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PHOSPHORYLASE KINASE FROM HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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Summary

Phosphorylase kinase from human polymorphonuclear leukocytes was investigated in a gel filtered crude preparation (17 000 × *g* supernatant). It was found to exist in two forms, one (the phosphorylated form) more active than the other (the dephosphorylated form). Interconversion between the two forms was carried out by a cyclic AMP dependent protein kinase and phosphoprotein phosphatase, respectively. The ratio of activity measured at pH 8.0 and 6.0 was 0.36 for the non-activated and 0.83 for the activated form, which is in contrast to the behaviour of phosphorylase kinase from muscle. K_m^{app} for the substrate phosphorylase b was 650 U/ml and 85 U/ml for the non-activated and activated form, respectively, whereas K_m^{app} for ATP was 0.03 mM and identical for the two forms. The non-activated form of phosphorylase kinase was activated by Ca^{2+} in the range 10^{-7}—$5 \cdot 10^{-6}$ M, which may have physiological importance, whereas the activated form was insensitive to variations in Ca^{2+} concentration between 10^{-9} and 10^{-3} M.

Introduction

Phosphorylase kinase (EC 2.7.1.38) is the enzyme that converts phosphorylase b to a by a phosphorylation reaction and thereby facilitates glycogen breakdown. The enzyme from rabbit muscle has been extensively investigated by several groups and the action mechanism is known down to the molecular plan. The enzyme has been found to exist in activated and non-activated forms,

Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; Pipes, piperazine-*N,N'*-bis(2 ethanesulfonic acid).

which are interconvertible by phosphorylation-dephosphorylation reactions [1—4]. Much less is known about the enzyme from liver tissue. Apart from being of interest from a pure biochemical point of view, a study of phosphorylase kinase from human tissues is particularly interesting, because it may enlighten some aspects of glycogen storage diseases in which phosphorylase kinase activity has been found low in liver and leukocytes [5—8]. It has also been suggested that the activation of glycogen breakdown in leukocytes [9] by phenylephrine is secondary to activation of nonactivated phosphorylase kinase by Ca^{2+} released from intracellular pools. Since little is known about basic properties of phosphorylase kinase from leukocytes, this study was initiated to give knowledge of characteristics that may provide a safe basis for assaying the enzyme.

Experimental procedures

Buffers. The pH of buffers were adjusted at 22° C and the assay of phosphorylase kinase run at this temperature.

Preparation of a gel filtered homogenate. Human polymorphonuclear leukocytes were prepared as previously described [10] and suspended in ice-cold 50 mM Tris-HCl (pH 7.2)/50 mM mercaptoethanol/5 mM EDTA buffer, using 2 ml buffer per gram of leukocytes. It is essential to use freshly withdrawn blood, as phosphorylase kinase activity decreases with storage, as also noted by Hujing [7]. The cells were sonicated (20 kHz, 2 × 20 s), centrifuged at 17 000 × *g* for 15 min and the supernatant gel filtered on a Sephadex G-25 fine column equilibrated in 25 mM Tris-HCl (pH 7.2), 25 mM mercaptoethanol and 0.2 mM EDTA, all procedures at 2° C. The gel-filtrate was used as enzyme source without further purification.

Activation of phosphorylase kinase. Immediately after preparation of the gel filtrate, phosphorylase kinase was in its inactive form, presumably due to the action of endogenous protein phosphatase during preparation. However, routinely, and additional 20 min of incubation on a waterbath (22° C) was employed. The activation was then accomplished by endogenous protein kinase activity by adding (final concentrations) 10 μM cyclic AMP and 30 s later 0.3 mM ATP/6 mM $MgCl_2$ (pH 7.2) in a total volume of 0.1 ml to 0.9 ml of the enzyme preparation, and further phosphatase activity was prevented by 50 mM NaF, unless otherwise specified. After 15 min, the activation was completed and the reaction mixture placed at 0° C and assayed within 2 min for phosphorylase kinase activity.

