Activators of Phosphorylase Kinase Alter the Cross-Linking of Its Catalytic Subunit to the C-Terminal One-Sixth of Its Regulatory α Subunit†

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ABSTRACT: Phosphorylase kinase, a regulatory enzyme of glycogenolysis in skeletal muscle, is a hexadecameric oligomer consisting of four copies each of a catalytic subunit (γ) and three regulatory subunits (α, β, and δ, the last being endogenous calmodulin). The enzyme is activated by a variety of effectors acting through its regulatory subunits. To probe the quaternary structure of nonactivated and activated forms of the kinase, we used the heterobifunctional, photoreactive cross-linker *N*-5-azido-2-nitrobenzoyloxysuccinimide. Mono-derivatization of the holoenzyme with the succinimidyl group, followed by photoactivation of the covalently attached azido group, resulted in intramolecular cross-linking to form two distinct heterodimers: a major ($\alpha\gamma$) and a minor ($\beta\delta$) conjugate. Formation of both conjugates was significantly altered in activated conformations of the enzyme induced by phosphorylation, alkaline pH, and several allosteric activators (ADP, exogenous calmodulin/Ca^{2+}, and Ca^{2+} alone). Of these activating mechanisms, all increased formation of $\alpha\gamma$, except Ca^{2+} alone, which inhibited its formation. When cross-linking was carried out at alkaline pH or in the presence of ADP or exogenous calmodulin/Ca^{2+}, the cross-linked enzyme remained activated following removal of the activators; however, cross-linking in the presence of Ca^{2+} resulted in sustained inhibition. The results indicate that perturbations in the subunit cross-linking forming the $\alpha\gamma$ dimer reflect the subsequent extent of sustained activation of the holoenzyme that is measured. The region cross-linked to the catalytic γ subunit was confined to the C-terminal $^1/_6$th of the α subunit, which contains known regulatory regions. These results suggest that activators of the phosphorylase kinase holoenzyme perturb interactions between the C-terminal region of the inhibitory α subunit and the catalytic γ subunit, ultimately leading to activation of the latter.

Phosphorylase kinase (PhK)[1] modulates glycogenolytic flux in skeletal muscle by integrating neural (Ca^{2+}), hormonal (cAMP and Ca^{2+}), and metabolic (ADP) signals via allosteric and covalent modification sites on its three regulatory subunits (reviewed in *1*, *2*). The three regulatory subunits α [138.4 kDa (*3*)], β [125.2 kDa (*4*)], and δ [16.7 kDa (*5*)], the last being an intrinsic molecule of tightly bound calmodulin (CaM) (*6*), act in concert to control the activity of the catalytic γ subunit [44.7 kDa (*7*)]. In addition to the stimulation by Ca^{2+} that occurs through the CaM subunit (δ), PhK is further activated by the Ca^{2+}-dependent binding of exogenous CaM, which is termed δ' (*8*, *9*). The PhK holoenzyme contains four copies each of its intrinsic subunits, $(\alpha\beta\gamma\delta)_4$ (*6*), for a total mass of 1.3×10^6 Da. When δ'/Ca^{2+} binds, it is in the stoichiometry of one per $\alpha\beta\gamma\delta$ protomer (*10*, *11*).

Numerous activators of the PhK holoenzyme cause common conformational changes in the β subunit (*12*−*14*), and phosphorylation (*15*) or selective proteolysis (*13*, *16*) of the α subunit causes increased activity of the catalytic γ subunit. Until recently, however, little was known about the changes in quaternary interactions within the $(\alpha\beta\gamma\delta)_4$ hexadecamer that occur upon activation. Recent studies using mAbs against epitopes on the β and γ subunits reported simultaneous increases in accessibility of the two epitopes during activation (*17*). Also, use of phenylenedimaleimide cross-linkers suggested changes in the interactions between these two subunits in response to several allosteric activators (*18*). Additionally, cross-linkers having distinct chemistries and cross-linking spans, including 4-phenyl-1,2,4-triazoline-3,5-dione (*19*) and the three geometric isomers of phenylenedimaleimide (*18*, *20*), have provided evidence suggesting that there are also changes in α−γ interactions that are associated with activation through several mechanisms. In these last studies (*18*−*20*), all activators of the holoenzyme promoted increased cross-linking of the α and γ subunits; however, the limited extent of formation of the conjugates, either $\alpha\gamma$ or $\alpha\gamma\gamma$, precluded analysis of the regions cross-linked in either subunit.

In our search for cross-linkers that produce sufficient quantities of $\alpha\gamma$ conjugates that are also markers of activated forms of the holoenzyme, we have identified a new photoactivatable, heterobifunctional cross-linker of PhK, *N*-5-

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[1] Abbreviations: PhK, phosphorylase kinase; CaM, calmodulin; δ'/Ca^{2+}, exogenous calmodulin; ANB·NOS, *N*-5-azido-2-nitrobenzoyloxysuccinimide; mAbs, monoclonal antibodies; BtCaM, biotinylated calmodulin.

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azido-2-nitrobenzoyloxysuccinimide (ANB·NOS), that forms sufficient quantities of an $\alpha\gamma$ dimer in activated forms of the kinase to permit localization of the region of the α subunit that is cross-linked. The results herein demonstrate the cross-linking of the γ subunit to the C-terminal $^1/_6$th of the α subunit, which contains known regulatory sites (*2*, *21*). Formation of this $\alpha\gamma$ complex by ANB·NOS is altered by autophosphorylation and the allosteric activators ADP, Ca^{2+}, and δ'/Ca^{2+}. Because of the nature of the cross-linker, we were able to prepare a mono-derivatized intermediate of PhK, which was subsequently photo-cross-linked at pH 6.8 and pH 8.2, pH values at which the native PhK holoenzyme behaves as nonactivated and activated, respectively. The cross-linking results suggest that slightly alkaline pH induces a conformation of PhK that is structurally similar to those caused by other activation mechanisms.

EXPERIMENTAL PROCEDURES

Enzymes and Proteins. PhK was isolated from fast-twitch skeletal muscle of New Zealand White rabbits (*22*), dialyzed against Hepes buffer (50 mM, pH 6.8)/sucrose (10%)/EDTA (0.2 mM) and used immediately, or stored frozen at −80 °C. All experiments described in this study were repeated a minimum of 3 times using three different PhK preparations. When autophosphorylated PhK was used for cross-linking, the phosphorylation was carried out at pH 8.2 in Hepes buffer for 1 min using the methodology of King et al. (*23*). To determine the ^{32}P content of the subunits, each band was excised, solubilized, and decolorized by heating in 250 μL of 30% H_2O_2 for 2 h at 80 °C. The samples and blanks, which contained equivalent amounts of polyacrylamide and H_2O_2, were diluted with 7 mL of Ecoscint scintillation cocktail (ICN), and their ^{32}P content was measured. The extent of phosphate incorporation with different kinase preparations ranged from 0.8 to 1.1 mol of phosphate/mol of β subunit and 1.5−2.4 mol/mol of α subunit. Prior to cross-linking, the autophosphorylated enzyme was purified by gel filtration over a Sepharose 6B column (1.5 × 112 cm) developed with Hepes buffer (50 mM, pH 6.8)/EDTA (0.2 mM)/sucrose (10%). Fractions that eluted at the position of native holoenzyme were collected; buffer was exchanged and the enzyme concentrated to 4.5 mg/mL by ultrafiltration at 3000*g*. Phosphorylase *b* was isolated from rabbit skeletal muscle (*24*), and residual AMP was adsorbed with activated charcoal (Sigma, C-4386). The concentrations of PhK and phosphorylase *b* were determined spectrophotometrically using their respective absorbance indices (*25*, *26*).

