Physicochemical changes in phosphorylase kinase associated with its activation

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Abstract

Phosphorylase kinase (PhK) regulates glycogenolysis through its Ca^{2+} -dependent phosphorylation and activation of glycogen phosphorylase. The activity of PhK increases dramatically as the pH is raised from 6.8 to 8.2 (denoted as $\uparrow\text{pH}$), but Ca^{2+} dependence is retained. Little is known about the structural changes associated with PhK's activation by $\uparrow\text{pH}$ and Ca^{2+} , but activation by both mechanisms is mediated through regulatory subunits of the $(\alpha\beta\gamma\delta)_4$ PhK complex. In this study, changes in the structure of PhK induced by $\uparrow\text{pH}$ and Ca^{2+} were investigated using second derivative UV absorption, synchronous fluorescence, circular dichroism spectroscopy, and zeta potential analyses. The joint effects of Ca^{2+} and $\uparrow\text{pH}$ on the physicochemical properties of PhK were found to be interdependent, with their effects showing a strong inflection point at $\text{pH} \sim 7.6$. Comparing the properties of the conformers of PhK present under the condition where it would be least active ($\text{pH} 6.8 - \text{Ca}^{2+}$) versus that where it would be most active ($\text{pH} 8.2 + \text{Ca}^{2+}$), the joint activation by $\uparrow\text{pH}$ and Ca^{2+} is characterized by a relatively large increase in the content of sheet structure, a decrease in interactions between helix and sheet structures, and a dramatically less negative electrostatic surface charge. A model is presented that accounts for the interdependent activating effects of $\uparrow\text{pH}$ and Ca^{2+} in terms of the overall physicochemical properties of the four PhK conformers described herein, and published data corroborating the transitions between these conformers are tabulated.

Keywords: phosphorylase kinase; Ca^{2+} ; Tyr environment; secondary structure; tertiary structure; surface electrostatics; zeta potential

Phosphorylase kinase (PhK), a complex regulatory enzyme in the cascade activation of glycogenolysis, is the only known kinase to phosphorylate and activate glycogen phosphorylase. By far, the most is known about the PhK from skeletal muscle (for reviews, see Heilmeyer Jr. 1991; Brushia and Walsh 1999), and that is the subject

of this study. This PhK is a 1.3-MDa hexadecameric complex composed of four copies each of a single catalytic protein kinase subunit (γ , 44.7 kDa) and three different regulatory subunits (α , 138.4 kDa; β , 125.2 kDa; and δ , 17.7 kDa). Thus, its regulatory subunits account for 86% of the mass of the $(\alpha\beta\gamma\delta)_4$ PhK complex. The α and β subunits are homologous and inhibitory, whereas the δ subunits, which are endogenous molecules of calmodulin, are stimulatory. It is through allosteric sites on these regulatory subunits that PhK integrates metabolic (ADP), hormonal (cAMP and Ca^{2+}), and neural (Ca^{2+}) signals to control the rate of glycogen breakdown through large increases in the kinase activity of its catalytic γ subunits. Ca^{2+} ions presumably exert their stimulatory effect through binding to the δ subunits, which in muscle couples contraction with energy production to supply

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Abbreviations: CD, circular dichroism; $\uparrow\text{pH}$, an increase in pH from 6.8 to 8.2; PhK, phosphorylase kinase; SE-HPLC, size exclusion-high performance liquid chromatography.

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the contraction's "short-term power demands" (Shulman 2005). Activation by cAMP-dependent protein kinase or ADP is effected through sites on the α and/or β subunits and can be thought of most simply as deinhibition. Activation through these sites on the α and β subunits apparently still requires participation of the δ subunits, because the catalytic activity of all forms of PhK retains an absolute requirement for Ca^{2+} ions.