Assay of phosphorylase kinase. The assay was based on the rate of conversion of rabbit muscle phosphorylase b to a. Samples of non-activated or activated enzyme preparation were first diluted three times with 25 mM Tris-HCl (pH 7.2)/25 mM mercaptoethanol/0.2 mM EDTA, and 50 mM NaF. A 5 μl sample containing about 20 mg protein per ml was then added to 45 μl of an assay mixture containing 13.9 mM Tris-glycerophosphate buffer (pH 7.2)/110 U/ml rabbit muscle phosphorylase b/11.1 mg · ml^{-1} glycogen/0.33 mM ATP/6.6 mM $MgCl_2$/5.5 mg · ml^{-1} protein kinase inhibitor/55 mM NaF/1.1 mM EGTA, and 1.1 mM $CaCl_2$. The assay was run at 22° C, and followed every 15 min for 45 min. The phosphorylase kinase reaction was stopped by diluting

10-μl samples with 500 μl ice-cold 50 mM Tris-HCl (pH 7.2)/50 mM NaF/5 mM EGTA/1 mM DTT and the phosphorylase a and b activity measured. The progress curve of phosphorylase kinase activity was linear for 120 min provided less than 30% phosphorylase b was used. The activity of phosphorylase kinase was calculated from the slope of the progress curve. One unit of phosphorylase kinase is the activity, which converts 1 unit of phosphorylase b to a in one min at 22° C. For means of comparison to activities determined at 30° C, it should be noted that one unit of activity at 22° C corresponds to 2.5 units at 30° C, cf. Results.

The concentration of ATP in the reaction was 0.3 mM, which is lower than used by most authors. The low concentration combined with the subsequent 51-fold dilution ensured that no carry-over effect of adenine nucleotides on phosphorylase b activity occurred. The inclusion of 1 mM EDTA/1 mM $CaCl_2$ in the assay mixture gives a minimum concentration of $2 \cdot 10^{-5}$ M Ca^{2+} [28], which ensures full activity of the non-activated form of phosphorylase kinase. It was necessary to include a very high concentration of protein kinase inhibitor (5 mg/ml) in order to block completely any activation of phosphorylase kinase during the assay.

Appropriate blanks for phosphorylase kinase activity were always included. The presence of phosphorylase phosphatase activity in the reaction mixture was investigated in a control experiment, where phosphorylase a was substituted for phosphorylase b and the mixture incubated for 45 min at 22° C with either activated or non-activated phosphorylase kinase. Under these conditions no phosphorylase phosphatase activity was observed.

Assay of phosphorylase. Phosphorylase was assayed in the direction of glycogen synthesis without or with 1 mM AMP, as previously described [11]. One unit incorporates one μmol glucose 1-phosphate into glycogen per min at 30° C. Protein was determined by the method of Lowry et al. [12].

Chemicals. Phosphorylase a and b from rabbit muscle, protein kinase holoenzyme from rabbit muscle (P 3891), and protein kinase inhibitor (P 5015) were all products of Sigma and were gel filtered on Sephadex G-25 (fine) before use. Glycogen rabbit liver type III from Sigma was passed through a mixed ion exchange resin (Amberlite MB 3) before use. [U-^{14}C]glucose 1-phosphate was from the Radiochemical Centre, Amersham, U.K.

Results

Interconversion of activated and non-activated phosphorylase kinase

After 20-min incubation of a gel filtered 17 000 × *g* supernatant, phosphorylase kinase from leukocytes is in its non-activated form (Fig. 1). Addition of cyclic AMP and MgATP leads to rapid activation of the enzyme, which is stable to gel filtration. However, in the absence of added NaF, which blocks the activity of endogenous phosphatases of the enzyme preparation, the activation was slowly reversed over a period of 40 min. The activation by cyclic AMP and MgATP is completely prevented by addition of protein kinase inhibitor. These results suggest that phosphorylase kinase of polymorphonuclear leukocytes exists in two forms, which are interconvertible by phosphorylation-dephosphorylation reactions, the former being catalyzed by a cyclic AMP-dependent protein kinase.