Monoclonal antibodies (mAbs) against the α, β, and γ subunits of PhK were those previously generated in mice against the holoenzyme as antigen (*17*, *27*). The anti-CaM mAb was purchased from Signal Transduction Laboratories. Detection conjugates for immunoblots were from Southern Biotechnology.

Chymotrypsin was from Worthington, and bovine serum albumin (A-9647) was from Sigma. Bovine brain CaM was isolated as previously described (*28*). Biotinylated CaM (BtCaM, biotinylated at Lys 94) was prepared by biotinylation of CaM at pH 6.0 with *N*-hydroxysuccinimidylbiotin and purified over DEAE-spherogel by the procedure of Mann and Vanaman (*29*). Concentrations of bovine brain CaM and BtCaM were determined as previously described (*29*).

Chemical Cross-Linking. For standard cross-linking, PhK was initially incubated with ANB·NOS for 30 min in the dark at 30 °C; cross-linking was initiated by irradiating with UV light (360 nm) for 30 s at 4 °C. Final concentrations in the reactions were PhK (0.43 μM), Hepes (50 mM, pH 8.2), EDTA (1.0 mM), and ANB·NOS (17.2 μM; 10 mol of ANB·NOS/mol of $\alpha\beta\gamma\delta$ protomer). The effects of Ca^{2+} (1.25 mM $CaCl_2$), δ'/Ca^{2+} (1.72 μM/1.25 mM), BtCaM/Ca^{2+} (1.72 μM/1.25 mM), ADP (400 μM), and autophosphorylation on subunit cross-linking were also tested under the conditions described above. Cross-linking was quenched by dilution of an aliquot of the assay mixture into an equivalent volume of SDS buffer (0.125 M Tris, pH 6.8/20% glycerol/5% β-mercaptoethanol/4% SDS) followed by brief mixing. After heating at 80 °C for 10 min, 1.5 or 11.2 μg protein samples were run on SDS−polyacrylamide gradient gels (4−20%) for electroblotting onto nitrocellulose or staining with Coomassie blue, respectively (*20*, *30*). All gels were destained in 40% methanol/10% acetic acid (2 h) and 7% acetic acid/4% methanol (15 h). The integral optical density of the protein bands was determined on a Bio Image whole band analyzer.

When the initial chemical mono-derivatization and subsequent photo-cross-linking were performed in a two-step process, such as when examining the effects of pH, the kinase was first modified with ANB·NOS at pH 8.2 by the methods above and purified by gel filtration HPLC as previously described (*31*), using a BioSep-SEC-S4000 column (Phenomenex Inc., 600 × 7.8 mm, 2 × 10^6 Da exclusion limit) developed with Hepes buffer (50 mM, pH 6.8)/200 mM NaCl/0.2 mM EGTA. This treatment desalted the protein while separating the hexadecameric PhK holoenzyme from aggregated forms. Aliquots of the purified, arylazido-labeled kinase (400 μL, 61.9 nM) were then cross-linked at either pH 6.8 or pH 8.2 after adding equal volumes of 50 mM Hepes (pH 6.8) or 0.5 M sodium borate (pH 9.3), respectively, prior to photoactivation (see above). Equal amounts of the cross-linked kinase from each reaction (pH 6.8 and 8.2) were then exchanged into 50 mM Hepes (pH 6.8) by ultrafiltration (4500*g*) using a Centricon 30 cell (Amicon) and analyzed by SDS−PAGE and densitometry as described above.

Cross-linking by ANB·NOS of PhK and its complexes with activators was intramolecular, as judged by comigration of cross-linked and native forms of the holoenzyme over gel filtration HPLC, as described above.

Identification of Cross-Linked Species. Apparent masses of cross-linked complexes were determined from comparison with the migration on 4−20% linear gradient PAGE (20) of protein standards: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) (all from Sigma). No attempt was made to estimate the mass of oligomers having apparent masses greater than 205 kDa. The extent of cross-linking for individual subunits was determined by optical densitometry.

The subunit composition of cross-linked complexes was analyzed by Western blotting as previously described using subunit-specific mAbs and BtCaM (*20*) and by N-terminal sequencing. With the latter approach, samples were electrophoresed, electrophoretically transferred to PVDF membranes, and stained with amido black, and the bands of

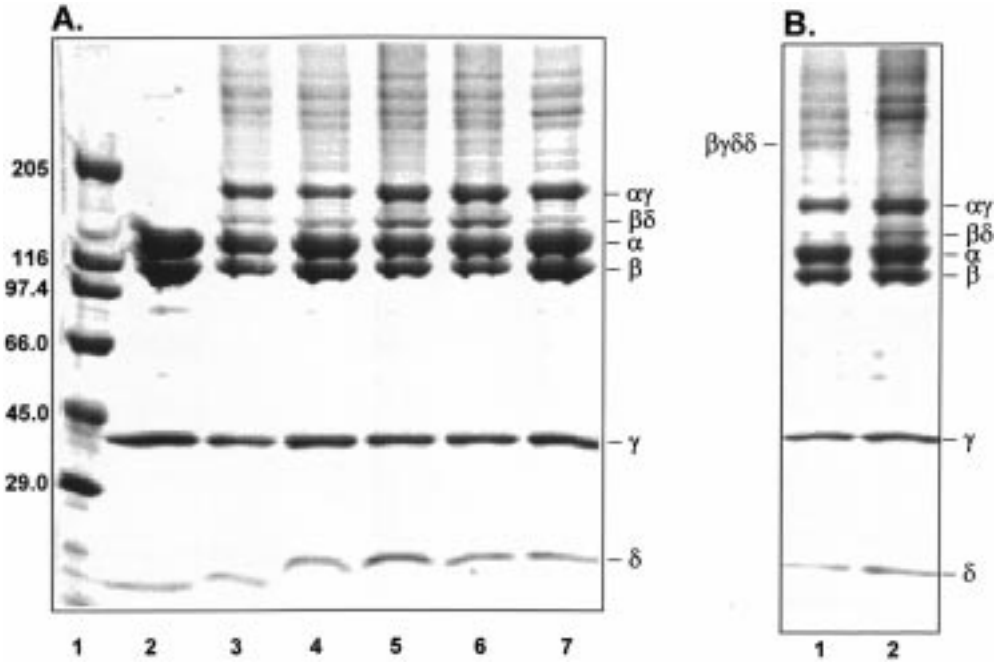

FIGURE 1: Cross-linking of native and activated forms of PhK by ANB·NOS. (A) Lane 1, mass standards (5 μg of each) listed from top to bottom: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa); lane 2 is non-cross-linked PhK. Native PhK was cross-linked with ANB·NOS alone (lane 3), or in the presence of Ca^{2+} (lane 4), δ′/Ca^{2+} (lane 5), or ADP (lane 6) as described under Experimental Procedures. Lane 7 is autophosphorylated PhK (2.3 and 1.1 mol of P incorporated/mol of α and β, respectively) purified as described under Experimental Procedures and cross-linked as above. (B) PhK was first chemically modified with ANB·NOS, purified by gel filtration, and then cross-linked either at pH 6.8 (lane 1) or at pH 8.2 (lane 2) as described under Experimental Procedures.

interest were excised. Amino acid analysis and N-terminal sequencing from the PVDF blots were carried out by the Harvard Microchemistry Facility. For the detection of BtCaM, streptavidin−alkaline phosphatase (Southern Biotechnology) was exposed to blots and assayed with an alkaline phosphatase kit from Bio-Rad, following the supplier's suggested protocol.

