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Activation of Phosphorylase Kinase by Physiological Temperature

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Abstract

In the six decades since its discovery, phosphorylase kinase (PhK) from rabbit skeletal muscle has usually been studied at 30 °C; in fact, not a single study has examined functions of PhK at a rabbit's body temperature, which is nearly 10 °C greater. Thus, we have examined aspects of the activity, regulation, and structure of PhK at temperatures between 0 and 40 °C. Between 0 and 30 °C, the activity at pH 6.8 of nonphosphorylated PhK predictably increased; however, between 30 and 40 °C, there was a dramatic jump in its activity, resulting in the nonactivated enzyme having a far greater activity at body temperature than was previously realized. This anomalous change in properties between 30 and 40 °C was observed for multiple functions, and both stimulation (by ADP and phosphorylation) and inhibition (by orthophosphate) were considerably less pronounced at 40 °C than at 30 °C. In general, the allosteric control of PhK's activity is definitely more subtle at body temperature. Changes in behavior related to activity at 40 °C and its control can be explained by the near disappearance of hysteresis at physiological temperature. In important ways, the picture of PhK that has emerged from six decades of study at temperatures of 30 °C does not coincide with that of the enzyme studied at physiological temperature. The probable underlying mechanism for the dramatic increase in PhK's activity between 30 and 40 °C is an abrupt change in the conformations of the regulatory β and catalytic γ subunits between these two temperatures.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b01032. Temperature-dependent cross-linking of PhK by DFDNB (Figure S1) (PDF)

In their first papers describing phosphorylase kinase (PhK) some 60 years ago, Fischer and Krebs chose a temperature of 30 °C to assay its kinase activity.^{1,2} Those studies were on the hexadecameric enzyme from fast-twitch skeletal muscle of New Zealand White rabbits, and virtually everything we know about the functions of PhK and its regulation has been learned from studying the enzyme from that same tissue, species, and breed and at that same temperature of 30 °C. In fact, we have identified 115 papers on the structure, function, or properties of rabbit muscle PhK in solution at 30 °C that have been published during the past 6 decades. An additional 22 related studies were conducted at temperatures of <30 °C, but only five were performed at greater temperatures. Of these five outliers, only one actually examined the effects of temperature, albeit incidentally, as it reported a variety of biophysical measurements using instrumentation connected to linear temperature ramps from 30 to 70 °C; however, no functional tests on PhK were conducted in that study.³ The threshold of 30 °C is meaningful because the body temperature of a rabbit is nearly 10 °C greater than that. Repeated carefully controlled measurements of the body temperatures of 24 New Zealand White rabbits at different times, under different conditions and using different methods, showed a range from 39.1 to 40.3 °C, with an average temperature of 39.7 °C.⁴ Given that the properties of enzymes, including regulatory, are often affected by temperature in surprising ways (e.g., refs 5–9), we thought it would be beneficial to examine the effect of temperature on the properties of PhK. Of special interest were differences between properties at 30 °C, at which most everything has been defined, and 40 °C, close to the average physiological temperature of the rabbit, but at which absolutely nothing is known concerning PhK functions.

MATERIALS AND METHODS

Materials

Nonactivated PhK was purified from the psoas muscle of female New Zealand White rabbits as described previously¹⁰ and stored at –80 °C in 50 mM Hepes buffer (pH 6.8), 10% sucrose, and 0.2 mM EDTA at a concentration of 3.5 mg/mL. The enzyme was thawed for use no more than twice before being discarded. All experiments described herein were repeated with a minimum of two different enzyme preparations and usually more. The PhK concentration was determined using an absorbance index at 280 nm of 12.4 for a 1% solution.¹¹ Glycogen phosphorylase *b* (GP) was also isolated from the skeletal muscle of New Zealand White rabbits as described previously¹² and recrystallized with Mg²⁺ and AMP. The AMP was removed by adsorption onto charcoal, and the concentration of GP was

determined spectrally using an absorbance index at 280 nm of 13.0 for a 1% solution.¹³ The catalytic subunit of murine cAMP-dependent protein kinase (PKA) was from New England Biolabs (Ipswich, MA). All nucleotides were from Sigma-Aldrich Products (St. Louis, MO), but [γ -³²P]ATP was from NEN PerkinElmer (Boston, MA).

