The Structural Effects of Endogenous and Exogenous Ca$$^{2+}$$/Calmodulin on Phosphorylase Kinase*  

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The activity of phosphorylase b kinase (PbK) is stimulated by Ca$$^{2+}$$ ions, which act through its endogenous calmodulin subunit (δ), and further stimulated by the Ca$$^{2+}$$-dependent binding of exogenous calmodulin (δ'). In contrast to their highly characterized effects on activity, little is known regarding the structural effects on the δ/δ' PbK holoenzyme induced by Ca$$^{2+}$$ and δ/δ'Ca$$^{2+}$$. We have used mono- and bifunctional chemical modifiers as conformational probes to compare how the two effectors influence the structure of the catalytic γ subunit and the interactions among all of the subunits. As determined by reductive methylation and carboxymethylation, Ca$$^{2+}$$ increased the accessibility of the γ subunit; it also increased the formation by phenylenedi- maleimide of an αγγ conjugate that is characteristic of activated conformations of PbK (Nadeau, O. W., Sacks, D. M., and Carlson, G. M. (1997) J. Biol. Chem. 272, 26196–26201); however, Ca$$^{2+}$$ also had structural effects that were clearly distinct from other effectors. Moreover, similar structural effects of Ca$$^{2+}$$ were observed with PbK that had been activated by phosphorylation, consistent with the fact that such activation does not eliminate the catalytic dependence of the enzyme on Ca$$^{2+}$$. Our results suggest tiers of conformational transitions in the activation of PbK, with δ, which is fundamentally being induced by Ca$$^{2+}$$. Analysis of the various cross-linked conjugates formed in the presence of Ca$$^{2+}$$ by o-phenylenedimaleimide or m-maleimidobenzoyl-N-hydroxy succinimide ester showed that the binding of Ca$$^{2+}$$ to the δ subunit triggers changes in the interactions among all subunits, including between protomers, indicating an extensive communication network throughout the PbK complex. Most of the structural effects of δ'/Ca$$^{2+}$$ were qualitatively similar to, but quantitatively greater than, the effects of Ca$$^{2+}$$ alone; but δ'/Ca$$^{2+}$$ also had distinct effects, especially involving cross-linking of the δ subunit. 

Phosphorylase b kinase (PbK)* is a regulatory enzyme of 

glycogenolysis that integrates metabolic, hormonal, and neural signals (for review, see Refs. 1 and 2). In skeletal muscle, its dependence on Ca$$^{2+}$$ ions for activity couples contraction with energy production (3). The enzyme has four copies each of four different subunits (αβγδ). The γ subunit, with a mass of 44.7 kDa (4), is catalytic; and the α, β, and δ subunits, with masses of 138.4, 125.2 (5, 6), and 16.7 (7) kDa, respectively, are regulatory. The δ subunit is an endogenous molecule of tightly bound calmodulin (CaM) (8) that is undoubtedly responsible for the Ca$$^{2+}$$-dependence of the enzyme activity, given that complexes containing the δ subunit (γδ, αγδ, and PbK) are stimulated by Ca$$^{2+}$$ (9), whereas the free γ subunit is not (10). Two distinct, high affinity binding domains for CaM/Ca$$^{2+}$$ have been identified near the COOH terminus of the γ subunit (11); the δ subunit has been shown to interact with γ in the holoenzyme (12); and, CaM/Ca$$^{2+}$$ stimulates the activity of free isolated γ subunit (10). Thus, the primary site of interaction for the δ subunit within the holoenzyme is presumed to be on the catalytic γ subunit. PbK can be activated through a variety of mechanisms, including phosphorylation (13), proteolysis (14), and allosterically by ADP (15), but none of the variously activated forms of the enzyme loses the capacity to be stimulated by Ca$$^{2+}$$ ions. Although there have been a large number of studies on the relationship of Ca$$^{2+}$$ to activity, little is known about the effect of Ca$$^{2+}$$ ions on the structure of the holoenzyme, either activated or nonactivated. A recent communication has suggested that Ca$$^{2+}$$ increases the accessibility of specific loci within the COOH-terminal region of the γ subunit of nonactivated enzyme (16). 

