphate, pH 7.1, 10mM-magnesium acetate, 5mM-ATP and 0.5mM-EGTA- Ca^{2+} buffer to yield a free Ca^{2+} concentration of 10 μ M. The preincubation period was 15 min at 25°C. In the incubations without 50mM-phosphate, 100mM-Tris-acetate, pH 7.1, was substituted.

Omission from preincubation mixture	Activity (units/mg of protein)
None	5.5
P_i	0.8
Mg^{2+}	2.5
ATP	5.0
Ca^{2+} -EGTA	5.0
Mg^{2+} , ATP	2.5
ATP, Ca^{2+} -EGTA	5.6
Mg^{2+} , Ca^{2+} -EGTA	3.0
Mg^{2+} , Ca^{2+} -EGTA, ATP	3.1

required for the activation of phosphorylase *b* kinase. In other experiments (not illustrated) the presence or absence of 1 μ M-cyclic AMP had no influence on the activation of the kinase. Thus these findings contrast the activation of phosphorylase *b* kinase from blowfly flight muscle with that from rabbit leg muscle (DeLange *et al.*, 1968; Walsh *et al.*, 1971); no evidence was found in the insect preparation for the presence of a protein kinase that phosphorylated phosphorylase *b* kinase. The possibilities that these flight-muscle extracts possessed an exceptionally potent phosphorylase kinase phosphatase or inhibitor of the protein kinase have not been excluded, however. As described in Table 2, the omission of free Ca^{2+} from the preincubation medium had little effect. However, maximal activation of the kinase in the preincubation medium required Mg^{2+} , in addition to P_i .

The effect of the concentration of Mg^{2+} in the preincubation medium on the activation of phosphorylase *b* kinase is illustrated in Fig. 3. Activity of the enzyme was maximal with 5 mM- Mg^{2+} . A Hill plot of the data indicated a coefficient of 3.8 ± 0.06 and an apparent K_m ($s_{0.5}$) of about 3 mM. Other experiments, shown in Fig. 4, indicate that the activation of the kinase by a given concentration of P_i was dependent on the concentration of Mg^{2+} in the preincubation medium and, conversely, that the activation of the enzyme by a given concentration of Mg^{2+} was dependent on the concentration of P_i in that medium. The data suggest a positive interaction between the two effectors.

The activation of the kinase by P_i was rapidly reversed by dilution in P_i -free media. Fig. 5 describes

Fig. 3. Effect of the concentration of Mg^{2+} in the preincubation medium on the activity of blowfly flight-muscle phosphorylase b kinase

Phosphorylase b kinase activity was measured by assaying the phosphorylase a formed in a 5 min incubation at 25°C of kinase with phosphorylase b in an incubation mixture described in the text. The preincubation mixture contained 50mM-potassium phosphate buffer, pH 7.1, and magnesium acetate as indicated. The preincubation period was 15 min at 25°C.

Fig. 4. Effects of P_i and Mg^{2+} on the activation of blowfly flight-muscle phosphorylase b kinase

Phosphorylase b kinase activity was measured by assaying the phosphorylase a formed in a 5 min incubation at 25°C of kinase with phosphorylase b in an incubation mixture described in the text. The preincubation mixture contained potassium phosphate buffer, pH 7.1, and magnesium acetate, as indicated. Osmolarity was maintained with Tris-acetate, pH 7.1. The preincubation period was 15 min at 25°C. The $[Mg^{2+}]$ values (mM) were: □, 0; △, 2; ●, 3; ○, 10.