Activity Assays

PhK activity for GP conversion measured the incorporation of ³²P into GP by a filter paper assay.¹⁴ Assays were initiated by the addition of PhK that had been equilibrated for a minimum of 2 min at the appropriate temperature, and unless a time course was run, the reactions were stopped after 5 min. The standard assay contained Tris (50 mM), β -glycerophosphate (50 mM), Hepes (9–15 mM), β -mercaptoethanol (9–15 mM), CaCl₂ (0.2 mM), EGTA (0.1 mM), Mg(CH₃CO₂)₂ (10 mM), [γ -³²P]ATP (1.5 mM), GP (5.6–8.2 mg/mL), and PhK (0.5–1 μ g/mL) at pH 6.8 or 8.2. Any deviations from these conditions are listed in individual figure legends.

The autophosphorylation activity of PhK was also measured using a filter paper assay. Unless a time course was run, these assays were also conducted for 5 min and contained CaCl₂ (0.25–0.3 mM), EGTA (0.1 mM), Mg(CH₃CO₂)₂ (10 mM), and PhK (0.5 or 0.85 mg/mL), which initiated the reaction after having been equilibrated at the same temperature as the reaction mixture. The concentrations of buffer (pH 6.8), reductant, and ATP are listed in the individual figure legends. Quantification of subunit phosphorylation by PKA was conducted as previously described,¹⁵ but with subunits resolved on polyacrylamide slab gels.

Cross-Linking

PhK (437 μ g/mL) was cross-linked by DFDNB (67 μ M) at 10 °C intervals between 0 and 40 °C in the presence of 50 mM Hepes (pH 6.8) and 0.1 mM EGTA. The reaction was quenched after 6 min in a reducing SDS buffer, resulting in final concentrations of 219 μ g/mL PhK, 63 mM Tris (pH 6.8), 10% glycerol, 2.5% β -mercaptoethanol, 2% SDS, and trace Coomassie. Aliquots were run on 6 to 18% gradient polyacrylamide gels, followed by staining with R250 Coomassie (0.1%) and Bismark Brown (0.02%) in 7% acetic acid and 40% methanol. Gels were destained in 7% acetic acid and 5% methanol. Cross-linking reactions were conducted in quintuplicate to confirm reproducibility.

RESULTS AND DISCUSSION

Temperature Dependence of Basal Activity

By basal activity, we refer to the Ca²⁺-dependent phosphorylation of GP by nonactivated PhK at physiological pH, which is generally considered to be pH 6.8 for studies with this enzyme. Initial comparisons of this basal activity at 30 and 40 °C showed the activity at the latter temperature to be enhanced more than expected. To determine if this large enhancement was indicative of a specific temperature-dependent effect, as opposed to the normal increase in reaction rates one would expect with an increase in temperature, we compared the activities at 10 °C intervals between 0 and 50 °C (Figure 1A). The Q₁₀ temperature dependence was relatively constant, averaging 3.5-fold between 0 and 30 °C; but between 30 and 40 °C, there was a >8-fold increase in activity (Figure 1B). Between 40

and 50 °C, the activity decreased, suggesting the onset of thermal inactivation. The unpredicted nature of the activity change between 30 and 40 °C is further indicated by a break at 40 °C in the otherwise linear Arrhenius plot (Figure 1C) when the data of Figure 1A are replotted. Attempting to more narrowly define the temperature at which this unexpected activation occurs, we measured the basal activity at 2 °C intervals between 30 and 40 °C; however, the five Q_2 values were relatively constant, showing a 1.5-fold rate enhancement for each interval (Figure 1D). So, with an increase from 30 °C to the physiological temperature of 40 °C, there is a steadily progressive increase in PhK's basal activity that greatly exceeds that caused by the predictable effect of temperature on reaction rates. We reasoned that this unexpected rate enhancement could be due to a protein conformational change, and if so, the effect would likely be reversible. Thus, we evaluated the reversibility of activation by performing a 5 min preincubation at 40 °C and running a time course assay at 30 °C, and vice versa. The enzyme activity was found to be dependent only on the assay temperature and independent of the preincubation temperature (Figure 1E); thus, the hypothetical temperature-dependent conformational transition leading to activation is fully reversible.