In addition to the stimulatory effect of Ca$$^{2+}$$ mediated by the endogenous CaM (δ subunit), which is essentially bound irreversibly to PbK, Ca$$^{2+}$$ is also required for the reversible binding of exogenous CaM to a different site on the holoenzyme (17–19). This exogenous CaM is termed δ', and it binds in a stoichiometry of one δ' molecule/each αβγδ protomer (12, 20). This binding of δ'/Ca$$^{2+}$$ further stimulates activity past that obtained with Ca$$^{2+}$$ alone, especially for nonactivated PbK (19, 21). Based on cross-linking and peptide binding studies (12, 20, 22), both the α and β subunits apparently contribute to the binding site for δ'/Ca$$^{2+}$$. Although it is again the γ subunit that is ultimately stimulated. Even though the binding sites for δ and δ' are distinct and on different subunits, skeletal muscle troponin C can substitute for both δ in activating isolated γ subunit (23) and δ' in activating the holoenzyme (21, 24, 25). The structural effects induced by the binding of δ'/Ca$$^{2+}$$ to PbK are only slightly more fully characterized than the effects of Ca$$^{2+}$$ alone. This laboratory has found that δ'/Ca$$^{2+}$$ increases the binding to nonactivated PbK (26) of a monoclonal antibody specific for an epitope (27) that occurs at the base of the peptide.
binding lobe of the γ subunit (28) and also increases the incorporation of putrescine into that subunit by transglutaminase (29). As in the case of the structural influence of Ca\(^{2+}\) alone cited above (16), both of these effects were interpreted as manifestations of increased accessibility of particular regions of the γ subunit induced by the binding of δ/Ca\(^{2+}\) (26, 29). Inasmuch as the transglutaminase used in that study required Ca\(^{2+}\), the effect of δ/Ca\(^{2+}\) on the structure of γ that it detected was necessarily greater than that caused by Ca\(^{2+}\) alone. The incorporation of putrescine into the α and β subunits was also influenced by δ/Ca\(^{2+}\), with modification of α decreased and β increased (29). Although the effect on α could be due to direct steric inhibition caused by the specific binding of δ/Ca\(^{2+}\), the stimulatory effect on modification of β indicates a conformational change in that subunit induced by δ/Ca\(^{2+}\).

In this study, we have used mono- and bifunctional modifying agents as conformational probes to compare the effects of Ca\(^{2+}\) alone versus δ/Ca\(^{2+}\) on the structure of PbK. Using the monofunctional reagents, we have further addressed the issue of relative changes in the conformation of the γ subunit induced by the two activators. The bifunctional reagents have allowed a screening for relative changes in the interactions of all subunits (as detected by cross-linking) initiated by the binding of Ca\(^{2+}\) to the δ subunit or of δ/Ca\(^{2+}\) to the α/β subunits. These conformational probes have also been used to evaluate whether activation of the enzyme through other mechanisms alters the structural changes induced by Ca\(^{2+}\) and δ/Ca\(^{2+}\). The results obtained indicate that, although Ca\(^{2+}\) has structural effects characteristic of other activators, it also has distinct effects, and these are observed with both nonactivated and activated enzyme; δ/Ca\(^{2+}\) appears, for the most part, to amplify the structural changes brought about by Ca\(^{2+}\) alone. A preliminary account of this work has been published (30).

**EXPERIMENTAL PROCEDURES**

**Enzymes and Proteins**—Nonactivated and autophosphorylated PbK used in this study were described in the accompanying report (31). All experiments described herein were repeated a minimum of three times using three different PbK preparations. Phosphorylase b and bovine serum albumin were obtained as described (31), as were the four mAbs used in this study were described in the accompanying report (31). All modifications, which were performed at both pH 6.8, where the mono-subunit (as detected by cross-linking) initiated by the binding of Ca\(^{2+}\) to the δ subunit or of δ/Ca\(^{2+}\) to the α/β subunits. These conformational probes have also been used to evaluate whether activation of the enzyme through other mechanisms alters the structural changes induced by Ca\(^{2+}\) and δ/Ca\(^{2+}\). The results obtained indicate that, although Ca\(^{2+}\) has structural effects characteristic of other activators, it also has distinct effects, and these are observed with both nonactivated and activated enzyme; δ/Ca\(^{2+}\) appears, for the most part, to amplify the structural changes brought about by Ca\(^{2+}\) alone. A preliminary account of this work has been published (30).