Fig. 5. Reversal of the P_i -activated blowfly flight-muscle phosphorylase b kinase by dilution in P_i -free media

Phosphorylase b kinase was activated by preincubation with 50mM-potassium phosphate buffer, pH 7.1, and 10mM-magnesium acetate for 10 min at 25°C. At zero time, the enzyme was diluted 1:10 with (a) 50mM-phosphate buffer or (b) 100mM-Tris-acetate buffer. In (c) the enzyme was preincubated with 5mM-potassium phosphate buffer and 10mM-magnesium acetate, and at zero time diluted 1:10 with 5mM-phosphate buffer. Activity is expressed as units of phosphorylase a formed/ml of kinase. Phosphorylase a was measured as detailed in the text.

experiments in which the enzyme activated by 50mM- P_i was diluted 1:10 in 100mM-Tris-acetate buffer (curve b). Within 5 min most of the enzyme had reverted to the non-activated form. Control experiments, in which the enzyme was diluted similarly, but in 50mM- P_i , retained full activation (curve a). A control experiment, in which the kinase was preincubated in 5mM- P_i and diluted in 5mM- P_i is shown (curve c). As reported in Table 1, dilution of the activated kinase in a medium free of P_i but containing the constituents of the incubation medium, namely phosphorylase b, Mg^{2+} , Ca^{2+} -EGTA and ATP, prevented reversal of the activation.

In view of the striking activation of blowfly flight-muscle phosphorylase b kinase by P_i , the effect of P_i on the enzyme from rabbit leg muscle was examined. Surprisingly (Table 3), when potassium phosphate, matched with respect to pH and ionic strength, was substituted for Tris-acetate a marked inhibition, rather than a stimulation, of the mammalian kinase was found. With the rabbit enzyme, however, the principal inhibitory ion was K^+ , although a moderate inhibition was observed with P_i (in the form of Tris-phosphate). Besides K^+ , inhibition of rabbit skeletal-muscle enzyme was observed with Na^+ and Li^+ . Only a slight inhibition was seen with NH_4^+ , relative to the activity of the enzyme with Tris ion. The effect of increasing concentrations of K^+ on the activity of rabbit skeletal-muscle phosphorylase b kinase is shown in Fig. 6. In these experiments osmolarity

Table 3. Comparison of the effects of P_i on phosphorylase *b* kinase of blowfly flight and rabbit leg muscles

Phosphorylase *b* kinase activity was measured by assaying the phosphorylase *a* formed in a 5 min incubation at 25°C. The reaction mixture contained, in addition to the buffer or salt noted, 10mM-magnesium acetate, 5mM-ATP, 2mM-EGTA- Ca^{2+} buffer to yield a free Ca^{2+} concentration of 10 μM , 1 unit of phosphorylase *b*/ml isolated from either blowfly flight or rabbit leg muscles, and 10 μl of crude phosphorylase *b* kinase in a total volume of 100 μl . In those experiments containing KCl and NaCl, 100mM-imidazole, pH 7.1, was added.

Experiment	Kinase activity (units/ml)
Blowfly flight muscle	
100mM-Tris-acetate, pH 7.1	5.0
50mM-Potassium phosphate, pH 7.1	31.0
Rabbit leg muscle	
100mM-Tris-acetate, pH 7.1	2.1
50mM-Potassium phosphate, pH 7.1	0.6
100mM-Tris-acetate, pH 7.1	1.6
50mM-KCl	0.3
50mM-NaCl	0.5
100mM-Tris-acetate, pH 7.1	3.7
50mM-Tris-phosphate, pH 7.1	2.8
50mM-Potassium phosphate, pH 7.1	1.7

Fig. 6. Effect of the concentration of KCl on the activity of rabbit skeletal-muscle phosphorylase *b* kinase

Phosphorylase *b* kinase activity was measured by assaying the phosphorylase *a* formed in a 5 min incubation at 25°C of kinase with rabbit muscle phosphorylase *b*. The reaction mixture contained 10mM-magnesium acetate, 5mM-ATP, 2mM-EGTA- Ca^{2+} buffer to yield a free Ca^{2+} concentration of 10 μM , 1 unit of phosphorylase *b*/ml, and KCl, as indicated, in imidazole buffer, pH 7.1. Osmolarity was maintained with Tris-acetate buffer, pH 7.1. Activity is expressed as units of phosphorylase *a* formed/ml of kinase.