As demonstrated in Figure 1E, the rate of product formation by the basal activity of PhK is not linear. This hysteretic behavior,¹⁶ which was noted in a very early study of PhK,¹⁷ has been attributed to autophosphorylation causing autoactivation¹⁸ and later to a time-dependent synergistic activation of PhK by Ca^{2+} and Mg^{2+} ions.¹⁰ Kim and Graves¹⁹ studied this hysteresis phenomenon in detail at temperatures of 30 °C and noted that the lag in product formation was more pronounced at 5 °C than at 30 °C. Because the short time courses of Figure 1E also suggested a shorter lag period at higher temperatures, we studied the lag in more detail over longer times and a broader temperature range. When GP conversion was followed at 30, 35, and 40 °C, a diminution of the lag time was observed with an increase in temperature, and by 40 °C, it was nearly abolished (Figure 2A). When assays were conducted at 45 °C, PhK activity began to diminish after 5 min, suggesting denaturation, but prior to that, the progress of the reaction appeared to be linear with no apparent lag (data not shown). Plotting estimated lag times against temperatures between 20 and 45 °C (Figure 2B) further confirmed the observation of Kim and Graves¹⁹ that the lag grows shorter with an increase in temperature. The key point here is that the hysteretic behavior of PhK nearly ceases to be a factor at the physiological temperature of 40 °C.

In an important early study that characterized factors affecting PhK activity, the pH dependence of its basal activity at 30 °C was determined.¹⁷ The nonactivated enzyme had only slight activity at neutrality, but that activity increased sharply with an increase in pH, reaching its maximum slightly above pH 8. Moreover, although the activity at neutral pH was hysteretic, as described above, the activity at the alkaline pH of ~8 was linear. From this work, the ratio of PhK activity at pH 6.8 to that at pH 8.2 in fixed-time GP conversion assays of 5 min under standardized conditions came to define the state of activation of PhK, a ratio that is used to this day for that purpose. Most laboratories consider a pH activity ratio of 0.05 indicative of nonactivated PhK; however, the ratio can often be as low as 0.01. To date, activators of PhK without exception have been shown to shorten or eliminate the hysteretic lag, causing a greater stimulation of the activity at pH 6.8 than at pH 8.2 and a concomitant increase in the pH 6.8:pH 8.2 activity ratio. Because the lag at 40 °C is

considerably shorter than at 30 °C (Figure 2), we expected the pH 6.8:pH 8.2 activity ratio to be significantly greater at the physiological temperature. For multiple PhK preparations having pH 6.8:pH 8.2 activity ratios of <0.02 at 30 °C, this ratio was, in fact, found to be on average 4-fold greater at 40 °C (data not shown). The ratio was greater simply because at pH 6.8 the Q_{10} between 30 and 40 °C was so large (Figure 1A) compared to an observed average Q_{10} of ~2 at pH 8.2 (data not shown), the latter being what one would expect for a typical linear enzyme-catalyzed reaction. (These effects of temperature and pH on activity of a single enzyme preparation can be observed in Table 1.)

Our initial definition of basal activity in this section was the Ca^{2+} -dependent activity of nonactivated enzyme at pH 6.8, because early studies from two laboratories showed that maximal PhK activity required Ca^{2+} ions;^{20–22} however, even at concentrations of the Ca^{2+} chelator EGTA well in excess of the concentration of Ca^{2+} , a small amount of apparent Ca^{2+} -independent activity remained.^{21,23} To examine the effect of temperature, specifically 40 versus 30 °C, on this Ca^{2+} -independent activity, standard GP conversion assays were performed in the presence of 0.2 mM EGTA with or without 0.3 mM Ca^{2+} (+, to simulate conditions of Figure 1; –, for Ca^{2+} -independent conditions).