**RESULTS**

**Ca\(^{2+}\) and CaM/Ca\(^{2+}\) Alter the Conformation of the Catalytic γ Subunit**—To screen for perturbation of the catalytic γ subunit induced by the binding of Ca\(^{2+}\) to endogenous CaM (δ) or by the binding of exogenous CaM/Ca\(^{2+}\) (δ' to the (αβδ)\(_{4}\) holoenzyme, PbK was incubated with radioactive, general chemical modifers as conformational probes either alone (control), with Ca\(^{2+}\), or with δ/Ca\(^{2+}\) (equimolar to αβγδ protomers), and the incorporation of label into the γ subunit was followed. Carboxymethylation by iodoacetate ([\(^{13}\)H]CH\(_{2}\)O), which is selective for thiols, and reductive methylation by formaldehyde ([\(^{13}\)H]CH\(_{2}\)O), which is selective for amines, were used for the modifications, which were performed at both pH 6.8, where the nonactivated enzyme has little activity, and pH 8.2, where it is nearly fully active. At either pH, there was a linear carboxymethylation of the γ subunit for 20 min (data not shown), which was measured by separating the photo-cross-linker and unreacted reagent by 2.1 × 2.8 × at pH 6.8 and by 1.8 × 2.4 × the pH 8.2 (Fig. 1A). Similarly, under conditions where reductive methylation of the γ subunit increased linearly with time, Ca\(^{2+}\) enhanced its modification by 1.5 × at pH 6.8 and by 2.0 × at pH 8.2; however, for this particular conformational probe, δ/Ca\(^{2+}\) had little effect over that of Ca\(^{2+}\) alone at either pH (Fig. 1B). These
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In addition to altering the rates of cross-linking, $\delta$/Ca$^{2+}$ also changed the patterns of subunit cross-linking, with new cross-linked species formed that contained CaM: $\alpha\gamma$ with o-PDM and $\beta$CaM with MBS (Fig. 2, B and C, and Figs. 3 and 4). In all such complexes, the CaM could, of course, represent either $\delta$ or $\delta'$; in many cases, as is described in a later section, we were able to distinguish between the two alternatives by utilizing derivatized CaM (BtCaM) as $\delta'$. As a result, whenever possible throughout this report, conjugates are specifically denoted as containing either $\delta$ or $\delta'$ (e.g., $\alpha\gamma$ below); if $\delta$ could be present with $\delta'$ or if the two are alternatively present in a conjugate (depending on cross-linking conditions), then the conjugate is simply denoted as containing CaM (e.g., $\beta$CaM above). In figures, for the labeling of a given band that might contain $\delta$ under one condition (one gel lane) but not another (a different gel lane), the “CaM” nomenclature is also used. The complication regarding the presence in conjugates of $\delta$ versus $\delta'$ is relevant, of course, only for cross-linking carried out in the presence of $\delta$/Ca$^{2+}$, not Ca$^{2+}$ alone, where only the $\delta$ subunit is present. Because cross-linking by o-PDM and MBS demonstrated the most significant changes in response to $\delta$/Ca$^{2+}$, we focused primarily on these cross-linkers as probes for targeting interactions in the holoenzyme mediated by $\delta$ and $\delta'$.

Cross-linking of Control PbK by o-PDM and MBS—To establish reference data against which to determine the effects of Ca$^{2+}$- and $\delta$/Ca$^{2+}$-dependent interactions, we characterized as fully as possible the cross-linking of nonactivated, control PbK by o-PDM and MBS. For o-PDM (4.8 Å cross-linking span), the conditions for cross-linking were as described previously (31), and correspondingly, the cross-linked species formed by a 10-fold molar excess of the cross-linker over protomers were before, namely predominant doublets of $\alpha\beta$ dimers and $\beta\gamma$ trimers, plus small amounts of an $\alpha\gamma$ trimer and a doublet with the mass of an $\alpha\delta$ dimer, but which cross-reacted only with anti-$\alpha$ mAb (Fig. 3, lane 2). As was discussed previously (31, 36), the presence of doublets might result from intramolecular cross-linking within the large $\alpha$ and $\beta$ subunits. In addition to the cross-linked complexes, the degradation product of $\alpha_{\text{tag}}$ (commonly occurs in small amounts in purified preparations of the enzyme (14) also showed the ability to cross-react with more than one mAb; its predominant interaction was, as expected, with the anti-$\alpha$ mAb, but it also showed highly variable cross-reactivity with the anti-CaM mAb (Fig. 3, lane 2). Because of this cross-reactivity, any species that, based on mass and cross-reactivity with the different mAbs, could have possibly contained the $\alpha_{\text{tag}}$ was eliminated from further analysis. The variability in the cross-reactivity may be related to epitope presentation in the blotting process itself because in all cases there was no cross-reactivity by the anti-CaM mAb with the intact $\alpha$ subunit, only with the $\alpha_{\text{tag}}$.