Fig. 7. Comparison of the effects of the concentration of Ca^{2+} on the activities of phosphorylase *b* kinases from blowfly flight muscle (●) and rabbit leg muscle (○)

The incubation mixture contained 100mM-Tris-acetate buffer, pH 7.1, 10mM-magnesium acetate, 5mM-ATP and 1 unit of flight-muscle or leg-muscle phosphorylase *b*/ml. Each incubation contained 2mM-EGTA and an amount of CaCl_2 sufficient to give the $[\text{Ca}^{2+}]$ indicated. The points marked 'EGTA' correspond to no added CaCl_2 . $\text{pCa}^{2+} = -\log [\text{Ca}^{2+} (\text{M})]$.

was maintained with Tris ions. A concentration of KCl as low as 10mM was strongly inhibitory and at 30mM-KCl the activity of the enzyme was inhibited by 70%.

At physiological concentrations, Ca^{2+} activated phosphorylase *b* kinase prepared from both blowfly flight and rabbit leg muscles (Hansford & Sacktor, 1970; Ozawa *et al.*, 1967; Heilmeyer *et al.*, 1970; Brostrom *et al.*, 1971). As shown in Fig. 7, however, the responses of the enzymes from the two tissues to Ca^{2+} were distinctly different when tested under identical conditions. The mammalian kinase had virtually no activity in the absence of Ca^{2+} , and showed a large increase in activity over a narrow range of Ca^{2+} concentrations. Flight-muscle kinase had appreciable activity in the absence of added Ca^{2+} , and had a much smaller increase, only approximately threefold, over a wide range of Ca^{2+} concentration. On the other hand, the blowfly kinase was more sensitive to low concentrations of Ca^{2+} . The concentration of Ca^{2+} required for half-activation of the flight-muscle enzyme was 0.1 μM , whereas 1 μM - Ca^{2+} was required to half-activate the mammalian leg-muscle enzyme. Activation of blowfly kinase by Ca^{2+} was observed only when the enzyme was incubated with phosphorylase *b*, in contrast with the activation of blowfly

Fig. 8. Effect of pH on the activity of phosphorylase *b* kinase from blowfly flight muscle in the presence and absence of Ca^{2+}

Phosphorylase *b* kinase activity was measured by assaying the phosphorylase *a* formed in a 5 min incubation at 25°C of kinase with phosphorylase *b*. The incubation mixture contained 50mM-potassium phosphate, 30mM-magnesium acetate, 60mM-Tris-chloride, 60mM-imidazole, 5mM-ATP, 1 unit of phosphorylase *b*/ml, adjusted to the indicated pH, and 4mM-EGTA- Ca^{2+} buffer to yield a free Ca^{2+} concentration of $1\mu\text{M}$ at that pH. Activity is expressed as units of phosphorylase *a* formed/ml of kinase. ○, $1\mu\text{M}-\text{Ca}^{2+}$; ●, with EGTA.

Fig. 9. Effect of pH on the activity of phosphorylase *b* kinase from rabbit leg muscle in the presence and absence of Ca^{2+}

Details of the experiment are the same as in Fig. 8. ○, $1\mu\text{M}-\text{Ca}^{2+}$; ●, with EGTA.

kinase by P_i , which was effected also in the absence of phosphorylase *b*.

Hansford & Sacktor (1970) reported that the activity of blowfly flight-muscle phosphorylase *b* kinase was enhanced by an increase of pH, though not as markedly as that of the mammalian muscle enzyme

Fig. 10. pH-rate profiles of the non-activated, phosphate-activated and phosphate+ Ca^{2+} -activated forms of phosphorylase *b* kinase from blowfly flight muscle

The incubation mixtures were as described in Fig. 8. In the experiments with Tris-EGTA (○), 100mM-Tris-acetate replaced the 50mM-phosphate and the EGTA concentration was 4mM with no added Ca^{2+} . In the experiments with phosphate-EGTA (●), 50mM-potassium phosphate and 4mM-EGTA were used. In the experiments with phosphate- Ca^{2+} (Δ), 50mM-phosphate and 4mM-EGTA- Ca^{2+} buffer to yield a free Ca^{2+} concentration of $10\mu\text{M}$ at the specified pH were used.