In contrast to the Ca^{2+} -dependent activity shown in Figure 1, we observed for multiple enzyme preparations that the Q_{10} between 30 and 40 °C approximately only doubled for the Ca^{2+} -independent activity at pH 6.8; moreover, the reactions were linear at both temperatures (data not shown). The fact that hysteresis is not observed in the absence of Ca^{2+} supports the hypothesis that hysteresis is caused by the synergistic actions of Ca^{2+} and Mg^{2+} on PhK.¹⁰ It might also be noted that at pH 6.8 and 40 °C, physiological conditions for resting muscle, the Ca^{2+} -independent activity of PhK is greater than has been appreciated, reaching ~2% of what has typically been assumed to be a maximal value for the Ca^{2+} -dependent activity of activated enzyme.

Temperature Dependence of Autophosphorylation

We evaluated the effect of temperature on autophosphorylation to eliminate the possibility that the temperature effects described above were due to an alteration of the properties of the substrate GP, rather than of the properties of PhK itself. If the anomalous temperature effect is truly on PhK, then one would expect PhK's self-phosphorylation at different temperatures to mirror its temperature-dependent phosphorylation of GP depicted in Figure 1. As was done for that figure, Ca^{2+} -dependent autophosphorylation was conducted at pH 6.8 for a fixed time of 5 min at temperature intervals of 10 °C between 0 and 40 °C. As before, there was a steady increase in the rate of phosphorylation between 0 and 30 °C, but between 30 and 40 °C, the rate dramatically increased (Figure 3A). As was the case for GP conversion, the Q_{10} values for autophosphorylation were relatively constant between 0 and 30 °C, with an average value of 1.7, and between 30 and 40 °C, there was once again a large increase in rate, with a Q_{10} value of 4.2 (Figure 3B). For whatever reason, both of these Q_{10} values are only half of those observed for GP conversion. The discontinuity in the temperature dependence of autophosphorylation is shown by the break at 40 °C in the otherwise linear Arrhenius plot (Figure 3C) when the data of Figure 3A are replotted. In summary, the

anomalous enhancement of PhK activity in going from 30 to 40 °C is due to the occurrence of an inherent change in the PhK complex itself, which we presume to be conformational.

In performing the experiments described above, we noted that there was no lag apparent in the autophosphorylation reaction at 40 °C; thus, we thought that this condition might be informative in evaluating the effects of an activator and an inhibitor known to shorten and lengthen, respectively, the lag at 30 °C. The activator used was ADP, which acts allosterically to stimulate PhK activity, including autophosphorylation, and ADP concentrations of 25 μ M abolish the lag in the progress curve of autophosphorylation at pH 6.8 and 23 °C.²⁴ We thus examined the effect of ADP at 23, 30, and 40 °C. Stimulation of autophosphorylation activity was greatest at 23 °C, lessened at 30 °C, and disappeared altogether at 40 °C, as did the lag (Figure 4A). The inhibitor used was orthophosphate, which is known to accentuate the autophosphorylation lag.²⁵ Autophosphorylation assays were performed at 30 and 40 °C with or without P_i from 2 to 50 mM. The maximal inhibition observed, which occurred at ~20 mM P_i at both temperatures, was 90% at 30 °C, but only 40% at 40 °C (Figure 4B). Examination of the time courses of autophosphorylation revealed the absence of a lag at 40 °C regardless of the P_i concentration, whereas an increase in the lag time with increasing concentrations of P_i was observed at 30 °C (data not shown). Therefore, at the physiological temperature, this particular activator at 30 °C failed to activate, and this inhibitor was much less effective in inhibiting.

Activity after Phosphorylation by PKA

The phosphorylation of PhK by PKA is recognized to cause a large increase in the rate of its conversion of GP at pH 6.8 and 30 °C²⁶ and to remove hysteresis, making the reaction linear. Both the α and β subunits become phosphorylated, but the activation is predominantly associated with phosphorylation of the latter near its N-terminus at Ser-26.^{27,28} We were curious if the activation by phosphorylation with PKA was less pronounced at 40 °C, thus matching the diminishing activation by ADP with increasing temperature. Phosphorylation was conducted at 30 °C with a relatively high concentration of PKA to achieve a high extent of phosphorylation (0.49 mol of P/mol of α and 0.61 mol of P/mol of β) and activation. Assaying the resultant phospho-PhK for GP conversion activity under standard conditions at pH 6.8 showed that at 30 °C the observed activation by phosphorylation was 26-fold; however, when the same enzyme was assayed at 40 °C, the activation was only 4.6-fold (Table 1). Once again, a dramatic effect observed at 30 °C is highly muted at the physiological temperature of 40 °C. Note that the pH 6.8:pH 8.2 activity ratios of the phospho-enzyme are the same at both temperatures, consistent with the linearity of all four reactions.

