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Conversion of Glycogen Phosphorylase *b* to *a* by Non-Activated Phosphorylase *b* Kinase: an *In Vitro* Model of the Mechanism of Increase in Phosphorylase *a* Activity with Muscle Contraction

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Abstract. Phosphorylase *b* kinase activity, as present in resting muscle in the non-activated form, appears to be ample to account for the fast appearance of phosphorylase *a* observed with muscle contraction. The kinase activity is repressed by free ATP and stimulated by free Mg^{2+}. Phosphorylase *b* kinase activity increases greatly when the Mg^{2+}:ATP ratio exceeds 1. It is proposed that the breakdown of ATP that occurs during muscle contraction may represent the triggering factor for the observed *in vivo* conversion of phosphorylase *b* into *a*.

Glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) is present in resting skeletal muscle entirely or almost entirely in the *b* form.[1] In the isolated muscle, electrically stimulated tetanic contractions result in a rapid conversion of phosphorylase *b* to the *a* form.[2] This conversion is catalyzed by a specific enzyme, phosphorylase *b* kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.37).[3] In the muscle at rest, phosphorylase *b* kinase is in a form which has been referred to as "non-activated kinase."[4] Under the usual conditions of assay, this form of the kinase shows little activity in the intracellular pH range; however, it can be converted to a different form, "activated kinase," which has 25–50 times more activity at pH 6.8 and twice as much activity at pH 8.2 as the non-activated form.[4] The ratio of activity *in vitro* at pH 6.8 to activity at pH 8.2 is used as an index of the ratio of the two forms of the kinase.

Several hypotheses have been advanced to explain the conversion of phosphorylase *b* to *a* as a result of muscle contraction. It has been shown[2] that an increase in activity of phosphorylase *b* kinase is responsible for this conversion, and that phosphorylase *a* phosphatase activity remains constant. Conversion of non-activated kinase to the activated form occurring as a result of Ca^{2+} liberation[5] or of Mg^{2+} liberation[6] during muscle contractions has been suggested as a possible mechanism. Recently, however, it has been found[7] that the increase in the percentage of phosphorylase *a* resulting from tetanic contraction of isolated muscle is not accompanied by the conversion of non-activated to activated kinase. Therefore, it appears possible that muscle contraction increases the *activity* of non-activated kinase *in vivo*, without conversion to the activated form.

"Non-activated" kinase is a misnomer, since it gives the impression that this form of the enzyme is inactive. On the contrary, it can be calculated† that the amount of non-activated kinase present in skeletal muscle, in the presence of a physiological concentration of glycogen (0.5%) and at the intracellular pH (6.9) would convert, under the usual conditions of assay, all of the phosphorylase in the muscle to the *a* form in less than 0.2 sec. The fact that in resting muscle phosphorylase is in the *b* form indicates that some type of metabolic control restrains the activity of non-activated kinase rather than that non-activated kinase is essentially inactive.

Atkinson has advanced the hypothesis[9] that the energy charge (half the average number of anhydride-bound phosphate groups per adenosine moiety) may be a major regulatory factor in processes which demand or produce energy. Phosphorylase *b* kinase, although it catalyzes an ATP-using reaction, participates in a sequence that is directed toward the regeneration of ATP. According to Atkinson, high energy-charge values would therefore be expected to inhibit the activity of phosphorylase *b* kinase, while a decrease in energy charge, such as occurs in muscle contraction, should result in an increase in kinase activity.

A second regulatory factor, related to but independent of the energy charge, has to be considered, namely the relative proportions of ATP and Mg^{2+} in the phosphorylase kinase system. Magnesium ion is a known activator of other protein kinases, and free magnesium ion may be required for activity. However, it is doubtful that in resting muscle there exists any appreciable free Mg^{2+}. Rabbit skeletal muscle has a concentration of free adenosine nucleotides (mostly as ATP) of 9.2 mM, while the concentration of free Mg^{2+} is only 8.5 mM.[11] Nanninga has calculated[12] that less than 90% of the ATP in frog muscle is present as ATP–Mg^{2+}, the remainder being free ATP. More recently, Rose has found[13] that only 70% of the ATP in red blood cells is present as the ATP–Mg^{2+} complex. An excess of ATP over Mg^{2+} concentration would be expected to inhibit kinase activity by reducing free Mg^{2+}. Muscle contraction, which results in decreased ATP levels, would increase the ratio of free Mg^{2+} to ATP.