The activation state of PhK is assessed by comparing its activity at pH 6.8 with that at pH 8.2, because activators bring about a very large increase in activity at the former pH and little at the latter; however, the activity at both pH values remains fully Ca^{2+} dependent (Krebs et al. 1964). In the absence of activators other than Ca^{2+} , the catalytic activity at pH 8.2 is well-behaved and linear; however, the activity at pH 6.8 is hysteretic, being characterized by a long lag time. At short assay times, the activity at the higher pH is ~ 100 -fold greater than that observed at the lower pH, and the activity vs. pH slope is sharp between the two values (Krebs et al. 1966). Activation, such as by phosphorylation, is manifested not only by a large increase in the pH 6.8 activity, but by that activity now becoming linear. Because activators have either no or little effect at pH 8.2 and because they make the activity at neutral pH linear, as occurs at pH 8.2, the increase in pH from 6.8 to 8.2 may be thought of as an *in vitro* mechanism for PhK's activation that mimics to some degree other mechanisms of activation. In fact, utilizing a photoreactive cross-linker as a conformational probe, it was concluded that pH 8.2 induces a conformation that is structurally similar to those caused by other activation mechanisms (Nadeau et al. 1999). In the present work, a battery of biophysical techniques has been used to evaluate the structural changes in PhK induced by increasing the pH from 6.8 to 8.2 (denoted herein as $\uparrow\text{pH}$), and whether structural changes induced by Ca^{2+} are pH dependent. From the results, a working model has been constructed that accounts for the interdependent activating effects of $\uparrow\text{pH}$, and Ca^{2+} in terms of PhK's overall physicochemical properties.

Results

Changes in the tertiary structure of PhK induced by Ca^{2+} and increasing pH

Second derivative UV absorption spectroscopy

PhK possesses a substantial number of aromatic residues: 428 Phe, 460 Tyr, and 124 Trp. Changes in the microenvironments of these residues induce alterations in their spectral properties, which can be resolved to 0.01 nm (Mach and Middaugh 1994), thus providing a sensitive means to monitor variations in PhK's tertiary

structure in response to Ca^{2+} or increasing pH. The second derivative spectrum of PhK at pH 6.8 shows six negative peaks having the following residue assignments: Phe (~ 245 and 251 nm), Tyr (~ 260 and 270 nm), overlapping Tyr/Trp signal (~ 276 nm), and Trp (~ 285 nm) (Fig. 1A). In general, exposure of aromatic amino acid side chains to a more polar environment causes a blueshift in their absorbance minima, whereas shifts to longer wavelengths suggest that the residues are on average incorporated into a more buried, less polar environment (Demchenko 1986). From Figure 1, one observes peak shifts upon $\uparrow\text{pH}$ or Ca^{2+} binding only for those peaks assigned to Tyr residues. When the pH is increased from 6.8 to 8.2, a small blueshift is observed in the Tyr peaks, suggesting increased solvent exposure of Tyr residues (Fig. 1A). In contrast, upon PhK's binding of Ca^{2+} at pH 6.8, the Tyr peak at ~ 270 nm is now shifted to a longer wavelength, suggesting that these residues are moved to a more apolar environment (Fig. 1B).

Comparing the opposite effects of Ca^{2+} and $\uparrow\text{pH}$ on the Tyr residues' microenvironment, the former activator seems to be dominant. For example, when the pH is increased to 8.2, the Tyr peak located at ~ 270 nm displays an even larger redshift when Ca^{2+} is added (Fig. 1C); however, when PhK is already in the Ca^{2+} -activated form, no significant changes are observed in the Tyr peaks by increasing the pH from 6.8 to 8.2 (Fig. 1D).

Further UV absorption studies were performed by increasing the pH by 0.2 unit increments from pH 6.8 to 8.2. The wavelength positions of PhK's Phe, Tyr, Tyr/Trp, and Trp second derivative minima are plotted in Figure 1F as a function of pH for Ca^{2+} -free and Ca^{2+} -bound PhK. The positions of the Phe, Tyr/Trp, and Trp peaks remained relatively unchanged with $\uparrow\text{pH}$ and were virtually identical $\pm \text{Ca}^{2+}$ at each pH value. In contrast, large changes were observed for the Tyr peak at ~ 270 nm: In the absence of Ca^{2+} , this peak progressively shifted to a shorter wavelength with $\uparrow\text{pH}$, indicating a stepwise exposure of Tyr residues to a more polar environment. At each pH value, the addition of Ca^{2+} caused a redshift of the Tyr peak. That is to say, at each pH the binding of Ca^{2+} led to a relative increase in the burial of Tyr residues into more apolar environments, and the higher the pH, the greater the change. Comparing the changes in the Tyr peak positions of the Ca^{2+} -free and Ca^{2+} -bound conformers of PhK, the latter was less sensitive to changes in pH, again suggesting a dominant effect of Ca^{2+} over $\uparrow\text{pH}$ with respect to overall Tyr microenvironments.