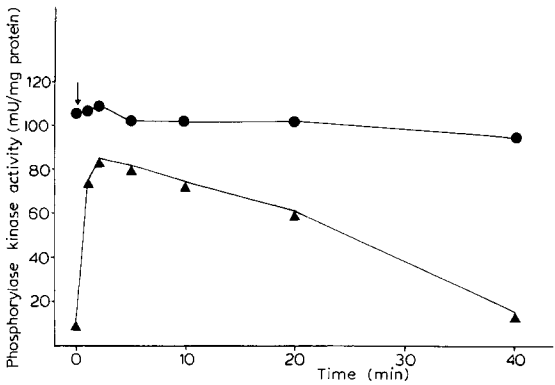


Fig. 1. 3 ml of a gel filtered 17 000 × *g* supernatant in 25 mM Tris-HCl (pH 7.2), 25 mM mercaptoethanol and 0.2 mM EDTA was preincubated for 20 min in the absence of NaF. Activation was then initiated by 10 μM cyclic AMP, 0.3 mM ATP, 6 mM Mg^{2+} (arrow) and the reaction (▲———▲), followed for 40 min by withdrawing 20-μl samples. The activation was stopped by mixing the samples with 10 mg/ml protein kinase inhibitor and 50 mM NaF to a total volume of 25 μl and the samples were then kept on ice until assay for phosphorylase kinase activity. During the activation period, total phosphorylase kinase activity was determined by withdrawing 100 μl samples (●———●) which were mixed with 50 mM NaF, 0.3 mM ATP, 6 mM $MgCl_2$, 5 μg protein kinase and 10 μM cyclic AMP in a final volume of 125 μl and incubated for 10 min at 22° C. Phosphorylase kinase activity was then assayed.

Thermal stability and activation

When activated and non-activated forms of phosphorylase kinase were incubated in the presence of 50 mM NaF, the enzyme activity was completely stable for 2 h, both at 22° C and 30° C, and thus not activated or inactivated by endogenous proteases. The thermal activation of both forms were studied at 22, 30 and 37° C. The Arrhenius plot gave straight lines in both cases and temperature coefficients of $2.06 \cdot 10^4$ cal/mol and $2.24 \cdot 10^4$ cal/mol were calculated for the activated and non-activated forms, respectively.

Effect of trypsin

Similar to phosphorylase kinase from liver [13] and muscle [14], the non-activated form of the leukocyte enzyme is rapidly activated by trypsin, whereas the activated form is not further activated (Fig. 2). Continued exposure to trypsin leads to inactivation of both enzyme forms.

K_m^{app} for muscle phosphorylase b and ATP

When studied at saturating concentrations of MgATP and at pH 7.2 (Fig. 3a), the apparent K_m for muscle phosphorylase b was 85 U/ml and 680 U/ml for the activated and non-activated forms of phosphorylase kinase, respectively, whereas the maximal velocity was the same for both forms. When the ATP concentration was varied and the Mg^{2+}-concentration kept constant at 6 mM, the apparent K_m for ATP was determined to 0.03 mM for both the activated and non-activated enzyme form at two constant concentrations of muscle phosphorylase b (Fig. 3b). It was not feasible to carry out these determinations at saturating concentrations of the substrate phosphorylase b, but the fact that the K_m^{app} for MgATP was the same at the two concentrations of phosphorylase b suggests that there is not allosteric interaction between the two substrates.