Evidence of a Conformational Change between 30 and 40 °C

Because of the anomalous sharp increase in the GP conversion and autophosphorylation activities of PhK between 30 and 40 °C, we inferred that a temperature-induced conformational transition occurs in this temperature range, resulting in a more active conformation. No physical evidence of such a change is obvious, however, from a variety of biophysical approaches: second-derivative UV absorption spectroscopy,³ intrinsic Trp fluorescence,³ extrinsic 1-anilino-naphthalene-8-sulfonate fluorescence,³ far-UV CD,³

dynamic light scattering,³ and differential scanning calorimetry (data not shown). One possible reason that a conformational transition was not observed is that these biophysical measurements did not include the full complement of cofactors and substrate that are present in PhK's activity assays, namely, Ca^{2+} , Mg^{2+} , and ATP. Perhaps one or more of these three additional components is necessary for a temperature-induced conformational transition to occur. A second possible reason is that a conformational transition does occur but is too small or too localized to be detected by the methods used. It should be noted that for many biophysical approaches the extremely large mass of PhK produces strong background signals that could readily obscure detection of conformational changes.

Inasmuch as PhK is able to differentiate between conformational states of GP,²⁹ we asked whether PKA might be able to do the same with its substrate PhK and distinguish between its conformations at 30 and 40 °C. Thus, we compared the rates of phosphorylation of PhK's α and β subunits at 30 and 40 °C by the catalytic subunit of PKA. The rate of phosphorylation of the α subunit was 276% faster at the higher temperature (Figure 5A) for a Q_{10} value of 2.8, which corresponds to a value one would normally expect for an enzyme-catalyzed reaction. In sharp contrast, the rate of phosphorylation of the β subunit in the same experiment was only 30% greater at the higher temperature compared to the lower (Figure 5B), giving a quite atypical Q_{10} value of merely 0.3. This 9-fold difference (2.8 vs 0.3) in the increased rate of phosphorylation of α versus β at the higher temperature suggests that a conformational change in PhK does occur between 30 and 40 °C and that it likely involves at least the phosphorylatable N-terminal regions of the β subunits, i.e., targets of PKA. We propose that a large increase in the rate of phosphorylation of β that one would normally expect in going from 30 to 40 °C is counterbalanced by a conformational change in β that simultaneously gives rise to activation but makes it a relatively worse substrate for PKA. A temperature-induced conformational change that leads to activation and involves the N-terminal region of β would be consistent with our recent structural model for activation of PhK by phosphorylation.³⁰ That report describes activation thusly: "the non-activated state of PhK is maintained by the interaction between the nonphosphorylated N-termini of β and the regulatory C-terminal domains of the γ subunits; phosphorylation of β weakens this interaction, leading to activation of the γ subunits." Perhaps the transition from 30 to 40 °C similarly weakens the interaction of the N-terminus of β with γ , bringing about activation and a conformational change in β .

Hoping to find evidence that more directly indicates a sharp conformational transition in the β subunits between 30 and 40 °C, we employed chemical cross-linking as a conformational probe. The short, homobifunctional cross-linker 1,5-difluoro-2,4-dinitrobenzene (DFDNB) has been shown to form heavy conjugates via cross-linking all of PhK's subunits except δ .³¹ Therefore, we incubated nonactivated PhK at pH 6.8 with a 50-fold molar excess of DFDNB (cross-linker, $\alpha\beta\gamma\delta$ protomer) for 6 min at 10 °C intervals between 0 and 40 °C, ran SDS-PAGE gels (Figure S1), and measured by densitometry the amount of each subunit remaining. There was a steady, unremarkable progression in the disappearance of the α subunit with an increase in temperature (Figure 6A), which serves as an internal control for the predictable effect of temperature on crosslinking kinetics. The behavior of the β subunit was in sharp contrast to that of α , in that very little β was consumed between 0 and 30 °C; however, at 40 °C, most of the β subunit had disappeared, surpassing even the consumption