Intrinsic fluorescence spectroscopy

Intrinsic fluorescence spectroscopy was used to further investigate changes in PhK's tertiary structure. The intrinsic fluorescence emission of proteins contains

Figure 1. High-resolution second derivative UV spectroscopy. (A–E) Absorption of PhK $\pm$ Ca²⁺ at pH 6.8 and 8.2. The second derivative minima framed represent the absorbance of Tyr residues. (F) Negative peak positions as a function of pH. Each data point is the second derivative minimum in the wavelength range characteristic of aromatic chromophores in proteins for Ca²⁺-free (○) and Ca²⁺-bound (△) PhK at the indicated pH values. Although discontinuous, the scale on the ordinate is kept the same.

overlapping peaks from Tyr and Trp residues; however, these can be distinguished by using the synchronous fluorescence technique (Lloyd 1971; Miller 1979). Synchronous fluorescence spectra are obtained through simultaneous scanning by the excitation and emission monochromators, and $\Delta\lambda$ represents the value of the difference between excitation and emission wavelengths (Liu et al. 2006; Hou et al. 2007). When $\Delta\lambda$ is set at either 60 nm or 15 nm, the shift of peak position and the change in fluorescence intensity imply an alteration in the polarity of microenvironments surrounding Trp and Tyr residues, respectively (Cui et al. 2006; Yuan et al. 2007). The synchronous fluorescence spectra of PhK $\pm$ Ca²⁺ at pH 6.8 and 8.2 are presented in Figure 2. Compared to the pH 6.8 spectrum, the $\lambda_{\max}$ of the Trp peaks show a decrease in fluorescence intensity and a small redshift by $\uparrow$ pH or

adding Ca²⁺; however, no further redshift occurs upon the addition of Ca²⁺ at pH 8.2 (Fig. 2A). Among the four conformers represented by the curves of Figure 2A, those at pH 6.8 + Ca²⁺ and at pH 8.2 – Ca²⁺ are the most similar, indicating the most similar microenvironments for PhK's Trp residues under those two conditions. The $\lambda_{\max}$ of the Tyr peaks behaved similarly to those of Trp in showing a decrease in fluorescence intensity and a small redshift by $\uparrow$ pH or adding Ca²⁺, with no further redshift occurring upon the addition of Ca²⁺ at pH 8.2 (Fig. 2B). In fact, the last condition resulted in a blueshift. Of the four conformers, the Ca²⁺-activated PhK at pH 8.2 has the lowest fluorescence intensity for both its Trp and Tyr residues, indicating that the collective signals from those residues in this conformation are subject to the most quenching by the effects of polar solvent, proximal charged or

Figure 2. Synchronous fluorescence spectra of PhK: (A) at $\Delta\lambda = 60$ nm (Trp) and (B) at $\Delta\lambda = 15$ nm (Tyr). The emission peaks displayed in curves 1–4 represent Ca^{2+} -free at pH 6.8, Ca^{2+} -bound at pH 6.8, Ca^{2+} -free at pH 8.2, and Ca^{2+} -bound at pH 8.2 forms of PhK, respectively.

uncharged polar side chains, and/or peptide backbone amides (Demchenko 1986).

Secondary structure characteristics

Circular dichroism (CD) was used to investigate changes in the secondary structure of PhK caused by the binding of Ca^{2+} and $\uparrow\text{pH}$. The spectrum of PhK at pH 6.8 shows two minima near 208 and 220 nm (Fig. 3A), which is characteristic of proteins containing a significant extent of α -helical content. Addition of Ca^{2+} at pH 6.8 did not change the positions of these two minima, but a little greater negative ellipticity at 208 nm was observed (Fig. 3A). The CD spectra of PhK observed at pH 8.2 are much different than those at pH 6.8: With the addition of Ca^{2+} at pH 8.2, the peak located ~ 220 nm is shifted to 223 nm, concomitant with an increase in the intensity of the CD signal, whereas the ellipticity at 208 nm is decreased dramatically with no change in the peak position (Fig. 3B).