Partial Proteolysis of Native and Cross-Linked PhK. To increase the yield of α−γ cross-linking, PhK was cross-linked with ANB·NOS (25 mol of ANB·NOS/mol of αβγδ) under the conditions described above. Native and cross-linked PhK (562 μg/mL) were digested with chymotrypsin (0.5 μg/mL) for 10 min at 30 °C. Proteolysis was quenched by adding equal volumes of SDS buffer, followed by brief mixing and heating at 80 °C for 10 min.

Activity Assays. PhK, with or without effectors, was cross-linked with ANB·NOS as previously described. The cross-linked protein was then purified by gel filtration HPLC, as described above, to remove effectors and assayed for catalytic activity. The activity of native and cross-linked PhK was determined by following the incorporation of ^{32}P from [γ-^{32}P]-ATP into phosphorylase *b* at 30 °C using phosphocellulose strips (32). Final concentrations in the assay mixture were as follows: PhK [0.7 μg/mL (pH 6.8) or 0.07 μg/mL (pH 8.2)], buffer (50 mM Tris/50 mM β-glycerophosphate, pH 6.8 or 8.2), phosphorylase *b* (6.0 mg/mL), EGTA (0.1 mM), $CaCl_2$ (0.2 mM), β-mercaptoethanol (13 mM), [γ-^{32}P]ATP (NEN DuPont) (1.5 mM, 0.17 Ci/mol), $Mg(CH_3CO_2)_2$ (10 mM), and sucrose (2−3%).

RESULTS

Effector-Induced Perturbations of ANB·NOS Cross-Linking of PhK. Optimal cross-linking of the PhK holoenzyme with the heterobifunctional cross-linker ANB·NOS occurred after chemical incorporation of the cross-linker into the kinase at pH 8.2, followed by photoactivation of the incorporated aryl azide. This cross-linking resulted in the formation of two major complexes of relatively low mass corresponding to dimers of αγ ($mass_{theor}$ = 183 kDa; 2.0% error) and βδ ($mass_{theor}$ = 142 kDa; 3.0% error) by apparent mass (Figure 1) and cross-reactivity against anti-α, -β, and -γ mAbs (Figure 2). Additionally, cross-linked complexes present in minor or trace quantities with apparent masses of greater than 205 kDa were also observed, but were not further characterized due to their probable complexity and limited extent of formation. Activators of PhK markedly perturbed the extent of formation of both the αγ and βδ dimers (Figures 1 and 3). With the exception of Ca^{2+}, which decreased formation of αγ by 12%, the other activators of PhK that were examined, namely, δ′/Ca^{2+}, ADP, and autophosphorylation, increased formation of αγ by 56, 62, and 68%, respectively (Figure 3). Significant increases in the formation of βδ were also observed with cross-linking in the presence of δ′/Ca^{2+} (72%) and ADP (147%); however, both Ca^{2+} and autophosphorylation decreased formation of βδ, by 15 and 10%, respectively. Cross-linking of the native enzyme in the absence of effectors resulted in a 67% combined loss in density of the α, β, and γ subunits, which was similar to their diminution when PhK was cross-linked in the presence of δ′/Ca^{2+} (58%) or ADP (61%). On the other hand, both Ca^{2+}, with a 35% overall loss, and autophosphorylation, with only a 17% overall loss, protected the α, β, and γ subunits against cross-linking. In summary, each effector tested altered the formation of αγ and βδ within the holoenzyme, and by different extents, suggesting that ANB·NOS is a sensitive probe of effector-induced conformational changes in PhK.