(Krebs *et al.*, 1959). We found (Fig. 8) that the non-activated as well as the Ca^{2+} -activated blowfly kinase had similar pH-rate profiles. For the non-activated enzyme, a ratio of 0.1 was calculated for the activity of the enzyme at pH6.8 to that at pH8.2. The Ca^{2+} -activated kinase had an identical pH6.8/pH8.2 activity ratio, despite the fact that the activity of the Ca^{2+} -activated kinase was about twice that of the non-activated form in this particular experiment at this concentration of Ca^{2+} . As the ratio of activities at pH6.8 to those at 8.2 was suggested as an index to measure the extent of conversion of the kinase from a non-activated into an activated form by various

effectors (Krebs *et al.*, 1964; Meyer *et al.*, 1964), it was apparent that this ratio was not a valid index for estimating the Ca^{2+} -induced activation of the kinase. This was especially evident with the rabbit leg-muscle enzyme (Fig. 9). The mammalian kinase had a seemingly absolute dependence on Ca^{2+} ; its activity was enhanced manyfold (30 times at pH 7.4) by Ca^{2+} and this activation was independent of the conversion of the kinase from a non-activated into an activated form. The rabbit muscle enzyme had negligible activity at pH 8.2 when the free Ca^{2+} concentration was less than 1.0 nM (i.e. in the presence of EGTA and in the absence of added Ca^{2+}), whereas the blowfly enzyme was still active.

The pH-rate profiles of the non-activated, P_i -activated, and P_i -activated+ Ca^{2+} forms of blowfly flight-muscle phosphorylase *b* kinase are compared in Fig. 10. The P_i -activated kinase as well as the non-activated enzyme increased in activity with increases in alkalinity, at least to pH 8.2. Activities of the enzyme above pH 8.2 were difficult to measure in the presence of Ca^{2+} and P_i because of precipitation of calcium and phosphate salts. In the experiments described in Fig. 10, the ratio of activities at pH 6.8 and 8.2 for the non-activated enzyme was 0.22. The ratio for the P_i -activated enzyme was elevated to 0.47, and this was increased slightly to 0.53 in the presence of Ca^{2+} . On the other hand, the activity at pH 7.4 of the non-activated kinase was increased sixfold by P_i -activation and this was doubled to 12-fold in the presence of P_i and Ca^{2+} .

Discussion

The activation of blowfly flight-muscle phosphorylase *b* kinase by P_i , as reported here, is quite distinct from previously described mechanisms of activation of the enzyme from mammalian tissues. It differs from the conversion of the non-activated into the activated form of the enzyme from rabbit skeletal muscle, as described by Krebs *et al.* (1964) and Huston & Krebs (1968), in that the blowfly kinase activated by P_i had an unmeasurably high K_m for phosphorylase *b* at neutral pH (Hansford & Sacktor, 1970), and the Ca^{2+} -dependent proteolysis should not have occurred here as EGTA or EDTA was always present in these experiments. Also, the presence of ATP and cyclic AMP had no effect on phosphorylase *b* kinase in crude flight-muscle extracts. Additionally, activation of blowfly kinase activity by P_i was found in the absence of these two nucleotides. Thus, the present results provide no evidence for the presence in blowfly flight muscle of a protein kinase that phosphorylates and activates phosphorylase *b* kinase. Moreover, the data are not consistent with the view that the activation of phosphorylase *b* kinase by P_i involves a phosphorylation process. Indeed, the requirement for high concentrations of P_i , the slow time-course of

activation, the rapidity with which the activation was reversed on dilution, and the energetics of the system tend to suggest that the activation by P_i is not the result of a covalently linked phosphorylation of the enzyme. The present data also show that the activation of phosphorylase *b* kinase by Mg^{2+} occurs by a mechanism different from the activation of the enzyme by Ca^{2+} . A possible role for free Mg^{2+} in triggering the activation of phosphorylase *b* kinase from rabbit skeletal muscle was proposed by Chelala & Torres (1968) and Villar-Palasi & Wei (1970).