Because the ratio of negative ellipticity at 222 nm to 208 nm is an index of the extent of interaction between α -helices and β -sheets (Levitt and Chothia 1976; Chothia et al. 1977; Manavalan and Johnson 1983; Arnold et al. 1992; Park et al. 1993), more detailed studies were carried out to investigate pH- and Ca^{2+} -induced changes in this ratio. At low pH values ($\text{pH} \leq 7.0$), the $\theta_{222}/\theta_{208}$ ratio did not significantly change upon addition of Ca^{2+} ; however, above this pH the $\theta_{222}/\theta_{208}$ ratio in the presence of Ca^{2+} progressively increased until exceeding unity at

pH 8.0 and above (Fig. 4). In contrast, for Ca^{2+} -free PhK, an increasing pH caused a small progressive decrease in the $\theta_{222}/\theta_{208}$ ratio (Fig. 4).

The secondary structure content of PhK was analyzed by the prediction program DICHROWEB (Lobley et al. 2002), with the results listed in Table 1. At pH 6.8 – Ca^{2+} , the secondary structure content is estimated to be 59% helix, 10% sheet, 14% turn, and 17% unordered regions, with the addition of Ca^{2+} inducing a small increase in α -helix and a decrease in sheet structure. At pH 8.2, which showed a higher amount of basal sheet structure (20%), the addition of Ca^{2+} caused very little change.

Electrostatic surface charge/zeta potential

Zeta potential measurements obtained by analyzing the shear surface electric potential of colloidal particles in solution provide an estimate of effective surface charge (Hunter 1981), and this value for PhK at the fixed pH of 6.8 was recently shown to be dramatically altered by its binding of Ca^{2+} (Priddy et al. 2007). Using phase analysis

Figure 3. CD spectra of Ca^{2+} -free (○) and Ca^{2+} -bound (△) PhK at pH 6.8 (A) and 8.2 (B). CD spectra were recorded from 190 to 260 nm, with standard errors of the mean ($n = 3$) shown. Each inset is an amplification of the region between 205 and 230 nm.

Figure 4. The $\theta_{222}/\theta_{208}$ (CD) ratios as a function of pH for Ca^{2+} -free (○) and Ca^{2+} -bound (●) PhK.

light scattering (McNeil-Watson et al. 1998), the zeta potential of Ca^{2+} -free and Ca^{2+} -bound PhK was measured in the current study as a function of pH. Increasing the pH from 6.8 to 8.2 caused the zeta potential values for both the Ca^{2+} -free and Ca^{2+} -bound forms of PhK to become progressively less negative; however, the greatest effects of pH were observed for Ca^{2+} -free PhK below pH 7.6 (Fig. 5). The most negative zeta potential was observed for Ca^{2+} -free PhK at pH 6.8, and that became significantly less negative upon the binding of Ca^{2+} , corroborating previous results (Priddy et al. 2007). Above pH 7.6 there was little change in zeta potential for either Ca^{2+} -free or Ca^{2+} -bound PhK, but the least negative zeta potential was observed for the latter form at pH 8.2.

Discussion

The α and β subunits of the PhK complex account for 81% of its 1.3-MDa mass (42.6% α and 38.5% β), 82% of its 460 Tyr residues (39.1% α and 42.6% β), and 87% of its 124 Trp residues (41.9% α and 45.1% β) (Kilimann et al. 1988). Thus, most of the physicochemical changes observed in this study likely reflect changes in PhK's α and β subunits. Moreover, the changes induced by $\uparrow$ pH are most likely triggered by deprotonation of residue(s) on the α and/or β subunits, because the pH 6.8/8.2 activity ratio of the $\gamma\delta$ dimer approaches unity (Chan and Graves 1982; Kumar et al. 2004), whereas that of the $(\alpha\beta\gamma\delta)_4$ PhK complex is dramatically lower at ~ 0.01 . Even though the changes induced by Ca^{2+} are presumably triggered by PhK's endogenous calmodulin (δ) subunit, the affinity of PhK for Ca^{2+} is different at pH 6.8 and 8.2 (Brostrom et al. 1971; Cohen 1980), and phosphorylation of its α and β subunits also alters PhK's affinity for Ca^{2+} (Brostrom et al. 1971; Cohen 1980). Thus, there is considerable communication among PhK's four different subunits, with α and β being inhibitory, particularly at pH 6.8: Not only does perturbation of their structures through