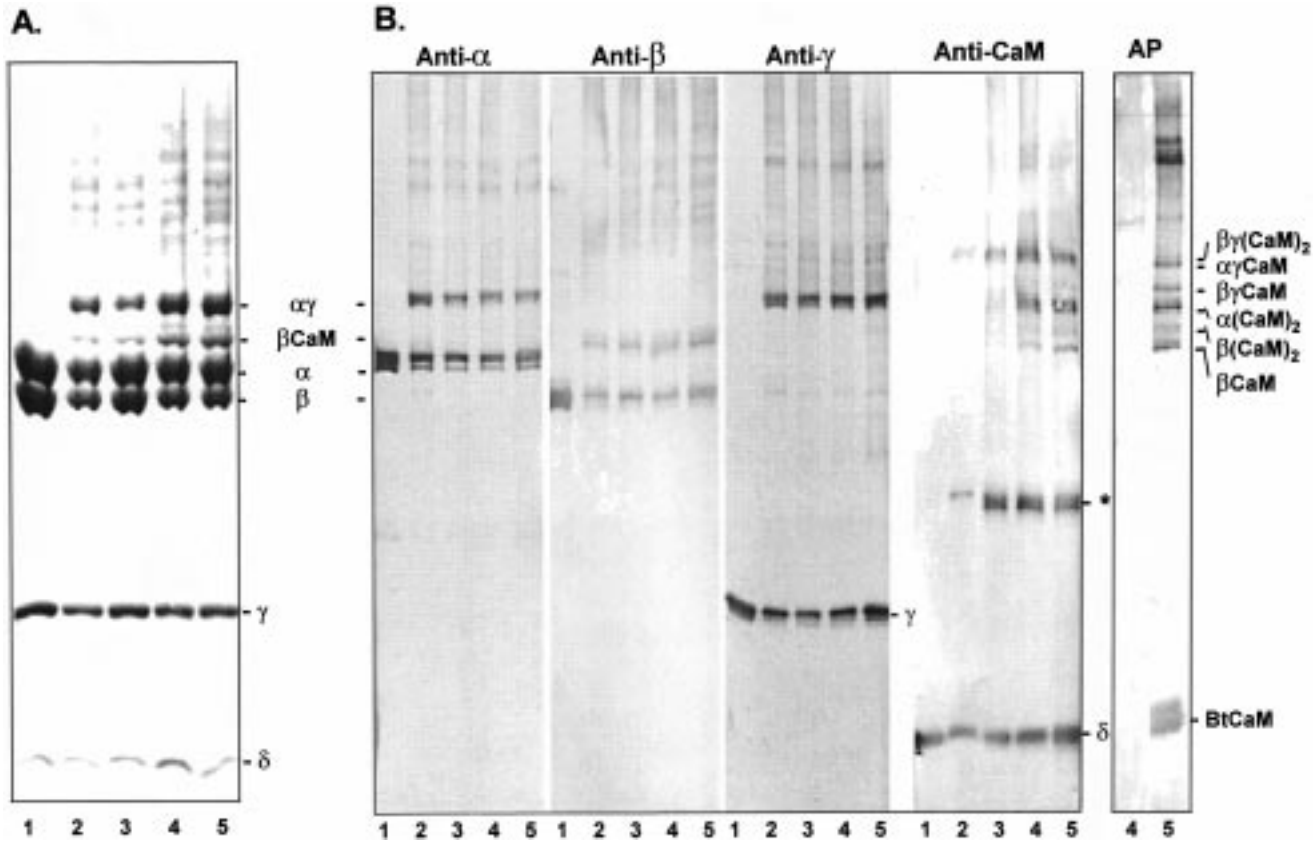

FIGURE 2: Identification of cross-linked species by Western blots. (A) Nonactivated PhK (control, lane 1) was cross-linked by ANB·NOS in the absence of effectors (lane 2), or in the presence of Ca^{2+} (lane 3), CaM(δ')/Ca^{2+} (lane 4), or BtCaM/Ca^{2+} (lane 5). Aliquots were then subjected to SDS−PAGE (4−15% acrylamide) and stained for protein to determine the extent of cross-linking, or (B) electroblotted onto nitrocellulose and probed with anti-α, anti-β, anti-γ and anti-CaM mAbs as described under Experimental Procedures. The asterisk in the anti-CaM blot denotes a degradation product of the α subunit that commonly occurs in small amounts in purified preparations of PhK and that has previously been shown to nonspecifically cross-react with this particular anti-CaM mAb (20). The panel labeled AP shows cross-reactivity against the streptavidin−alkaline phosphatase conjugate used to detect BtCaM.

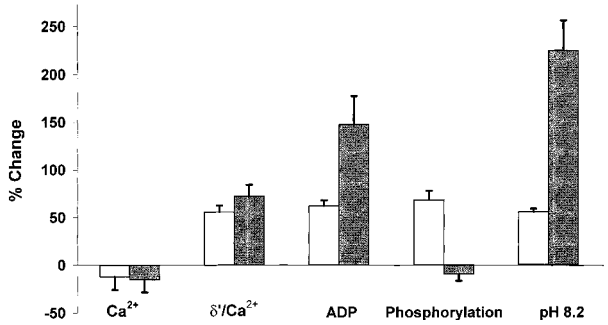

FIGURE 3: Effector-induced changes in the cross-linking of PhK by ANB·NOS. PhK was cross-linked in the presence of the indicated activators as described under Figure 1. Following their separation by SDS−PAGE, integrated optical densities were determined for each cross-linked complex using a Bio Image whole band analyzer. Bars represent the percent change in the formation of $\alpha\gamma$ (open) and $\beta\delta$ (filled) dimers caused by the indicated activator, as compared to cross-linking in its absence. For the bars labeled pH 8.2, the control for the percent change caused by cross-linking at that pH was cross-linking carried out at pH 6.8 after the initial chemical modification at pH 8.2. For all other conditions, both steps (modification and cross-linking) were performed at pH 8.2. Error bars represent the SEM of three independent experiments with different preparations of PhK.

Of the activators tested, Ca^{2+} had the most distinctive effects on cross-linking, suggesting that it may induce a conformation having different structural features than those induced by the remaining activators.

There are qualitative differences in the catalytic activity of PhK at pH 6.8 versus pH 8.2. The time course of product formation is characterized by a pronounced lag at pH 6.8, whereas it is linear at pH 8.2. The influence of activators is most pronounced at pH 6.8, where they shorten or abolish the lag, leading to the ratio of activity at pH 6.8 to pH 8.2 being taken as an index of the degree of activation of the enzyme (reviewed in *1*). Because of these differences in activity, we wished to determine whether cross-linking by ANB·NOS could provide evidence for conformational differences in PhK at these two pH values. To accomplish this, chemical modification of PhK by the succinimidyl functional group of the heterobifunctional ANB·NOS was carried out at pH 8.2 in the dark to avoid photolysis of the azido functionality and subsequent cross-linking. The modified enzyme was then purified by gel filtration at pH 6.8 to obtain homogeneous preparations of mono-derivatized, non-cross-linked enzyme. Aliquots of this singly modified kinase were then photolyzed at either pH 6.8 or pH 8.2 (Figure 1B). Photo-cross-linking at either pH resulted in similar decreases in subunit density for the α, β, and γ subunits (42% at pH 6.8 and 35% at pH 8.2), suggesting that the efficiency of photo-cross-linking of the subunits by ANB·NOS does not change significantly from one pH value to the other. The subunit cross-linking pattern was also similar at the two pH values; however, there were significant differences in the extent of formation of several complexes. A minor conjugate

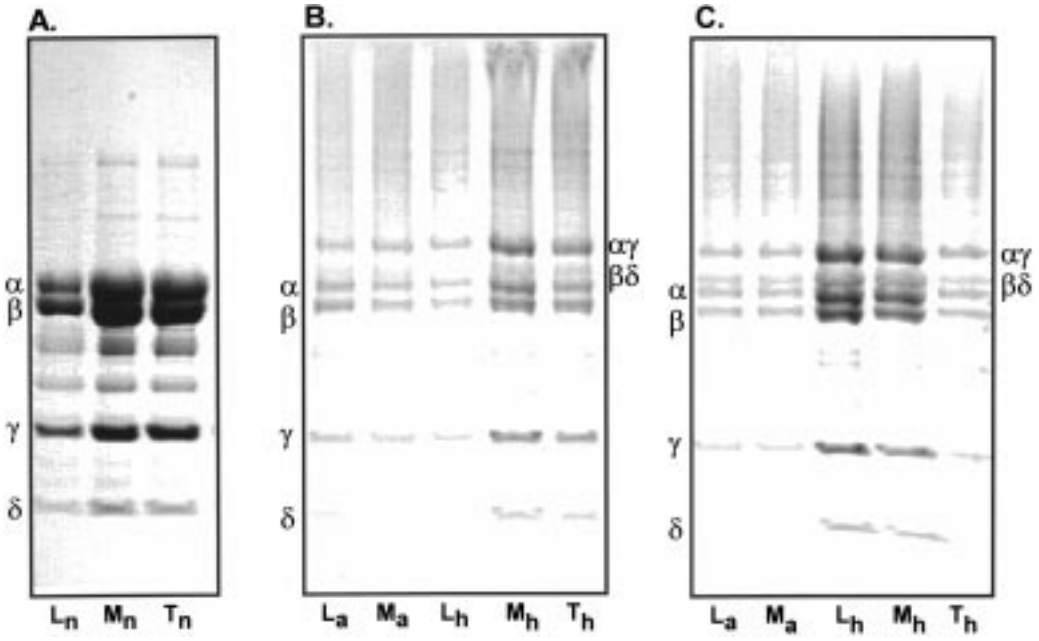

FIGURE 4: SDS−PAGE of fractions from gel filtration HPLC of native and cross-linked PhK. PhK, before and after cross-linking, was subjected to nondenaturing gel filtration HPLC developed with 50 mM Hepes buffer (pH 6.8)/200 mM NaCl/0.2 mM EGTA, as described under Experimental Procedures. Fractions were collected, dialyzed against 10 mM Hepes (pH 6.8), and lyophilized, before being dissolved in SDS buffer, heat-treated, and electrophoresed on 4−20% polyacrylamide gels. Coomassie-stained gels of fractions from the leading edge (L), middle edge (M), and trailing edge (T) of the major protein peaks are shown. (A) Non-cross-linked native hexadecameric holoenzyme (subscript n). (B) Representative fractions of cross-linked PhK from the early eluting aggregate peak (subscript a) that is typically observed in preparations of the purified native enzyme, independent of cross-linking (25, 35−37), and from the major peak, which comigrates with native hexadecameric holoenzyme (subscript h). (C) Representative fractions of PhK cross-linked in the presence of ADP and taken from the aggregate peak (subscript a) and from the major peak comigrating with native hexadecameric enzyme (subscript h).