of α at that temperature (Figure 6B). Thus, between 30 and 40 °C, the β subunit undergoes a dramatic temperature-dependent change in its conformation. The consumption of the γ subunit mirrored that of β , in that there was a dramatic increase at 40 °C in its cross-linking to form heavy conjugates (Figure 6C).

PhK is arranged as a dimer of $(\alpha\beta\gamma\delta)_2$ octamers, with the two octamers interconnected by four discrete, centrally located bridges.³² These four bridges are composed entirely of the four β subunits.³³ When the enzyme becomes activated by phosphorylation, the $(\alpha\beta\gamma\delta)_4$ PhK complex as a whole becomes less stable, but the four β subunits in the phospho-activated conformer avidly interact with one another.³⁴ With the nonactivated enzyme, the cationic activator Ca^{2+} causes noteworthy structural changes in the β bridges,^{35,36} and other activators of PhK, such as Mg^{2+} and ADP, have been shown to cause conformational changes in the β subunits.^{37–39} It appears that the temperature-dependent activation described herein can be added to the list of activating mechanisms that affect the conformation of the β subunits.

As for the parallel changes in the disappearance of the β and γ subunits, that was not a complete surprise, as these subunits have previously been shown to be functionally and structurally linked with each other. Immuno-electron microscopy revealed that monoclonal antibodies against the two subunits bound to adjacent regions on the interior lobe face of the complex, and activation of PhK by a variety of methods (phosphorylation, pH 8.2, and binding of ADP or Mg^{2+}) caused similar increases in the level of binding of the two antibodies.³⁷ Thus, the structures of both subunits change in parallel upon the activation of PhK through various mechanisms. Moreover, monovalent fragments of both antibodies stimulated the activity of nonactivated PhK. A $\beta\gamma$ dimer has been observed by native MS of the nonactivated enzyme,³⁷ and chemical cross-linking has shown linkage between the N-terminal region of the β subunit and the C-terminal regulatory region of the γ subunit,⁴⁰ which could provide a means of interaction and communication between the two subunits. Additionally, a synthetic peptide corresponding to the N-terminus of β has been shown to inhibit the catalytic activity of phospho-activated PhK,³⁰ further supporting the interaction and communication between these two subunits. Thus, the activation of nonactivated PhK by physiological temperature may occur with structural changes in the β and γ subunits similar to those that occur in other mechanisms of activation.

There are significant differences between the properties of PhK at the physiological temperature of the rabbit versus the enzyme that has been characterized over the past six decades at temperatures of 30 °C. The nonactivated enzyme has much greater activity than previously appreciated, and that activity is stimulated considerably less by phosphorylation. In fact, effectors, whether activating or inhibiting, are less effective at the physiological temperature. In general, the allosteric control of PhK's activity is definitely more subtle at body temperature. The apparent underlying reason for these differences in activity and its control (stimulatory and inhibitory) is the near loss of hysteresis at body temperature. An unanswered question is whether the transition from hysteretic to linear activity at temperatures of 30 °C occurs concomitantly with a change in the conformation of the β and γ subunits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

DFDNB	1,5-difluoro-2,4-dinitrobenzene
GP	glycogen phosphorylase <i>b</i>
PhK	phosphorylase kinase
PKA	cAMP-dependent protein kinase

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Figure 1.

Temperature dependence of GP conversion at pH 6.8. (A) Amount of GP converted at a fixed time at each indicated temperature. (B) Fold change in activity between each 10 °C temperature interval (Q_{10}), calculated by dividing the value at the higher temperature by that at the lower. (C) Arrhenius plot for the activity shown in panel A. (D) Fold change in activity at 2 °C temperature intervals (Q_2) between 30 and 40 °C, calculated as in panel B. (E) Effects of preincubation and assay temperatures on activity. PhK was preincubated for 5 min at either 30 or 40 °C and immediately assayed at both of the same temperatures. The preincubation and assay temperatures were 30 and 30 °C (□), 40 and 30 °C (○), 30 and 40 °C (■), and 40 and 40 °C (●), respectively. Where present, the error bars (red) indicate the standard error of triplicate assays and generally did not exceed the symbol dimensions.