When nonactivated, control enzyme was cross-linked with MBS (9.9 Å cross-linking span), the majority of the cross-linked complexes contained the $\beta$ subunit (Fig. 4, lane 2). The predominant species formed were a $\beta\gamma$ trimer (5.0% error) and an $\alpha\beta$ dimer (2.3% error) and in smaller amounts, a $\beta\beta$ dimer (mass$\text{ theor} = 250$ kDa; 3.0% error) and an only partially classified conjugate termed $\beta\times\alpha\beta$ that contained $\beta$ and $\delta$ and migrated with a mass of 225 kDa, slightly slower than $\beta\gamma$. The mass and cross-reactivity of this last complex do not correspond to any straightforward combination of subunits. Several other complexes containing the $\beta$ subunit that were present in only small amounts had masses that most closely corresponded to a $\beta\beta$ dimer (mass$\text{ theor} = 142$ kDa) but did not cross-react with the anti-CaM mAb. One of these, designated $\beta\delta^\ast$ (Fig. 4, lane 2; 9.8% error), migrated slightly below the $\alpha$ subunit; another,


$\beta\delta^*2$ (Fig. 4, lane 2, designated $\beta CaM^*2$ in the figure; -5.6% error), migrated just above the $\alpha$ subunit. The faster migrating $\beta\delta^*1$ may represent intramolecular cross-linking of the $\beta$ subunit, in that such cross-linking has been shown previously to cause more rapid migration of this subunit (31, 36). Even though these putative $bd$ complexes did not cross-react with the anti-$CaM$ mAb and even though a relatively large number of theoretical permutations of subunit cross-linking are possible, there are no other combinations of subunits that are consistent with the observed masses and cross-reactivities of these complexes. Apparently the epitope recognized by the anti-$CaM$ mAb, which is at the COOH terminus of $CaM$ (37), is masked in the blots of these particular complexes containing the $d$ subunit, the smallest of the four PbK subunits.

Influence of $Ca^{2+}$ Alone on Cross-linking—Inclusion of $Ca^{2+}$ caused two significant changes in the cross-linking of PbK by $o$-PDM (Fig. 3, lane 3): a large increase in the formation of $\alpha\gamma\gamma$ trimer (2.2% error), which was formed in only trace amounts in the absence of $Ca^{2+}$; and formation of a new conjugate, tentatively identified as an $\alpha\gamma\delta$ tetramer (mass$_{theor}$ = 217 kDa; -3.2% error). Although this latter complex cross-reacted with only the anti-$\alpha$ and anti-$\gamma$ mAbs, it comigrated exactly with the better defined $\alpha\gamma\delta$ complex formed in the presence of $d^*/Ca^{2+}$ (lane 4). The results with $o$-PDM indicate that the binding of $Ca^{2+}$ by

**Fig. 2.** Effect of $d^*/Ca^{2+}$ on the cross-linking of PbK by reagents of variable spans and chemistries. Panel A, listed from top to bottom, lane $M$ contains 5 $\mu$g each of myosin (205 kDa), $\beta$-galactosidase (116 kDa), phosphorylase $b$ (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa) as mass standards; lane $C$ contains PbK prior to cross-linking. PbK (1.73 $\mu$M $\alpha\beta\delta\gamma$ protomers) was cross-linked at pH 8.2 in the absence of effectors (lane 1) and in the presence of $d^*/Ca^{2+}$ at 1.73 $\mu$M/250 $\mu$M (lane 2) or 4.32 $\mu$M/250 $\mu$M (lane 3) with 17.3 $\mu$M $o$-PDM (panel B), 8.6 $\mu$M MBS (panel C), 86 $\mu$M ANB-NOS (panel D), or 17.3 $\mu$M mdPDM (panel E) as described under “Experimental Procedures.” The span of the cross-linkers is listed under their structures. After resolution of the cross-linked complexes by SDS-PAGE, the gel was stained for protein with Coomassie Blue.