corresponding to $\beta\gamma\delta\delta$ was preferentially formed at pH 6.8 (Figure 1), whereas photo-cross-linking at pH 8.2 increased the extent of formation of $\alpha\gamma$ and $\beta\delta$ by 55 and 224%, respectively, over the amounts formed at pH 6.8 (Figure 3). Therefore, as observed for several of the activators tested above, alkaline pH increased the formation of $\alpha\gamma$ and $\beta\delta$, suggesting that this activating pH may promote a conformational change in the holoenzyme similar to that induced by certain allosteric activators. Although it is also possible that differences in cross-linking at either pH simply reflect different extents of protonation of nucleophilic side chains, which are known to react with highly electrophilic dehydroazepine photolysis intermediates of aryl azides over a broad pH range (*33*), the increased formation of $\beta\gamma\delta\delta$ at pH 6.8 and the similar total extent of cross-linking observed at either pH would appear to be more consistent with conformation-dependent cross-linking.

Characterization of βδ Cross-Linking by ANB·NOS. Cross-linking of the native holoenzyme in the absence of effectors resulted in the formation of an anti-β cross-reactive conjugate with a mass corresponding only to a $\beta\delta$ dimer; however, cross-reactivity against the anti-CaM mAb was not observed for this conjugate (Figure 2B, lane 2), although it did cross-react with the anti-β mAb. To gain further information about its subunit composition, this putative $\beta\delta$ conjugate was electroblotted onto PVDF and subjected to N-terminal sequencing; however, no sequence was observed, despite the presence of picomole quantities of protein in the blot, as measured by amino acid analysis. Because both the β and δ subunits are N-terminally blocked (*5*, *34*), we expected to detect sequences from the conjugate only if the unblocked α or γ subunits (*7*, *34*) were present. Thus, as judged by apparent mass, cross-reactivity against the anti-β mAb, and the probable absence of α or γ, the observed complex is almost certainly a $\beta\delta$ conjugate in which the epitope for the anti-CaM mAb on the C-terminus of δ is masked by cross-linking. When the enzyme was cross-linked in the presence of Ca^{2+}, however, weak anti-CaM cross-reactivity was observed for a complex migrating at the position of $\beta\delta$, suggesting that binding of Ca^{2+} by the δ subunit promotes a conformational change that alters its cross-linking with β; i.e., the epitope on δ for the anti-CaM mAb is less masked in this complex.

Cross-linking the holoenzyme in the presence of δ'/Ca^{2+} further increased the extent of formation and anti-CaM cross-reactivity of a β−CaM complex. To determine whether this β−CaM complex represented $\beta\delta$ or $\beta\delta'$, we employed as δ' a mono-biotinylated form of CaM (BtCaM), which mimics the actions of wild-type CaM on the PhK holoenzyme and can be distinguished from native CaM through use of streptavidin−alkaline phosphatase (*20*). The cross-linking of CaM(δ')/Ca^{2+} and BtCaM/Ca^{2+} complexes of PhK by ANB·NOS exhibited the same cross-linking patterns and cross-reactivities for all conjugates against the anti-subunit mAbs (Figure 2, lanes 4 and 5). The major β-containing complex formed in the presence of BtCaM/Ca^{2+} was the dimeric βCaM, which contained BtCaM; however, we do not know what fraction of the CaM in this dimer is actually δ' and what fraction is δ. Several larger conjugates containing BtCaM and combinations of the α, β, and γ subunits were also formed in minor amounts (Figure 2B). In contrast to the binding of Ca^{2+} to the δ subunit of PhK, the binding of

δ'/Ca^{2+} to PhK increases the extent of formation of $\alpha\gamma$ (Figure 2B), providing further evidence that Ca^{2+} and δ'/Ca^{2+} induce distinct conformations in the holoenzyme (*20*).

Molecularity of Subunit Cross-Linking by ANB·NOS. To determine whether cross-linking of PhK and its complexes with effectors was intramolecular or intermolecular, cross-linked enzyme formed under all conditions was purified by gel filtration HPLC, as described under Experimental Procedures, to determine if it comigrated with native, hexadecameric, holoenzyme (intramolecular cross-linking) or with heavier forms of the enzyme, which could possibly indicate intermolecular cross-linking. All cross-linked forms of the enzyme, shown, for example, by cross-linked native PhK and its complex with ADP (Figure 4), coeluted with non-cross-linked, native PhK, indicating intramolecular cross-linking. The perturbations in $\alpha\gamma$ and $\beta\delta$ cross-linking induced by each effector (Figures 1−3) were also observed to occur in the same manner in the major peak comigrating with native PhK. Purified preparations of PhK routinely contain small amounts of aggregated enzyme (*25*, *35*−*37*). Cross-linked subunits were also observed in the minor aggregate peak for all cross-linked forms of the enzyme; however, the pattern of cross-linking was the same as for the major hexadecameric peak, further supporting intramolecular cross-linking. The results indicate that the ability of activators to alter the intramolecular formation of $\alpha\gamma$ and $\beta\delta$ dimers by ANB·NOS results from perturbations in subunit interactions induced within the holoenzyme by the given effectors.

Effect of Cross-Linking Activated Conformers of PhK on Their Subsequent Activity. Two recent reports from our laboratory demonstrated that when PhK was cross-linked by phenylenedimaleimide in the presence of the activators Ca^{2+}, δ'/Ca^{2+}, or ADP, the enzyme remained partially activated after removal of the effectors (*18*, *20*), suggesting that certain structural elements of the activated conformations were stabilized by the cross-linking. Because ANB·NOS produces in PhK different subunit cross-linking patterns than phenylenedimaleimide in response to activation by the above indicated effectors, we wished to determine if sustained activation of the holoenzyme also occurred following its cross-linking by ANB·NOS in the presence of activators, which were removed by gel filtration HPLC before the activity assays. All comparisons for the activity, measured at pH 6.8, of each form of the kinase were made following their purification by gel filtration. The activity of the holoenzyme was not significantly affected either by mono-derivatization (chemical modification only by ANB·NOS at pH 8.2, denoted as 'Labeled') or by the subsequent photo-cross-linking of this intermediate at pH 6.8 (Figure 5). On the other hand, cross-linking at pH 8.2 resulted in a 69% enhancement of the activity measured at pH 6.8 (Figure 5), suggesting that cross-linking traps some structural elements of the activated conformation induced by the alkaline pH. Cross-linking in the presence of δ'/Ca^{2+} or ADP at pH 8.2 caused additional increases in the subsequent activity of the purified enzyme, by 97 and 118%, respectively (Figure 5), again suggesting that cross-linking by ANB·NOS traps some structural elements of the active conformations induced by these effectors in the holoenzyme. In direct contrast, the subsequent activity of enzyme cross-linked in the presence of Ca^{2+} was decreased by 26% in comparison to the control cross-linked at pH 8.2 in the absence of effectors. Because Ca^{2+} was the only activator to inhibit the formation of $\alpha\gamma$ with respect to cross-linked native enzyme (Figure 3), the activity results suggest that the amount of $\alpha\gamma$ dimer formed by ANB·NOS reflects the subsequent extent of sustained activation observed in cross-linked enzyme.