Methods. We have used an *in vitro* model system to test the possibility that energy charge and (or) the relation between [Mg^{2+}] and the concentration of adenosine nucleotides provide an effective control of the activity of non-activated phosphorylase *b* kinase. Conditions similar to those known to occur *in vivo* were utilized, but for simplicity of interpretation, purified enzymes were used. Phosphorylase *b*, three times recrystallized and free of AMP, was prepared as described[10] by Fischer and Krebs. Rabbit muscle non-activated phosphorylase *b* kinase (ratio of activity at pH 6.8/activity at pH 8.2 = 0.03 to 0.05) was obtained according to Krebs *et al.*[4] Mixtures of ATP, ADP, and AMP (48 mM total adenosine nucleotide concentration) at the several energy-charge values were prepared according to Atkinson[9]; the proportions of the nucleotides were at each value equal to the final composition which would be obtained with adenylate kinase present. Magnesium chloride and, when used, 3′:5′-cyclic adenylate were added, and the pH was adjusted with Tris base (20 mM) and NaOH to 6.9. Muscle phosphorylase *b* kinase kinase (the 3′:5′-cyclic adenylate-dependent enzyme which activates phosphorylase *b* kinase) was purified by the method of Walsh *et al.*[14]

The standard reaction mixture for determining conversion of phosphorylase *b* to *a* consisted of 50 μl of purified non-activated phosphorylase *b* kinase (diluted 1:15 to 1:30 with 50 mM Tris–2 mM EDTA containing 5 mg of nucleotide-free rabbit liver glycogen per ml, pH 6.9); 100 μl of phosphorylase *b* solution in 50 mM Tris–50 mM mercapto-

ethanol–2 mM EDTA, pH 6.9; and 30 μl of adenosine nucleotide mixture. After 3–5 minutes of incubation at 30°C, the reaction was stopped by the addition to each mixture of 5 ml of ice-cold 40 mM glycerophosphate–30 mM mercaptoethanol–30 mM glucose 6-phosphate–2 mM EDTA, pH 6.8. Control reactions, to which 5-ml portions of this stopping mixture were added before incorporating the adenosine nucleotides, were run for each energy charge point. Phosphorylase activity (minus AMP) was measured using 0.1 ml aliquots of these mixtures by a method previously described.[15] Glucose 6-phosphate is a powerful inhibitor of the activation of phosphorylase *b* by AMP.[16] Therefore, this sugar phosphate was added in order to minimize the high blank readings due to the AMP carried over in the phosphorylase *a* determination. The difference in inorganic phosphate released from glucose 1-phosphate by incubated and control reaction mixtures was considered as a measure of increase in phosphorylase *a*, and was expressed as micromoles of P_i per 5 min of incubation.

Results. Conversion of phosphorylase *b* to *a* was tested at energy charge values between 0.6 and 1, and at a concentration of Mg^{2+} of 5.6 mM, equivalent to 0.7 of the molarity of the adenosine nucleotides. The amount of phosphorylase *a* formed increased with decreasing energy charge, reaching a maximum at an energy charge of 0.7, and decreased below this value (Fig. 1). Addition of

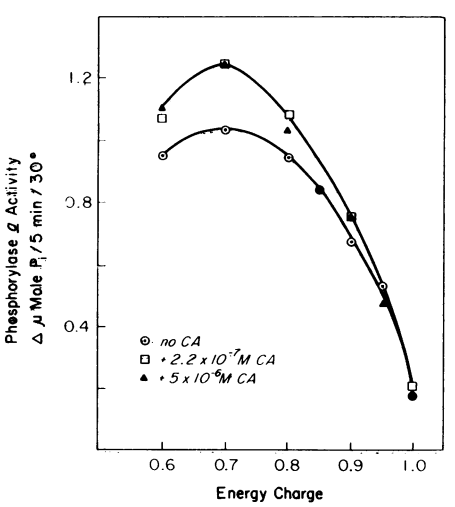

FIG. 1. Energy charge and 3′:5′-cyclic adenylate (CA) dependency of the conversion of phosphorylase *b* into *a* by non-activated phosphorylase *b* kinase. Assay as described in the text. Final concentration of Mg^{2+}, 5.6 mM. Ratio (in weight of protein) of phosphorylase *b* to phosphorylase *b* kinase, 10:1.