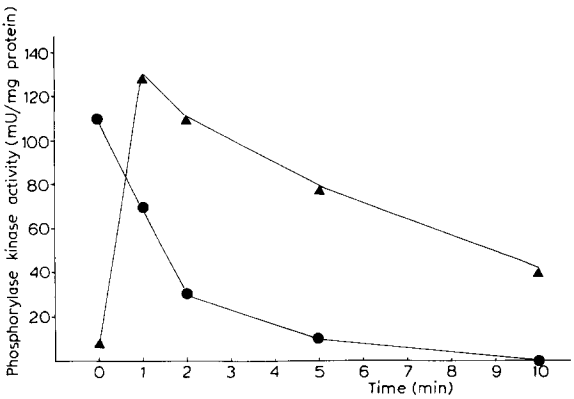


Fig. 2. 1 ml of activated (●———●) or non-activated (▲———▲) phosphorylase kinase of a gel filtered 17 000 × *g* supernatant was incubated at 22°C with 20 μg/ml trypsin. At time indicated 100-μl samples were withdrawn and mixed with 1 mg/ml soy bean trypsin inhibitor and assayed.

Effect of Ca^{2+}

Ca^{2+} profoundly affects the activity of phosphorylase kinase in its non-activated form, which is essentially inactive at Ca^{2+} concentrations below $3 \cdot 10^{-7}$ M and becomes fully active at concentrations above $5 \cdot 10^{-6}$ M (Fig. 4). The activated form is fully active over the range of Ca^{2+} concentrations used. It was not possible to abolish the activity of the activated enzyme form completely by adding EGTA to the assay mixture. However, in the presence of 20 mM EGTA corresponding to a calculated concentration of Ca^{2+} below 10^{-10} M, the activity of the activated enzyme decreased to 50% of the maximal activity.

Effect of pH (Fig. 5)

Both forms of phosphorylase kinase had maximal activity at pH 6.0. The non-activated form showed a steep decline in activity with a minimum at pH

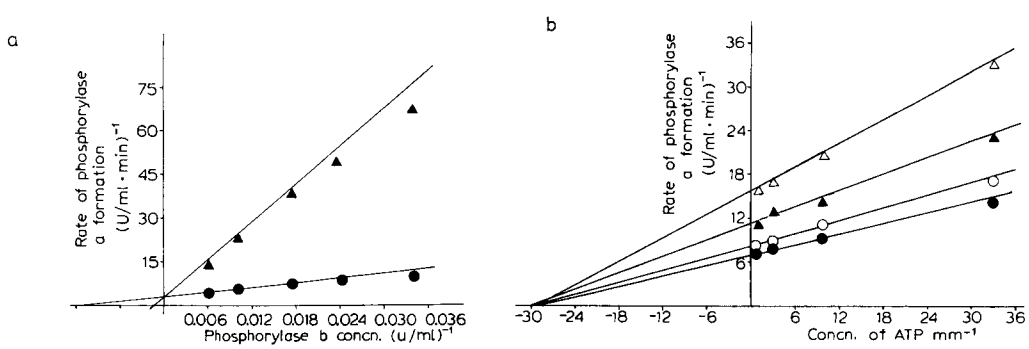
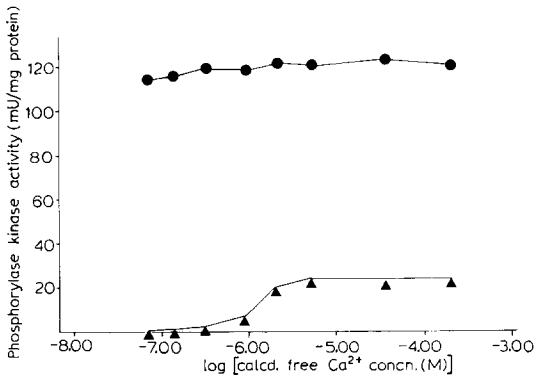


Fig. 3. a. Reciprocal plot of activity of activated (●———●) and non-activated (▲———▲) phosphorylase kinase as a function of muscle phosphorylase b concentration at constant concentration of 6 mM $MgCl_2$ and 0.3 mM ATP. b. Reciprocal plot of activity of activated (circles) and non-activated (triangles) phosphorylase kinase at varied concentrations of ATP. Mg^{2+} concentration was kept constant at 6 mM. Experiments were conducted at two constant concentrations of muscle phosphorylase b. Open symbols, 96 U phosphorylase b/ml, closed symbols, 167 U phosphorylase b/ml.