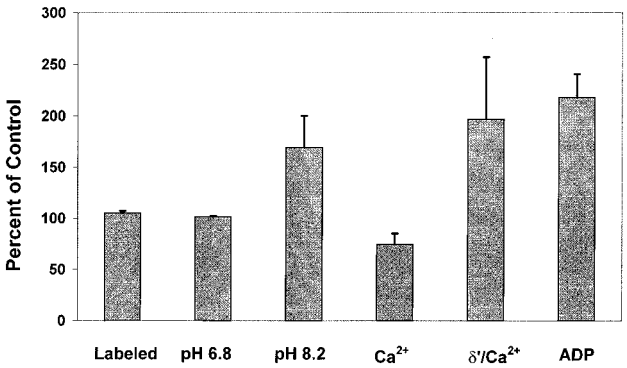

FIGURE 5: Influence of cross-linking different activated conformers of PhK on their subsequent catalytic activity. All forms of PhK were incubated for 30 min at 30 °C in the dark in the absence or presence of ANB·NOS ('Labeled') to modify nucleophilic side chains. Cross-linking was then carried out by photoactivation of the modified protein at pH 6.8 or at pH 8.2. When the effects of Ca^{2+}, δ'/Ca^{2+}, or ADP were evaluated, they were present throughout at a pH of 8.2. All forms of the enzyme (nonlabeled, non-cross-linked; labeled; and cross-linked) were purified by gel filtration HPLC before being measured for catalytic activity at pH 6.8 as described under Experimental Procedures. The ratio of activity at pH 6.8 versus pH 8.2 for three separate preparations of control PhK passed over the gel filtration column was ≤0.05, demonstrating that the purification process did not cause activation of the holoenzyme (1). The amount of product formed in 5 min in a 20 μL aliquot is plotted as the percent change with respect to the control for that sample. The control for labeled enzyme, as well as that cross-linked at pH 6.8 and 8.2 without effectors, was the PhK that was incubated in the absence of ANB·NOS before being subjected to gel filtration HPLC. The control for PhK cross-linked in the presence of Ca^{2+}, δ'/Ca^{2+}, or ADP was the enzyme cross-linked at pH 8.2 in the absence of effectors. Error bars represent the SEM of three independent experiments with different preparations of PhK.

Catalytic γ Subunit Is Cross-Linked to the C-Terminal Regulatory Region of the α Subunit. Because of the relationship between increased cross-linking of the $\alpha\gamma$ dimer and increased activity of the cross-linked enzyme, we wished to determine which region of the regulatory α subunit was cross-linked to γ. To narrow the region, we relied on the known ability of chymotrypsin to selectively cleave the α subunit within the PhK holoenzyme (*13*, *16*). Chymotryptic cleavage of α in native non-cross-linked PhK resulted in the formation of several major fragments, including two near its C-terminus that have been previously shown to cross-react with the anti-α mAb used in this study (*27*): α_{Cf1} (mass = 58 kDa) and α_{Cf2} ($mass_{theor}$ = 24.1 kDa; 1.5% error) (Figure 6, lane 2). The 24.1 kDa fragment, which contains within it the epitope for the anti-α mAb used in this study, has been shown to result from cleavage at Phe-1014 and to correspond to the C-terminal $^1/_6$th of the α subunit (*27*). This portion of α contains known regulatory regions (*2*, *21*). A comparison of the partial chymotryptic cleavage patterns of native and cross-linked PhK revealed the presence of two new fragments in the cleavage pattern of the latter (Figure 6, lane 4): a minor fragment termed $\gamma\alpha_{Cf1}$ (mass = 102 kDa) and a major fragment termed $\gamma\alpha_{Cf2}$ (mass = 68.7 kDa), whose masses correspond, respectively, to intact γ subunit