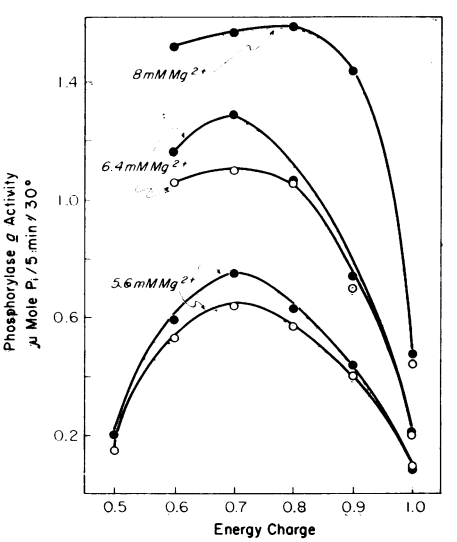

FIG. 2. Energy charge and Mg^{2+} concentration dependency of the phosphorylase *b* to *a* conversion by non-activated phosphorylase *b* kinase. Assay as described in the text.

3′:5′-cyclic adenylate and phosphorylase *b* kinase kinase to the reaction mixtures resulted in only a small further increase in the production of phosphorylase *a*. Conversion of non-activated into activated phosphorylase *b* kinase was determined in parallel reaction mixtures incubated under the same conditions with and without phosphorylase *b* kinase kinase and 3′:5′-cyclic adenylate (5×10^{-6} M). No conversion to activated kinase was found under any of these

conditions at high energy-charge values, and only a very small conversion (from a pH 6.8/8.2 activity ratio of 0.03 to one of 0.04) at an energy charge of 0.7. At this energy charge, the increase in phosphorylase *a* formation was over 5 times that at energy charge 1.0, and addition of 3′:5′-cyclic adenylate and phosphorylase *b* kinase kinase resulted in only about a 20% further stimulation.

At equivalent energy-charge values, the amount of phosphorylase *b* converted to *a* increased with the proportion of free Mg^{2+} to adenosine nucleotides (Fig. 2). A change in the Mg^{2+} concentration from 5.6 to 6.4 mM (from 0.7 to 0.8 saturation of adenine nucleotides) resulted in the appearance of larger amounts of phosphorylase *a*, but the general shape of the response to energy charge was similar. When the concentration of Mg^{2+} was brought to 8 mM (ratio Mg^{2+}: adenosine nucleotides = 1) there was a further increase in the formation of phosphorylase *a*, and a displacement of the response to energy charge. A ratio Mg^{2+}:adenosine nucleotides = 1, according to the existing data, is probably higher than in the cell. Therefore, in the absence of an appropriate determination of the *in vivo* ratio, it can be concluded that the response of non-activated phosphorylase *b* kinase activity to variations in energy charge resides somewhere in the region of one of these experimental curves.

In going from 5.6 mM Mg^{2+} to 6.4 mM Mg^{2+} (0.8 mM difference) there is only a 2-fold increase in phosphorylase *a* formation at energy charge 1.0. When the energy charge is reduced from 1.0 to 0.9, there is a decrease in ATP concentration of less than 0.8 mM, and a more than 4-fold increase in phosphorylase *a* formation. Thus, an increase in Mg^{2+} concentration produced by addition of free Mg^{2+} is less effective in increasing the activity of non-activated phosphorylase *b* kinase than the liberation of an equivalent amount of ATP-bound Mg^{2+} (Fig. 2). This fact can be interpreted as an indication that phosphorylase *b* kinase has an active site for its substrate, ATP–Mg^{2+}, which can be occupied by free ATP, resulting in an inhibition of the enzyme activity. Besides this active site, the enzyme probably has a second site for stimulation by free Mg^{2+}, on which ATP-bound Mg^{2+} is ineffective or inhibitory. Such a combined mechanism would explain the fact that breakdown of ATP, with only partial liberation of Mg^{2+} (ADP and even AMP also have Mg^{2+} complexing capacity) appears to be a more effective way of stimulating kinase activity than the simple increase in free Mg^{2+} concentration.‡

Identical responses of the phosphorylase *b* kinase activity to variations in energy-charge values were found when glucose 6-phosphate (3.6 mM), KCl (100 mM), or both, were added to the phosphorylase conversion mixtures.