**Fig. 3.** Effect of $Ca^{2+}$ and $d^*/Ca^{2+}$ on the cross-linking of PbK by $o$-PDM. Cross-linking was carried out as described under “Experimental Procedures,” and the resultant gel from SDS-PAGE was electrophobbed onto nitrocellulose and stained for cross-reactivity against the indicated mAbs. The various lanes represent non-cross-linked, nonactivated control PbK (lane 1) and the same enzyme cross-linked in the absence of effectors (lane 2) or in the presence of $Ca^{2+}$ (lane 3) or $d^*/Ca^{2+}$ (lane 4). The bands denoted as $a$ and $b$ contain all four subunits but in indeterminate stoichiometries.
the δ subunit of PbK promotes a conformational change in the holoenzyme that, as a minimum, perturbs interactions among the α, γ, and δ subunits. To ask whether Ca\(^{2+}\) also perturbs interactions involving the δ subunit, cross-linking was performed with MBS, which preferentially forms conjugates containing this subunit.

Including Ca\(^{2+}\) in the cross-linking with MBS caused significant changes mostly in the anti-CaM blot of the cross-linked products (Fig. 4, lane 3), although two of the observed changes are not readily interpretable. One of these two is a new cross-reactive band that migrates in the area of the α\(_{\text{frag}}\) but, as was discussed previously, species that could possibly contain this fragment were eliminated from further analysis because of its anomalous cross-reactivity. The second concerns the previously described βX\(_{225}\) conjugate containing at least the β and δ subunits, which shows enhanced anti-CaM cross-reactivity when the cross-linking includes Ca\(^{2+}\). The interpretable changes in the cross-linked products containing δ involve the α and β subunits. The heaviest of this is an αδδ trimer (mass\(_{\text{theor}}\) = 172 kDa; 2.9% error), whose formation is slightly increased by Ca\(^{2+}\); for the related αδ dimer (mass\(_{\text{theor}}\) = 155 kDa; 5.8% error), Ca\(^{2+}\) caused an increase in cross-reactivity. The remaining change was in βδ2, which now cross-reacted with the anti-CaM mAb, unlike the situation when cross-linking was carried out in the absence of Ca\(^{2+}\). These last results suggest that Ca\(^{2+}\) promotes a conformational change that results in the cross-linking of different regions of δ to the β subunit, no longer masking the epitope on δ for the anti-CaM mAb.

**Influence of 8′/Ca\(^{2+}\) on Cross-linking**—Compared with the results observed with Ca\(^{2+}\) alone, including 8′/Ca\(^{2+}\) in the cross-linking of PbK by MBS caused several changes (Fig. 4, lane 4); new bands were formed, and there were changes in the cross-reactivities of other bands, especially in the anti-CaM blot. There was an increase in the cross-reactivities of the bands corresponding to CaM, α(CaM)\(_2\), βδδ, and βCaM*2, as well as of βX\(_{225}\), and a decrease in the cross-reactivity of the βγ band. New bands formed in the presence of 8′/Ca\(^{2+}\) included several that migrated between the β subunit and the α\(_{\text{frag}}\), which as discussed previously, were not considered further, plus a new βCaM dimer, *3 (1.4% error). The three differently migrating βCaM complexes presumably result from different amounts or regions of inter- and intramolecular cross-linking, particularly of the β subunit in the latter case.

To determine if the CaM in the CaM-containing complexes represented δ or δ′ (i.e. endogenous or exogenous CaM), we used as the source for δ′ a tagged (monobiotinylated) CaM derivative, BtCaM (33), which activates the PbK holoenzyme in parallel with bovine brain CaM (data not shown). Even though the exchange rate of the endogenous δ subunit for exogenous CaM has been shown to be barely detectable, especially in the presence of Ca\(^{2+}\) (12), we nevertheless incubated the BtCaM/Ca\(^{2+}\) with equimolar PbK for only 2 min prior to cross-linking to eliminate further any possibility of exchange. Cross-linking in the presence of BtCaM/Ca\(^{2+}\) resulted in the same cross-
linking pattern and cross-reactivities against the anti-CaM mAb as were observed with nondervatized CaM as δ' (Fig. 4, lane 5). Avidin-alkaline phosphatase cross-reacted with βCaM*δ, suggesting that this band may be entirely βδ', especially given that it is not observed in the absence of exogenous CaM. Some bands that were initially observed to form in the absence of exogenous CaM, such as βδ*2, cross-reacted with the avidin probe when formed in the presence of BtCaM/Ca ²⁺; this indicates either that δ'/Ca²⁺ flips the cross-linking of β from δ to δ' or that these bands contain both βδ and βδ'. In contrast, the βδ*1 band, which is formed both in the presence and absence of BtCaM/Ca²⁺, did not appear to cross-react with avidin, suggesting that it is composed entirely of βδ. It is noteworthy that although δ'/Ca²⁺ causes only a small increase over Ca²⁺ alone in the amount of βδ*1 observed with anti-β, it causes a dramatic increase in the amount of βδ*1 that cross-reacts with anti-CaM. These results suggest that in the βδ*1 dimer different regions of δ are cross-linked in the presence of δ'/Ca²⁺ than in the presence of Ca²⁺ alone, i.e. that exogenous CaM affects the endogenous CaM of PbK.