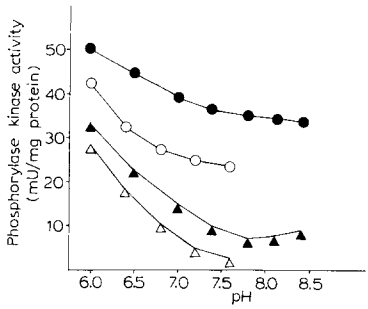


Fig. 4. The activity of activated (●——●) and non-activated (▲——▲) phosphorylase kinase was assayed at different Ca^{2+} concentrations. The standard assay conditions were used, but $CaCl_2$ was omitted from the buffer and added as indicated. The free Ca^{2+} concentrations were calculated using the values given by Portzehl et al. [28].

Fig. 5. The activity of activated (circles) and non-activated (triangles) phosphorylase kinase was assayed at different pH of the assay mixture described in Methods, however, with the omission of EGTA and $CaCl_2$ (closed symbols), and at different pH of an assay mixture where 12.5 mM Pipes/10 mM mercaptoethanol was substituted for Tris-glycerophosphate and EGTA and $CaCl_2$ were omitted (open symbols).

8.0 as the pH increased, whereas the activated form showed a slight decrease in activity with no minimum as the pH increased. It has been shown that protein kinase inhibitor is equally effective at pH 6.0—9.0 (Juhl, H., personal communication).

Discussion

The present experiments have shown that phosphorylase kinase from human polymorphonuclear leukocytes, as has been found for the enzyme from muscle [1—4] and liver [13,15—18], exists in two forms, one more active than the other. The results are compatible with the assumption that activation is accomplished by a phosphorylation reaction by cyclic AMP dependent protein kinase since: (1) The activation is initiated by adding cyclic AMP and MgATP and can be completely blocked by protein kinase inhibitor. (2) The activation is stable to gel filtration, provided NaF is added. (3) The activation is reversible, and inactivation is completely prevented by the addition of NaF, which is known to block phosphorylase kinase phosphatase activity [20]. The fact that activation could take place in the absence of added cyclic AMP, is explained by the observation that cyclic AMP dependent protein kinase in a 17 000 $\times g$ supernatant from human polymorphonuclear leukocytes, is partially dissociated in its regulatory and catalytic subunits, even though no cyclic AMP has been added (Juhl, H., personal communication).

It has been shown that, as for the muscle enzyme [14], activation results in a higher affinity towards the substrate phosphorylase b with no change in either maximal catalytic capacity or affinity towards the other substrate. The affinity towards the substrate MgATP was not influenced by varying the concentration of the other substrate, phosphorylase b (Fig. 3b). Similar information about the liver enzyme has, to our knowledge, not been published.

Several workers have reported that in glycogenosis tp VIa, which is characterized by deficiency of phosphorylase kinase in the liver, the activity of the corresponding leukocyte enzyme is very low [6,7,20,21]. Although it can be concluded, that the leukocyte enzyme is always affected in this disease, whether it is the X-linked or the autosomal recessive form, a residual activity of about 20% can, however, always be demonstrated, whereas in the liver the enzyme activity is almost completely absent [20]. This, however, does not justify the conclusion that the liver and leukocyte enzymes are different since the way of obtaining tissue for assay is different and the exposure to proteolytic activity during preparation may be different for the two tissues. Therefore, positive evidence should be presented before any conclusion about the identity of the liver and leukocyte enzymes is made.