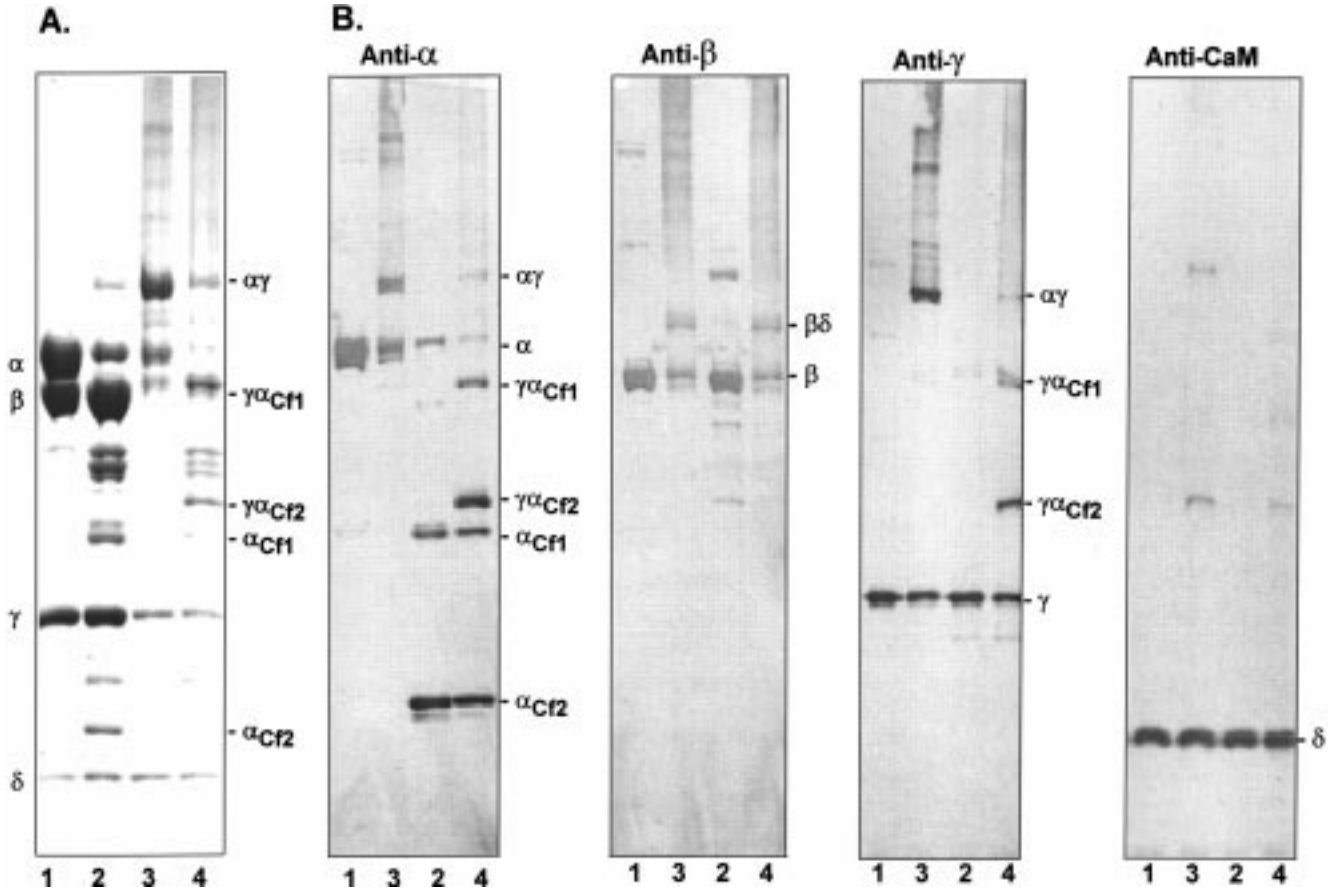

FIGURE 6: Partial proteolysis of native and cross-linked PhK as analyzed by SDS−PAGE and Western blotting. (A) Non-cross-linked PhK (lane 1) was partially digested with chymotrypsin (lane 2), or cross-linked with ANB·NOS (lane 3) and then partially digested with chymotrypsin (lane 4). Samples were subjected to SDS−PAGE and stained for protein. (B) Parallel samples were electroblotted onto nitrocellulose and probed with anti-α, anti-*β*, anti-*γ*, and anti-CaM mAbs as described under Experimental Procedures. The bands termed αC_{f1} (58 kDa) and αC_{f2} (24.1 kDa) are previously characterized chymotryptic fragments of α containing the known C-terminal eptitope for the anti-α mAb (27). The order of lanes 2 and 3 is reversed in the blots of panel B to more easily compare the anti-α and anti-*γ* cross-reactive species.

cross-linked to α_{Cf1} and α_{Cf2} ($mass_{theor}$ of $\gamma\alpha_{Cf2} = 68.8$ kDa). Correspondingly, two new bands with the masses of $\gamma\alpha_{Cf1}$ and $\gamma\alpha_{Cf2}$ were observed in blots of the partially digested cross-linked kinase as anti-α and anti-*γ* cross-reactive species (Figure 6B, lane 4). Based on the identity of the bands in the anti-*γ* blot of partially digested cross-linked PhK, the cross-linking of *γ* appears to be confined solely to the indicated region of α at its C-terminus. Partial chymotryptic digestion of PhK cross-linked in the presence of the activators gave the same results, indicating that *γ* is cross-linked to the same region of α in these complexes of the holoenzyme.

DISCUSSION

Native hexadecameric PhK undergoes effector-induced, conformation-dependent, intramolecular cross-linking by ANB·NOS, resulting in varied extents of formation of two primary conjugates: *αγ* and *βδ*. Effectors known, or thought, to act through the enzyme's regulatory α and *β* subunits (phosphorylation, ADP, alkaline pH, and δ'/Ca^{2+}) increased formation of the major *αγ* conjugate, whereas Ca^{2+}, which targets the regulatory endogenous CaM subunit (*δ*), decreased *αγ* formation. The finding that Ca^{2+} had an opposite effect on *αγ* formation than the other activators supports the conclusion based on cross-linking with phenylenedimaleimide that, although 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 (*20*). Based on the correspondence in this study between the amount of *αγ* formed under different conditions and the subsequent activity of the cross-linked enzyme, *α*−*γ* cross-linking by ANB·NOS appears to reflect the extent of sustained activation that is measured. Previously, other studies have provided indirect evidence suggesting the possibility of *α*−*γ* cross-talk associated with the activation of PhK (*38*−*40*). Recently, we have observed that all of the activators used in this study, including Ca^{2+}, also caused increased formation of a cross-linked *αγ* complex by 4-phenyl-1,2,4-triazoline-3,5-dione (*19*) and of an *αγγ* complex by phenylenedimaleimide (*18*, *20*). Thus, these latter two chemical cross-linkers, in addition to ANB·NOS, provide direct structural evidence that alterations in the interactions between the regulatory α and catalytic *γ* subunits occur during the activating allosteric transitions induced by effectors of PhK. Cross-linking of the *γ* subunit within the C-terminal $^1/_6$th of the α subunit by ANB·NOS represents the first region of any of the three regulatory subunits of PhK that has been directly shown to interact with the catalytic subunit within the holoenzyme. This region of α is regulatory in that it contains multiple phosphorylation sites (*2*), as well as a binding site for exogenous CaM (*21*). Because cross-linking in the *αγ* dimer is essentially confined to this region of α in all activated forms of the kinase examined, changes

in the extent of α−γ cross-linking promoted by activators reflect altered interactions of this region of α with γ, regardless of the location of the site within the holoenzyme that is directly targeted by the activator. An additional region within the C-terminal half of the α subunit that may interact with the γ subunit in the PhK holoenzyme has been previously suggested based on the chromatographic comigration of a 29 kDa proteolytic fragment of α with frayed fragments of γ representing its first 290−298 residues (*41*, *42*). This fragment of the α subunit was shown to begin at residue 724 and would be calculated to stretch through approximately residue 981 (*42*), and thus would not overlap with the region of the α subunit identified in the current study.

In the case of βδ formation by ANB·NOS, no clear pattern was observed for differences in the extent of its formation induced by the effectors tested. For example, alkaline pH and ADP increased its formation, whereas autophosphorylation and Ca^{2+} caused decreased formation. Besides decreasing the amount of βδ formed, Ca^{2+} also appeared to qualitatively alter the cross-linking between these two subunits, by causing in blots of the βδ dimer exposure of the epitope on δ for the anti-CaM mAb that is masked in the dimer formed in the absence of the cation. We have also recently observed this same Ca^{2+}-induced effect on the cross-linking of δ within the holoenzyme using a heterobifunctional cross-linker of different chemistry than ANB·NOS, namely, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (*20*). Cross-linking of the holoenzyme by ANB·NOS in the presence of δ′/Ca^{2+} also caused increased formation of a β−CaM dimer, although an unknown fraction of this increase was due to the cross-linking of δ′ (exogenous CaM) to the β subunit. Thus, the β subunit of the PhK holoenzyme is ultimately targeted by both endogenous CaM (by the actions of Ca^{2+} acting through the δ subunit) and exogenous CaM (δ′/Ca^{2+}).

As was discussed earlier, there are significant differences in the catalytic activity of the PhK holoenzyme (influence of activators, time course of product formation, and specific activity) at pH 6.8 versus pH 8.2, with the activity at slightly alkaline pH resembling that of activated enzyme. There has been little evidence, however, that the conformations of the enzyme at the two pH values are different or that the conformation at pH 8.2 is similar to that of enzyme activated by allosteric effectors or phosphorylation. The strongest previous evidence to support these suppositions is that the epitopes for mAbs against the β and γ subunits are more accessible in activated PhK and in enzyme probed at pH 8.2 (*17*). It has been difficult to obtain structural evidence for pH-dependent conformational differences based on cross-linking, because nucleophilic side chains of proteins are considerably more reactive when deprotonated. Thus, with conventional chemical cross-linkers, it is problematic to interpret changes in cross-linking at pH 6.8 versus 8.2 solely on the basis of conformational differences. With the phenyltriazolinedione cross-linker, we did suggest that increased formation of an αγ complex was due to a conformational change in PhK rather than deprotonation of a reactive nucleophile, because allosteric activators also enhanced formation of this complex at pH 8.2 (*19*), as was observed with ANB·NOS. The evidence for a pH-dependent conformational change is stronger in the case of ANB·NOS, however, because the initial chemical modification step (nucleophilic attack at the carbonyl carbon of the *N*-succinimidyl leaving group) can be carried out at a different pH than the final photo-cross-linking step, allowing a homogeneous preparation of labeled kinase to be used for cross-linking at either pH 6.8 or pH 8.2. Moreover, photoactivation of the aryl azide to form highly reactive dehydroazepines (*43*), which ultimately leads to the intramolecular cross-linking of the kinase, can be achieved over a broad pH range. Thus, the increased formation of αγ observed when cross-linking the enzyme at pH 8.2 likely results from a conformational change, more so because cross-linking at pH 8.2, but not 6.8, leads to sustained activation of PhK. The results with ANB·NOS suggest that alkaline pH induces a conformational change in the holoenzyme that is structurally related to the conformation(s) promoted by autophosphorylation, ADP, and δ′/Ca^{2+}.