In contrast to its effects on the cross-linking by MBS, δ'/Ca²⁺ (compared with Ca²⁺ alone) caused relatively small changes in the cross-linking by α-PDM (Fig. 3, lane 4). The two most significant changes were a large increase in the amount of δδ' formed and in the number of cross-reactive bands observed in the anti-CaM blot. In the latter case, however, the mass and cross-reactivity of the bands other than δδ' and αγδδ did not allow their unambiguous identification. As with MBS, use of BtCaM as δ' did not cause additional changes; however, the δδ' and αγδδ formed in its presence did not cross-react with the avidin probe (data not shown), suggesting that these species did not contain δ', only δ. Consequently, δ' increases the formation of δδ' by α-PDM and apparently alters the cross-linked regions in both δδ' and αγδδ, allowing greater cross-reactivity with the anti-CaM mAb.

Activation of PbK by Cross-linking in the Presence of Ca²⁺ and δ'/Ca²⁺.—In the previous report (31) we demonstrated that when PbK is cross-linked by PDM in the presence of the allosteric activators ADP and GDP it remains activated even after dilution of the allosteric effectors to ineffective concentrations, i.e. the active conformers were trapped by cross-linking. In addition, formation of αγδ increased along with activation. Because both Ca²⁺ and δ'/Ca²⁺ are activators that also caused increased formation of αγδ by α-PDM (Fig. 3), we asked whether cross-linking in the presence of these effectors could similarly lock the enzyme in an active conformation. When enzyme was cross-linked at pH 8.2 in the presence of Ca²⁺ or δ'/Ca²⁺ and then assayed at pH 6.8, there was a 4.2- and 5.9-fold increase, respectively, in its activity, following dilution of the effectors (Fig. 5, closed bars). In contrast, the non-cross-linked control enzyme did not show significant activation when assayed with identical carryover concentrations of Ca²⁺ and δ'/Ca²⁺ (Fig. 5, open bars). Therefore, the irreversible activation in response to cross-linking results from the direct action of α-PDM on the Ca²⁺ and δ'/Ca²⁺ complexes of PbK. Cross-linking with MBS, which does not form αγγ complexes, did not activate PbK or its Ca²⁺ or δ'/Ca²⁺ complexes (data not shown). These results, now obtained with different activators, corroborate the previous finding that formation of the αγγ trimer by PDM is a marker for active conformers of PbK (31).

Influence of Ca²⁺ and δ'/Ca²⁺ on the Conformation of Autophosphorylated Enzyme.—The maximal activation of PbK is achieved through its autophosphorylation (38), but even this activity remains Ca²⁺-dependent, which suggests that Ca²⁺-induced effects on conformation, which were observed with nonphosphorylated, nonactivated PbK, may also occur with autophosphorylated enzyme. We first tested the abilities of Ca²⁺ and δ'/Ca²⁺ to stimulate the carboxymethylation of the γ subunit of autophosphorylated enzyme at pH 6.8. Similar to the results obtained with the nonactivated enzyme (Fig. 1A), Ca²⁺ and δ'/Ca²⁺ enhanced the carboxymethylation of the γ subunit by 1.6 × and 1.7 ×, respectively; however, autophosphorylation itself caused no increase in carboxymethylation above that observed with the control nonactivated enzyme (data not shown). Using cross-linking by α-PDM as the conformational probe, Ca²⁺ and δ'/Ca²⁺ had the same effects on the cross-linking of both nonactivated and autophosphorylated PbK, namely increased formation of αγγ and δδ and induction of αγδδ (Fig. 6A). Furthermore, as with nonactivated PbK, when the latter two complexes were formed in the presence of BtCaM as δ', they did not cross-react with avidin (Fig. 6A), suggesting that in phosphorylated and in nonactivated PbK, δ' has similar effects on δ. With MBS as the cross-linker, the behavior of autophosphorylated and nonactivated PbK was again very similar, but with some minor differences. The cross-linking patterns in the absence of effectors were the same, and Ca²⁺ and δ'/Ca²⁺ had the same influence on the cross-linking of both forms of the enzyme; but, with the phosphorylated enzyme, there was diminished cross-reactivity in the anti-CaM blot for the βδ*2, αδ, and αδδ complexes formed in the presence of δ'/Ca²⁺ (Fig. 6B). In summary (see Table I), Ca²⁺ significantly alters the structure of the γ subunit of autophosphorylated PbK, as indicated by the apparent accessibility difference detected by carboxymethylation, and perturbs the cross-linking of the enzyme. These effects are very similar to those observed with nonactivated PbK and suggest, therefore, that the effects of Ca²⁺ on PbK are essentially independent of its state of activation. The results with δ'/Ca²⁺ indicate that autophosphorylation does not block the binding of exogenous CaM nor its being cross-linked to the regulatory subunits nor its ability to promote differences in the cross-linking of the δ subunit (Table I); however, the influence of δ'/Ca²⁺ on the chemical modification of the γ subunit was less with the autophosphorylated enzyme.