either phosphorylation or partial proteolysis cause dramatic activation (deinhibition) of the PhK complex (Brushia and Walsh 1999), they directly inhibit activity of the isolated γ subunit (Paudel and Carlson 1987, 1988). In the case of the α subunit, there is strong evidence of a Ca^{2+} -dependent $\alpha \leftrightarrow \gamma \leftrightarrow \delta$ communication network within the PhK complex (Nadeau et al. 2002; Rice et al. 2002); but much less is known about the interactions of the α and β subunits that are affected by pH.

In the absence of Ca^{2+} , the effects of $\uparrow$ pH on PhK's relative tertiary structure were relatively modest as monitored by the differential features exhibited in multiple spectra acquired using dual methods. Second derivative UV absorption showed only Tyr residues of PhK to be influenced by the deprotonation assumed to occur with $\uparrow$ pH, as evidenced by a small blueshift in their absorbance minimum at ~ 269 nm (Fig. 1A,F). Correspondingly, intrinsic fluorescence (synchronous) showed a small redshift for both Trp and Tyr residues upon $\uparrow$ pH (Fig. 2). Although opposite in direction, the structural changes that cause a blueshift in UV absorption and a redshift in fluorescence emission indicate the same phenomenon, namely exposure of chromophore/fluorophore to a more polar environment (Demchenko 1986). In the case of PhK, both occur as a function of $\uparrow$ pH in the absence of Ca^{2+} . It is also important to recall the utility of synchronous fluorescence for discerning the fluorescence emission of both types of fluorophores. Because the Trp (excitation) and Tyr (emission) peaks overlap, it is possible that the influence of Trp was not totally resolved from the Tyr spectrum, which often results in Tyr emission being quenched by proximal Trp residues (Demchenko 1986). The synchronous fluorescence data support the conclusion from the second derivative UV studies that, overall, Tyr residues are affected to a greater extent than Trp by $\uparrow$ pH. Changes in PhK's secondary structure induced by $\uparrow$ pH in the absence of Ca^{2+} were also relatively modest, with a slight decrease in the intensity of the negative ellipticity at 222 and 208 nm (Fig. 3), a small progressive decrease in the $\theta_{222}/\theta_{208}$ ratio (Fig. 4), and an increase in the content of sheet structure (Table 1). By far the most dramatic change observed in PhK's properties upon its deprotonation was in its surface charge as estimated by the measured zeta potential, which

Table 1. Secondary structure content of PhK $\pm \text{Ca}^{2+}$ analyzed by DICHROWEB

	Helix %	Sheet %	Turn %	Unordered %	Total
PhK 6.8	59 $\pm$ 2	10 $\pm$ 1	14 $\pm$ 2	17 $\pm$ 2	100
PhK 6.8 + Ca^{2+}	64 $\pm$ 2	6 $\pm$ 1	15 $\pm$ 1	15 $\pm$ 2	100
PhK 8.2	57 $\pm$ 2	20 $\pm$ 2	11 $\pm$ 1	12 $\pm$ 2	100
PhK 8.2 + Ca^{2+}	55 $\pm$ 2	21 $\pm$ 2	10 $\pm$ 1	14 $\pm$ 2	100

Figure 5. Zeta potential values as a function of pH for Ca^{2+} -free (Δ) and Ca^{2+} -bound ($\blacktriangle$) PhK.

sharply became less negative as the pH was increased from 6.8 to 7.4, and reached a plateau at pH 7.8 (Fig. 5).