The activation of phosphorylase *b* kinase by both P_i and Ca^{2+} provides additional support for the concept that in muscle the conversion of phosphorylase *b* into *a* represents an essential component in the mechanism coupling contraction to glycogenolysis (Cori, 1956; Hansford & Sacktor, 1970; Brostrom *et al.*, 1971). On initiation of flight, the concentration of P_i in blowfly flight muscle was increased almost instantaneously (Sacktor & Hurlbut, 1966), and Ca^{2+} was presumably released from sites of sequestration, i.e. the sarcoplasmic reticulum and mitochondria (Sacktor, 1970; Carafoli *et al.*, 1971). Significantly, in addition to phosphorylase *b* kinase, at least five enzymes participating in the regulation of the rest-to-flight transition (Sacktor & Wormser-Shavit, 1966) are now known to be sensitive to Ca^{2+} and P_i at concentrations present in the muscle. These are: actomyosin adenosine triphosphatase, phosphofructokinase, and the mitochondrial glycerol phosphate dehydrogenase, isocitrate dehydrogenase and proline dehydrogenase (see Sacktor, 1970). Three of these, phosphorylase *b* kinase (Hansford & Sacktor, 1970), actomyosin adenosine triphosphatase (Sacktor, 1953; vom Brocke, 1966; Maruyama *et al.*, 1968) and glycerol phosphate dehydrogenase (Hansford & Chappell, 1967; Klingenberg & Buchholz, 1970; Donnellan *et al.*, 1970) were activated by the same low concentration of Ca^{2+} , at sites external to the inner mitochondrial membrane. However, Ca^{2+} was reported to be an allosteric inhibitor of isocitrate dehydrogenase (Vaughan & Newsholme, 1969), but this enzyme was localized in the matrix compartment of the mitochondria. The opposing effects of Ca^{2+} on these enzymes, which must be activated coincidentally with the start of muscle contraction, may depend on their cytological location. Actomyosin adenosine triphosphatase activity, glycolysis and respiration would be maximal when the extramitochondrial concentration of Ca^{2+} is high and the intramitochondrial concentration low, and would be inhibited when the extramitochondrial Ca^{2+} concentration is low and the intramitochondrial concentration high.

The comparative study of the control of phosphorylase *b* kinase activity in blowfly flight muscle and rabbit skeletal muscle by Ca^{2+} indicates that there is a real difference between the two enzymes in their Ca^{2+} sensitivity. In contrast with the mammalian enzyme,

flight-muscle kinase had appreciable activity in the absence of added Ca^{2+} , and showed only a small increase in activity over a wide concentration range of Ca^{2+} . This low Ca^{2+} -sensitivity of the kinase in blowfly flight muscle may be physiologically important. Flight muscle of the blowfly showed contraction-relaxation cycles at a constant Ca^{2+} concentration (Jewell & Ruegg, 1966), which could be varied considerably without loss of oscillations (Pringle & Tregear, 1969). Moreover, blowfly muscle has very little sarcoplasmic reticulum and it would appear that rapid segregation of Ca^{2+} within membrane-bounded vesicles is not required for oscillations of the asynchronous flight muscle. Therefore a high Ca^{2+} -sensitivity for phosphorylase *b* kinase would be disadvantageous. On the other hand, rabbit skeletal muscle, having a well developed sarcoplasmic-reticulum system and possessing a relatively slow, synchronous pattern of contraction-relaxation, is able to segregate Ca^{2+} more effectively. Thus phosphorylase *b* kinase activity of rabbit skeletal muscle can be fully controlled by changes in Ca^{2+} concentration. It is probably significant that the distinction between the asynchronous blowfly flight muscle and the synchronous rabbit skeletal muscle in the Ca^{2+} -sensitivities of their phosphorylase *b* kinases has a precise counterpart in the distinction between asynchronous and synchronous types of muscle in the Ca^{2+} -sensitivities of their actomyosin adenosine triphosphatases (Maruyama *et al.*, 1968).

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