**DISCUSSION**

The influence of Ca²⁺ ions on the actions of the monofunctional and bifunctional chemical probes used in this study to monitor PbK conformation indicates extensive communication, either direct or indirect, between the δ subunit (endogenous CaM) and the remaining subunits of the holoenzyme, regardless of its state of activation. Because the activity of the ho-
loenzyme expressed by the \( \gamma \) subunit is \( Ca^{2+} \)-dependent and because direct interactions between the \( \gamma \) and \( \delta \) subunits have been documented (10, 12), we first evaluated the ability of \( Ca^{2+} \) to alter the conformation of the catalytic \( \gamma \) subunit, as monitored by chemical modification. Both carbamylation and reductive methylation of \( \gamma \) were increased by \( Ca^{2+} \). Because \( Ca^{2+} \) also influenced the formation of several cross-linked complexes containing \( \gamma \) (Table I), we think that the increased modification of \( \gamma \) likely represents increased accessibility of this subunit, rather than simply increased reactivity of particular side chains. An increased accessibility of \( \gamma \) in response to \( Ca^{2+} \) is also consistent with previous reports that \( Ca^{2+} \) enhances the binding to the holoenzyme of antipeptide antibodies against carboxyl-terminal regions of the \( \gamma \) subunit (16) and increases the affinity of PbK for its macromolecular substrate, phosphorylase \( b \) (39). With the bifunctional probes, \( Ca^{2+} \) influenced the formation of cross-linked species containing the inhibitory \( \alpha \) and \( \beta \) subunits (Table I). With respect to the \( \alpha \) subunit, \( Ca^{2+} \) promoted increased formation of \( \alpha \gamma \gamma \gamma \) (both by \( o \)-PDM) and a second new conjugate, \( \alpha \delta \delta \delta \) (by MBS). This influence by \( Ca^{2+} \) on the interactions among the \( \alpha \), \( \gamma \), and \( \delta \) subunits suggests an activation mechanism for the holoenzyme that includes a linkage in which the binding of \( Ca^{2+} \) to \( \delta \) perturbs constraining quaternary interactions imposed by \( \alpha \) upon \( \gamma \); support for such a linkage comes from the work of Chan and Graves (9), who reported that the activity of the \( \gamma \delta \) complex showed a less stringent requirement for \( Ca^{2+} \) than did the \( \alpha \gamma \delta \) complex. It should be noted, however, that \( Ca^{2+} \) also affects the \( \beta \) subunit, as indicated by the results with MBS (Table I) or by the use of partial proteolysis as a probe of conformation (40).