With the techniques utilized in this study, changes in PhK's physicochemical properties induced by Ca^{2+} were generally more apparent than the changes induced by $\uparrow\text{pH}$; however, the Ca^{2+} -induced changes in all properties were nevertheless influenced by pH. Second derivative UV absorption showed a Ca^{2+} -induced redshift (burial) in the absorbance minima of Tyr residues at all pH values, but the shift was greatest at the highest pH (Fig. 1F). The largest effect of Ca^{2+} on fluorescence was also at pH 8.2, where the intensities of both Trp and Tyr were greatly diminished and Tyr showed a considerable blueshift (Fig. 2). Regarding secondary structure, Ca^{2+} caused relatively large changes at pH 8.2 compared to pH 6.8 (Fig. 3). Likewise, it dramatically increased the $\theta_{222}/\theta_{208}$ ratio at pH 8.2, while having no effect at pH 6.8 (Fig. 4). In contrast, Ca^{2+} caused the zeta potential to become considerably less negative at pH 6.8, but not at pH 8.2 (Fig. 5).

The sum of the data in this study indicates that the Ca^{2+} -free and Ca^{2+} -bound forms of PhK at pH 6.8 (protonated) and pH 8.2 (deprotonated) represent four distinct conformations having distinct physicochemical properties. These forms are designated as H-E, H-E-Ca, E, and E-Ca, respectively, in Figure 6A, where they are vertically ordered according to their zeta potentials. In addition to the results of this current study, a number of previous reports using a wide variety of techniques provide more evidence for the conformational transitions numbered 1 through 3 in Figure 6A between pairs of the different forms of PhK studied herein, and this evidence is compiled for the first time in Table 2. We are unaware of any previous biophysical studies on conformational transition #4, H-E-Ca $\rightarrow$ E-Ca. Considering the four forms of PhK analyzed in this current study, the Ca^{2+} -bound conformers at pH 6.8 (H-E-Ca) and Ca^{2+} -free at pH 8.2 (E) are perhaps the most similar overall in their physicochemical properties: second derivative UV ab-

sorption spectra (Fig. 1E), intrinsic Trp fluorescence (Fig. 2A), $\theta_{222}/\theta_{208}$ ratios (Fig. 4), and zeta potentials (Fig. 5). The two most dissimilar conformers with respect to their physicochemical properties are the Ca-free form at pH 6.8 (H-E) and the Ca-bound form at pH 8.2 (E-Ca), which is not surprising given that these would be considered, respectively, to be the least active and most active forms of PhK evaluated in this study. The most active E-Ca conformer has a set of properties that clearly distinguish it from the other three conformers: intrinsic fluorescence of Trp and Tyr (Fig. 2), CD spectrum (Fig. 3B) with the greatest $\theta_{222}/\theta_{208}$ ratio (Fig. 4), and the least negative zeta potential (Fig. 5).

In a previous study on PhK conformations probed by a photoreactive cross-linker, it was noted that the binding of Ca^{2+} to deprotonated enzyme (E-Ca) had an opposite effect on α - γ dimer formation than observed with other activators of PhK (Nadeau et al. 1999). From this it was concluded that, even though "the activated conformation induced by Ca^{2+} shares structural features in common with conformations of PhK induced by other activators, it also has features that are clearly distinct" (Nadeau et al.

Figure 6. Effects of pH and Ca^{2+} on the structure of PhK. (A) Schematic depicting the various forms of PhK at pH 6.8 (H-E and H-E-Ca) and 8.2 (E and E-Ca) vertically arranged according to the values of their zeta potential, with the Ca^{2+} -free form at pH 6.8 (H-E) having the most negative value. The wavy line intersecting conformational transitions 3 and 4 represents the occurrence of the increased $\theta_{222}/\theta_{208}$ ratios. (B) The $\theta_{222}/\theta_{208}$ ratios and zeta potential values as a function of pH. The $\theta_{222}/\theta_{208}$ ratios are plotted on the lower abscissa: Ca^{2+} -free PhK ($\circ$) and Ca^{2+} -bound PhK ($\bullet$). The zeta potential values are plotted on the upper abscissa with the pH values reversed: Ca^{2+} -free PhK (Δ) and Ca^{2+} -bound PhK ($\blacktriangle$). This replot of Figures 4 and 5 uses their exact scaling, i.e., no normalization of data was performed for its construction.