Figure 2.

Effect of temperature on the hysteric behavior of GP conversion at pH 6.8. (A) Time course of GP conversion at 30 (○), 35 (□), and 40 °C (△). Assays were conducted in duplicate. Error bars representing the average deviation generally did not exceed the symbol dimensions. (B) Lag time of GP conversion as a function of temperature. The lag time was estimated from the interception on the abscissa of a tangent to the most linear portion of the time course of GP conversion. In these assays, the concentrations of Tris and β -glycerophosphate were 64 mM and Hepes was not present.

Figure 3.

Temperature dependence of autophosphorylation at pH 6.8. (A) Activities in stopped-time assays between 0 and 40 °C. Besides the components described in Materials and Methods, these assays also contained β -glycerophosphate (64 mM), Hepes (8 mM), and [γ - ^{32}P]ATP (3 mM). Assays were conducted in triplicate. Error bars depicting the standard error did not exceed the symbol dimensions. (B) Q_{10} of stimulation calculated as described in the legend of Figure 1B. (C) Arrhenius plot of the data in panel A.

Figure 4.

Temperature-dependent effects of ADP and P_i on autophosphorylation at pH 6.8. (A) Time course of phosphate incorporation at 23, 30, and 40 °C in the absence (○, □, and △, respectively) and presence (●, ■, and ▲, respectively) of 0.1 mM ADP. In addition to the components listed in Materials and Methods, these assays also contained [γ - 32 P]ATP (0.4 mM), β -mercaptoethanol (4 mM), and Hepes (50 mM). Assays were conducted in triplicate. Error bars depicting the standard error generally did not exceed the symbol dimensions. (B) Inhibition by orthophosphate at 30 (○) and 40 °C (●). In addition to the standard components, the assays also contained [γ - 32 P]ATP (1.5 mM), dithiothreitol (0.1 mM), and Hepes (50 mM). Assays were conducted in duplicate, with error bars representing the average deviation.

Figure 5.

Phosphorylation of the (A) α and (B) β subunits by PKA at 30 (○) and 40 °C (●). The reaction mixtures at pH 6.8 contained PhK (1 mg/mL), EDTA (0.5 mM), Tris (50 mM), β -glycerophosphate (50 mM), and PKA (0.04 μ g/mL), and the reactions were initiated with $\text{Mg}(\text{CH}_3\text{CO}_2)_2$ and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10 and 0.2 mM, respectively). At the indicated times, aliquots were removed, quenched by dilution into reducing SDS buffer, and run on SDS-PAGE, and phosphorylation was quantified. Assays were performed in triplicate. Error bars depicting the standard error generally did not exceed the symbol dimensions.

Figure 6.

Temperature-dependent subunit consumption by DFDNB cross-linking. The percent consumption of (A) α , (B) β , and (C) γ after cross-linking for 6 min with a 50-fold molar excess of DFDNB at the temperatures indicated. Percent consumption, derived from Figure S1, is based on the density remaining of the monomeric subunits relative to the density of the unmodified subunits from the same concentration of control non-cross-linked enzyme. Error bars represent the standard error of five replicate cross-linking reactions at each temperature.

Table 1

Activity of Control and Phospho-PhK at 30 vs 40 °C

enzyme	temp (°C)	specific activity ($\mu\text{mol of P min}^{-1} \text{mg}^{-1}$)		
		pH 6.8	pH 8.2	pH 6.8:pH 8.2 ratio
nonactivated	30	0.05 ± 0.01	2.5 ± 0.5	0.02
phospho-activated	30	1.30 ± 0.07	3.1 ± 0.7	0.42
nonactivated	40	0.67 ± 0.14	6.7 ± 1.1	0.10
phospho-activated	40	3.09 ± 0.07	7.4 ± 0.5	0.42