Although \( Ca^{2+} \) displays the property common to other activators of PbK of promoting formation by PDM of the \( \alpha \gamma \gamma \) trimer, it also has effects that are distinct from those of the other activators. For instance, neither ADP nor autophosphorylation caused formation of the \( \alpha \gamma \delta \delta \delta \) tetramer by PDM (31) or stimulated carbamylation of the \( \gamma \) subunit (41) or enhanced the affinity of PbK for phosphorylase \( b \) (39). Not only were the structural effects of \( Ca^{2+} \) observed in this study distinct, they were nearly the same for both nonactivated and autophosphorylated PbK: similar stimulation of carbamylation of the \( \gamma \) subunit and similar perturbation of cross-linking by either \( o \)-PDM or MBS (Table I). That the activator \( Ca^{2+} \) has unique structural effects that occur with both nonactivated and activated PbK is totally consistent with the fact that all other activators of the enzyme stimulate its \( Ca^{2+} \)-dependent activity but do not eliminate the requirement for \( Ca^{2+} \). One might envision a hierarchy of tiered conformational transitions leading to the activation of PbK, with the most fundamental being that induced by the binding of \( Ca^{2+} \) to the \( \delta \) subunit. Conformational transitions triggered by additional activating events targeted to the remaining subunits (phosphorylation and proteolysis) to \( \beta \) and \( \alpha \) (1); binding of \( \delta \) to \( \alpha \beta \) (12, 20, 22); binding of ADP, probably to \( \beta \) (42, 43); and binding of excess \( Mg^{2+} \), at least in part to \( \gamma \) (44, 45)) would then be incorporated into the \( Ca^{2+} \)-induced conformation. The resulting conformations of the holoenzyme would then exhibit different levels of activity, mirroring the different degrees of quaternary constraint on the \( \gamma \) subunit resulting from differences among the linkages of the \( \alpha \), \( \beta \), \( \gamma \), and \( \delta \) subunits. Although dependence on \( Ca^{2+} \) is not eliminated by activation, the extensive communication network between the \( \delta \) subunit and the remaining subunits that was shown in this study suggests that the \( Ca^{2+} \) requirement should at least be structurally coupled to and changed by activating events initiated at the remaining subunits. Such a change upon activation has, in fact, been reported by Cohen (21), who found that phosphorylation and proteolysis substantially decreased the \( K_{a} \) for \( Ca^{2+} \). It has been suggested that the activation by CaM of the full-length \( \gamma \) subunit is not due merely to the passive removal of autoinhibition, but also to an active stimulation of \( \gamma \) by CaM (46), which could explain why none of the other known activators completely removes the \( Ca^{2+} \) dependence of the PbK holoenzyme.

In contrast to the anchoring of the \( \delta \) subunit, which is presumed to occur largely through the \( \gamma \) subunit, previous evidence suggests that the binding site for \( \delta / Ca^{2+} \) comprises portions of both the \( \alpha \) and \( \beta \) subunits (12, 20, 22). Our cross-linking results with MBS and BtCaM as \( \delta \) support this view, in both the \( \alpha \delta \) and \( \beta \delta \) dimers were observed. We noted previously that \( \delta / Ca^{2+} \) inhibits the zero length cross-linking of Lys and Gln residues on the \( \alpha \) and \( \beta \) subunits to form an \( \alpha \beta \) dimer, but we were unable to determine whether that inhibition was, in fact, steric (29). With the exception of conjugates containing the \( \delta \) subunit, most of the effects of \( \delta / Ca^{2+} \) on conformation, as monitored by cross-linking, were qualitatively similar to, but quantitatively greater than, those induced by \( Ca^{2+} \) alone (Table I), consistent with tiered conformations. These effects occurred with both nonactivated and autophosphorylated enzyme.
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(indicating that \(\delta/\alpha\) still binds to phosphorylated PhK and induces conformational changes that are characteristic of its effects on nonactivated enzyme, although the activity of phosphorylated enzyme is relatively unaffected by \(\delta/\alpha\) (19, 21). Reflecting its relative effects on activity, we found that enhancement of the carboxymethylation of the \(\gamma\) subunit by \(\delta/\alpha\), compared with \(\alpha\) alone, was considerably less with phosphorylated PhK than with nonactivated enzyme. In other studies with nonactivated PhK, \(\delta/\alpha\) has also been shown to increase the incorporation of putrescine into the \(\gamma\) subunit by transglutaminase (29) and to enhance the binding of an anti-\(\gamma\) monoclonal antibody (26). These results concerning the carboxymethylation of \(\gamma\) and its interactions with transglutaminase and antibody are again consistent with an increase in its accessibility induced by the binding of \(\delta/\alpha\), an increase greater than that caused by \(\alpha\) alone. Besides its effects on the \(\gamma\) subunit described above, \(\delta/\alpha\) also influenced the formation of cross-linked conjugates involving all four subunits of the PhK holoenzyme (Table I). In the case of the \(\delta\) subunit, our results suggest that different regions of it cross-link to the \(\alpha\) and \(\beta\) subunits in the presence of \(\delta/\alpha\) than in the presence of \(\alpha\) alone, raising the possibility that exogenous CaM may influence the conformation of the endogenous CaM of PhK.

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