Table 2. Evidence for pH- and Ca^{2+} -dependent conformational transitions in PhK

Transition 1: H-E $\rightarrow$ H-E-Ca	
Transmission electron microscopy with 3D image reconstruction	Nadeau et al. (2002)
Small-angle X-ray scattering	Priddy et al. (2005)
Less negative zeta potential	Priddy et al. (2007); This study
Increased affinity for glycogen phosphorylase	Xu et al. (1996)
Decreased proteolysis of β subunit by trypsin	Trempe and Carlson (1987)
Increased proteolysis of α subunit by chymotrypsin	Rice et al. (2002)
Increased formation of α - γ dimer by formaldehyde	Rice et al. (2002)
Decreased formation of γ - δ dimer by zero-length cross-linker EEDQ	Jeyasingham et al. (2008)
Increased reductive methylation and carboxymethylation of γ	Nadeau et al. (1997)
Transition 2: H-E $\rightarrow$ E	
Less negative zeta potential	This study
Blueshift for Tyr residues	This study
Increased interaction with anti- β and anti- γ monoclonal antibodies	Wilkinson et al. (1997)
Increased formation of α - γ and β - δ dimers by photoreactive cross-linker ^a	Nadeau et al. (1999)
Transition 3: E $\rightarrow$ E-Ca	
Increase in $\theta_{222}/\theta_{208}$ (CD) ratio	This study
Decreased formation of α - γ and β - γ conjugates by photoreactive cross-linker	Nadeau et al. (1999)
Increased formation of α - γ dimer by phenyltriazolinedione	Ayers et al. (1998)
Increased formation of α - γ dimer by phenylenedimaleimide	Nadeau et al. (1997)
Increased reductive methylation and carboxymethylation of γ	Nadeau et al. (1997)
Transition 4: H-E-Ca $\rightarrow$ E-Ca ^b	
Increase in $\theta_{222}/\theta_{208}$ (CD) ratio	This study

^aCross-linking carried out so as to be relatively independent of pH.

^bDiscounting activity measurements, studies comparing Ca^{2+} -bound PhK at pH 6.8 and 8.2 have not been performed.

1997, 1999). That conclusion is consistent with the results observed using other chemical cross-linkers as conformational probes (Nadeau et al. 1997; Ayers et al. 1998), with the results herein, and with the fact that, no matter how it is otherwise activated, PhK still requires Ca^{2+} for catalytic activity. The deprotonation event(s) leading to a less negative zeta potential (Fig. 5) and to the ability of Ca^{2+} to increase the $\theta_{222}/\theta_{208}$ ratio (Fig. 4), the hallmarks of the most active E-Ca conformation, may well be triggered by deprotonation of the same residue(s), because plotting those two figures on the same plot shows a remarkable pH-dependent overlap and the same sharp inflection point at pH 7.6 (Fig. 6B). We consider it noteworthy that Ca^{2+} increases the $\theta_{222}/\theta_{208}$ ratio of only deprotonated PhK, which already exhibits a relatively high zeta potential. Thus, our working hypothesis is that full activation of inactive protonated PhK (H-E) first requires a conformational change that results in a less negative surface charge before the obligatory Ca^{2+} is able to induce an increase in the $\theta_{222}/\theta_{208}$ ratio, which is a manifestation of the most active conformation, i.e., that subject to the least quaternary constraint by the inhibitory α and β subunits. Considering activation by $\uparrow$ pH within the framework of this working hypothesis, we suggest that key residue(s) of the regulatory α and/or β subunits become deprotonated, causing a conformational change resulting in a less negative surface charge on the PhK complex, and that the binding of Ca^{2+} to the δ subunits of this deprotonated conformer indirectly decreases interactions between α -

helices and β -sheets within PhK (i.e., increased $\theta_{222}/\theta_{208}$ ratio) as part of the process of forming a more active conformer (Levitt and Chothia 1976; Chothia et al. 1977; Arnold et al. 1992; Park et al. 1993). We assume that the decreased interactions of sheets and helices largely involve the α and β subunits. Of course, the H-E conformer of PhK can be activated by other mechanisms than deprotonation, such as phosphorylation or partial proteolysis of the α and β subunits, or by the binding of ADP (Cheng et al. 1985), most likely to the β subunit (King and Carlson 1982; King et al. 1982). It will be of great interest to determine whether activation through these other mechanisms causes the zeta potential of the H-E form at pH 6.8 to also become less negative, and whether Ca^{2+} can then induce an increase in the $\theta_{222}/\theta_{208}$ ratio of this protonated conformer, such as observed herein for the highly active deprotonated conformer at pH 8.2.