REFERENCES

1. Pickett-Gies, C. A., and Walsh, D. A. (1986) *Enzymes* (*3rd ed.*) *17*, 395−459.
2. Heilmeyer, L. M. G., Jr. (1991) *Biochim. Biophys. Acta 1094*, 168−174.
3. Zander, N. F., Meyer, H. E., Hoffmann-Posorske, E., Crabb, J. W., Heilmeyer, L. M. G., Jr., and Kilimann, M. W. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2929−2933.
4. Kilimann, M. W., Zander, N. F., Kuhn, C. C., Crabb, J. W., Meyer, H. E., and Heilmeyer, L. M. G., Jr. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9381−9385.
5. Grand, R. J. A., Shenolikar, S., and Cohen, P. (1981) *Eur. J. Biochem. 113*, 359−367.
6. Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C., and Nairn, A. C. (1978) *FEBS Lett. 92*, 287−293.
7. Reimann, E. M., Titani, K., Ericsson, L. H., Wade, R. D., Fischer, E. H., and Walsh, K. A. (1984) *Biochemistry 23*, 4185−4192.
8. Shenolikar, S., Cohen, P. T. W., Cohen, P., Nairn, A. C., and Perry, S. V. (1979) *Eur. J. Biochem. 100*, 329−337.
9. DePaoli-Roach, A. A., Gibbs, J. B., and Roach, P. J. (1979) *FEBS Lett. 105*, 321−324.
10. Picton, C., Klee, C. B., and Cohen, P. (l980) *Eur. J. Biochem. 111*, 553−561.
11. Heilmeyer, L. M. G., Jr., Gerschinski, A. M., Meyer, H. E., and Jennissen, H. P. (1993) *Mol. Cell. Biochem. 127/128*, 19−30.
12. Fitzgerald, T. J., and Carlson, G. M. (1984) *J. Biol. Chem. 259*, 3266−3274.
13. Trempe, M. R., and Carlson, G. M. (1987) *J. Biol. Chem. 262*, 4333−4340.
14. Cheng, A., Fitzgerald, T. J., Bhatnagar, D., Roskoski, R., Jr., and Carlson, G. M. (1988) *J. Biol. Chem. 263*, 5534−5542.
15. Ramachandran, C., Goris, J., Waelkens, E., Merlevede, W., and Walsh, D. A. (1987) *J. Biol. Chem. 262*, 3210−3218.
16. Fischer, E. H., Alaba, J. O., Brautigan, D. L., Kerrick, W. G. L., Malencik, D. A., Moeschler, H. J., Picton, C., and Pocinwong, S. (1978) in *Versatility of Proteins* (Li, C. H., Ed.) pp 133−149, Academic Press, Orlando, FL.
17. Wilkinson, D. A., Norcum, M. T., Fitzgerald, T. J., Marion, T. N., Tillman, D. M., and Carlson, G. M. (1997) *J. Mol. Biol. 265*, 319−329.
18. Nadeau, O. W., Sacks, D. B., and Carlson, G. M. (1997) *J. Biol. Chem. 272*, 26196−26201.
19. Ayers, N., Nadeau, O. W., Read, M. W., Ray, P., and Carlson, G. M. (1997) *Biochem. J. 331*, 137−141.
20. Nadeau, O. W., Sacks, D. B., and Carlson, G. M. (1997) *J. Biol. Chem. 272*, 26202−26209.
21. James, P., Cohen, P., and Carafoli, E. (1991) *J. Biol. Chem. 266*, 7087−7091.
22. King, M. M., and Carlson, G. M. (1981) *J. Biol. Chem. 256*, 11058−11064.
23. King, M. M., Fitzgerald, T. J., and Carlson, G. M. (1983) *J. Biol. Chem. 258*, 9925−9930.

24. Fischer, E. H., and Krebs, E. G. (1958) *J. Biol. Chem. 231*, 65−71.
25. Cohen, P. (1973) *Eur. J. Biochem. 34*, 1−14.
26. Kastenschmidt, L. L., Kastenschmidt, J., and Helmreich E. (1968) *Biochemistry 7*, 3590−3608.
27. Wilkinson, D. A., Marion, T. N., Tillman, D. M., Norcum, M. T., Hainfeld, J. F., Seyer, J. M., and Carlson, G. M. (1994) *J. Mol. Biol. 235*, 974−982.
28. Gopalakrishna, R., and Anderson, W. B. (1982) *Biochem. Biophys. Res. Commun. 104*, 830−836.
29. Mann, D. M., and Vanaman, T. C. (1989) *J. Biol. Chem. 264*, 2373−2378.
30. Laemmli, U. K. (1970) *Nature (London) 227*, 680−685.
31. Nadeau, O. W., Traxler, K. W., and Carlson, G. M. (1998) *Biochem. Biophys. Res. Commun. 251*, 637−641.
32. Roskoski, R., Jr. (1983) *Methods Enzymol. 99*, 3−6.
33. Hermanson, G. T. (1996) in *Bioconjugate Techniques*, pp 252−255, Academic Press, Orlando, FL.
34. Crabb, J. W., and Heilmeyer, L. M. G., Jr. (1984) *J. Biol. Chem. 259*, 6346−6350.
35. Hayakawa, T., Perkins, J. P., Walsh, D. A., and Krebs, E. G. (1973) *Biochemistry 12*, 567−573.
36. Hessova, Z., Varsanyi, M., and Heilmeyer, L. M. G., Jr. (1985) *Eur. J. Biochem. 146*, 107−115.
37. Hinrichsen, A., Bockl, H. J., and Schramm, H. J. (1989) *Biol. Chem. Hoppe-Seyler 370*, 125−133.
38. Chan, K.-F. J., and Graves, D. J. (1982) *J. Biol. Chem. 257*, 5956−5961.
39. Paudel, H. K., and Carlson, G. M. (1988) *Arch. Biochem. Biophys. 264*, 641−646.
40. Newsholme, P., Angelos, K. L., and Walsh, D. A. (1991) *J. Biol. Chem. 267*, 810−818.
41. Malencik, D. A., and Fischer, E. H. (1982) in *Calcium and Cell Function, Vol. III* (Cheung, W. Y., Ed.) pp 161−188, Academic Press, San Diego, CA.
42. Harris, W. R., Malencik, D. A., Johnson, C. M., Carr, S. A., Roberts, G. D., Byles, C. A., Anderson, S. R., Heilmeyer, L. M. G., Jr., Fischer, E. H., and Crabb, J. W. (1990) *J. Biol. Chem. 265*, 11740−11745.
43. Li, Y.-Z., Kirby, J. P., George, M. W., Poliakoff, M., and Schuster, G. B. (1988) *J. Am. Chem. Soc. 110*, 8092−8098.

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