Huijing [21] has suggested that two forms of deficient phosphorylase kinase activity can be detected in leukocytes from patients with glycogen storage disease, one form with a very low maximal catalytic activity (deficient in enzyme) and one with a highly reduced affinity towards phosphorylase b (K_m^{app} 1000—2000 U/ml), but normal V. In the present study K_m^{app} for non-activated phosphorylase kinase from normal leukocytes was determined to 680 U/ml, which probably is not significantly different from the value reported by Huijing [7]. Huijing did not distinguish between activated and non-activated phosphorylase kinase but from an evaluation of his experimental procedure we believe that he worked with the activated form, provided the enzyme can be maximally activated. It therefore remains to be shown (1) whether the phosphorylase kinase in leukocytes from patients with glycogenosis tp VIa exists in a non-activated and an activated form both of which have abnormal kinetic properties, but where, by coincidence, the kinetic properties of the activated form from these patients is similar to the kinetic properties of the non-activated enzyme from normal subjects or (2) whether the enzyme from patients with glycogenosis tp VIa, despite normal content of protein kinase [8], cannot be activated and therefore is the kinetically normal non-activated phosphorylase kinase.

The fact that the non-activated form of phosphorylase kinase is activated by trypsin, similar to the enzyme from muscle [14] and liver [13], indirectly excludes that our enzyme preparation has been significantly modified by proteolysis.

The effect of Ca^{2+} on phosphorylase kinase from muscle is well known. Both the activated and non-activated forms are totally dependent on Ca^{2+} for activity and both are stimulated in the range 10^{-8} to 10^{-5} M [22]. Also, the activated and non-activated forms of liver phosphorylase kinase are stimulated in the same range of Ca^{2+} concentrations [13,18,23]. We found that the activity of the non-activated form of leukocyte phosphorylase kinase was totally dependent on Ca^{2+} with activation occurring in the concentration range 10^{-7}–$5 \cdot 10^{-6}$ M with half maximal activation at 10^{-6} M Ca^{2+}. In contrast, the activity of activated phosphorylase kinase was independent of variations in Ca^{2+} concentrations between 10^{-9} and 10^{-3} M. It is therefore suggested that the activity of non-activated phosphorylase kinase in leukocytes fluctuates in response to fluctuations in intracellular Ca^{2+} concentrations, whereas the activity of the activated form is insensitive to physiological variations in intra-

cellular Ca^{2+}. In experiments with intact leukocytes suspended in a Ca^{2+}-containing buffer, it has recently been shown [9] that the activation of phosphorylase by phenylephrine is not associated with measurable changes in the concentration of cyclic AMP in the cells, but is associated with an increased efflux of Ca^{2+} from ^{45}Ca prelabelled cells. The present finding supports the assumption, that an increase in intracellular Ca^{2+} concentration is responsible for the activation of phosphorylase through the action of non-activated Ca^{2+} dependent form of phosphorylase kinase.

One characteristic feature of phosphorylase kinase from rabbit muscle is that the activity changes in response to variation in pH [24,25]. Both activated and non-activated phosphorylase kinase is more active at higher pH. For the non-activated form the ratio of activity measured at pH 6.8 and 8.2 is 0.01, whereas the corresponding ratio for the activated form is 0.36 [2]. At all pH, the activated form being more active than the non-activated form. There has to our knowledge been nor report of the activity of the liver enzyme at different pH values. In leukocytes almost the inverse pH-dependency was found true. Both forms had maximum at the lowest pH investigated (pH 6.0). The non-activated form showed a steep fall in activity as the pH increased, whereas the activity of the activated form only decreased slightly. When measured in a Tris-glycerophosphate buffer, the pH 8.0/6.0 was determined to 0.36 ± 0.014 (S.E.M., $n = 5$) for the non-activated form, whereas the corresponding ratio for activated phosphorylase kinase was 0.83 ± 0.047 (S.E.M., $n = 5$). As shown in Fig. 5 the shape of the curve remains essentially the same, when using Pipes as buffer, but somewhat lower activity is obtained. The higher activity at low pH may have physiological importance since it is known that the intracellular pH in leukocytes decreases during phagocytosis [26], when at the same time glycogen breakdown is initiated [27].

Acknowledgements

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