Materials and Methods

PhK preparation

Nonactivated PhK was purified from skeletal muscle of female New Zealand white rabbits as previously described (King and Carlson 1981) and stored at -80°C in buffer containing 50 mM HEPES, 0.2 mM EDTA, and 10% (w/v) sucrose (pH 6.8). Frozen samples of PhK were thawed and immediately centrifuged for 30 sec at 10,000g to remove insoluble precipitates. Soluble aggregates were removed by size exclusion-high performance liquid chromatography (SE-HPLC) (Traxler et al.

2001), and the fractions containing only hexadecamers were retained for spectroscopic analyses after elution from a BioSep SEC-S4000 column (Phenomenex, Inc.) developed with a mobile phase containing 6 mM HEPES, 200 mM NaCl, 0.2 mM EGTA (pH 6.8) at a flow rate of 0.4 mL/min. The enzyme solution was subsequently dialyzed overnight against 6 mM HEPES, 0.2 mM EGTA at various pH values between 6.8 and 8.2. PhK concentration was determined for each sample by UV₂₈₀ using an absorption coefficient of 12.4 for a 1% protein solution (Cohen 1973). For analyses of PhK at different pH values, dialyzed samples were diluted with their corresponding dialysis buffers to 100 µg/mL; to achieve the same protein concentrations for Ca²⁺-activated PhK, enzyme solutions were diluted in the same buffers containing CaCl₂ to achieve a final Ca²⁺ ion concentration of 0.5 mM.

Second derivative UV absorption spectroscopy

High-resolution UV absorption spectra were collected on an Agilent 8453 diode-array spectrophotometer (Agilent technologies). At each pH, spectra were analyzed between 240 and 310 nm with 1.0-nm increments. Studies were conducted in 1-cm quartz cuvettes and a 5-min temperature equilibration time was incorporated before collection of each spectrum. Second derivatives of individual spectra were calculated applying a nine-point filter and fifth-degree Savitsky–Golay polynomial fitted to a cubic function with a 99-point interpolation per raw data point using the UV-Visible Chemstation software suite (Agilent technologies). Second derivative peak minima were assigned by peak picking within the OriginPro software program.

Intrinsic fluorescence spectroscopy

Fluorescence spectroscopy of PhK was carried out at ambient temperature with a PTI 814 fluorescence spectrometer (PTI Photon Technology International) interfaced to a microcomputer. The synchronous fluorescence technique was used to distinguish the overlapped fluorescence peaks of Trp and Tyr residues (Lloyd 1971; Miller 1979). The value of the difference between excitation and emission wavelengths ($\Delta\lambda$) was set at 15 and 60 nm, characteristic of Tyr and Trp residues, respectively (Joaquim et al. 1998; Hou et al. 2007). For each measurement, the excitation and emission slits were set at 1.5 nm.

Far-UV circular dichroism spectroscopy

Far-UV CD spectra were collected under constant nitrogen purge in 0.1-cm quartz cuvettes from 260 to 190 nm at 1-nm intervals with a 20 nm/min scan rate using a Jasco J-810 spectropolarimeter (Jasco, Inc.). Reference spectra were subtracted from a minimum of three replicates of raw protein spectra, and analyzed using Origin Professional v.7.0 Scientific Graphing and Analysis Software (OriginLab). Secondary structure content was estimated using the Dichroweb software package (Lobley et al. 2002), which permits analyses of secondary structure by CONTIN, SELCON, and CDSSTR algorithms (Sreerama and Woody 2000).

Surface of shear electrostatic potential

The zeta potential at each pH value $\pm$ Ca²⁺ was collected at 25°C on a Brookhaven Instrument Corp ZetaPALS analyzer.

Light scattering at 15° to the incident 676-nm, 25-mW, solid-state laser beam was measured to calculate the Smoluchowski approximation, which is appropriate for the typical colloid and salt concentrations that were used for these experiments.

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