Discussion. Two previous hypotheses have been presented to explain the increase in phosphorylase *a* in rapidly contracting muscle. One, suggested by Krebs *et al.*,[17] relates the release of Ca^{2+} that occurs during muscle contraction to the activation of phosphorylase *b* kinase (activated or non-activated) by this cation. Phosphorylase *b* kinase appears to have an absolute requirement for Ca^{2+} for activity; the K_a for the ion is approximately 10^{-7}M, and a similar concentration is released as a result of muscle contraction. Possibly, this liberation of Ca^{2+} triggers the activity of phosphorylase *b* kinase, which, however, would not be able to catalyze the conversion of phosphorylase *b* into *a unless the*

relative concentrations of ATP and Mg^{2+} were appropriate—that is, until some of the chelated Mg^{2+} had been released and some ATP had been degraded to ADP and AMP.

A second hypothesis, presented by Danforth,[18] postulates the alkalinization that in theory occurs when creatine phosphate is hydrolyzed to creatine and inorganic phosphate as the cause of the increased activity of non-activated phosphorylase *b* kinase. Danforth has shown indirectly that such a mechanism may be operating *in vivo*. When isolated muscle was incubated in the presence of high CO_2 concentrations in the gas phase, the conversion of phosphorylase *b* into *a* produced by tetanic contractions was delayed, possibly because the intracellular pH had been lowered. It is well established that non-activated phosphorylase *b* kinase *in vitro* is sensitive to minute variations in pH in the physiological range. Therefore, it appears also likely that under certain conditions the variation in the pH may be important *in vivo*. However, it must be pointed out that the conversion of phosphorylase *b* into *a* does not start in electrically stimulated muscle until most of the creatine phosphate has been hydrolyzed, that is, at the point when the ATP would start being effectively degraded, and bound Mg^{2+} would be released.[18]

In conclusion, we present the following hypothesis to explain the conversion of phosphorylase *b* into *a* that occurs in contracting muscle. Stimulation of the muscle releases into the sarcoplasma Ca^{2+} ions, required for phosphorylase *b* kinase activity. To supply the energy for contractions, creatine phosphate is utilized first, while the ATP levels are maintained. As a result of the hydrolysis of creatine phosphate the intracellular space becomes more alkaline, thus improving the pH requirement for activity of the non-activated phosphorylase *b* kinase. Finally, actual conversion of phosphorylase *b* into *a* occurs when ATP concentrations are effectively decreased and free Mg^{2+} simultaneously becomes available for the activation of non-activated phosphorylase *b* kinase. Therefore, even if Ca^{2+} ions are required for activity, and alkalinization of the intracellular space is also a factor in increasing the activity of non-activated phosphorylase *b* kinase, we suggest that the breakdown of ATP and release of bound Mg^{2+} are the triggering factors in the conversion of phosphorylase *b* to *a*.

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† According to Krebs and co-workers,[8] there is over half as much phosphorylase *b* kinase in skeletal muscle as phosphorylase itself on a protein basis (approximately 1% and 2% of soluble protein, respectively). 1 mg of purified non-activated phosphorylase *b* kinase catalyzes the conversion of 1.2×10^6 units of phosphorylase *b* into *a* per minute at pH 8.2 and 30°C, when assayed under standard *in vitro* conditions. At pH 6.9, and in the presence of a physiological concentration (0.5%) of its activator, glycogen, the activity of non-activated phosphorylase *b* kinase is over half that at pH 8.2 in the absence of glycogen.[4] As the specific activity of crystallized phosphorylase *b* is 1,600 units/mg protein,[9] 1 mg of non-activated phosphorylase *b* kinase, in the presence of 0.5% glycogen at pH 6.9, is therefore capable of phosphorylating 6.25 mg of phosphorylase *b* *per second* under standard assay conditions. At 30°C, all the phosphorylase *b* in muscle should be transformed by the phosphorylase *b* kinase present in less than 0.3 sec while at 37°C, assuming the usual increase in activity with temperature, that time would be less than 0.2 sec (this, not considering the reversal of the reaction catalyzed by phosphorylase *a* phosphatase).

‡ A similar mechanism appears to be present in the glycogen synthetase I kinase system *in vitro*. High energy charge and limiting Mg^{2+} concentrations (below those of ATP)

repress muscle synthetase I kinase activity, even in the presence of saturating concentrations of 3′:5′-cyclic adenylate, while a decrease in energy charge promotes an increase in kinase activity. It appears, then, that muscle stimulation may decrease glycogen synthesis and increase glycogen breakdown by a common mechanism of metabolic control.

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