Differential Affinity Cross-linking of Phosphorylase Kinase Conformers by the Geometric Isomers of Phenylenedimaleimide

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Phosphorylase b kinase (PbK) from skeletal muscle is a highly regulated oligomer consisting of four copies of four distinct subunits (αβδγ). The γ subunit is catalytic, and the remaining subunits are regulatory. To characterize effector-induced changes in the quaternary structure of the enzyme, we utilized the ortho-, meta-, and para-isomers of phenylenedimaleimide (PDM), which in addition to having different geometries, also vary 2.5-fold in their cross-linking spans. Even at concentrations equivalent to the αβδγ protomers of PbK, all three isomers caused specific, rapid, and extensive cross-linking of the holoenzyme to form primarily αβ dimers, plus smaller amounts of βγ and γγ trimers. The formation of these three conjugates was nearly totally inhibited by a 10-fold molar excess over PDM of N-(o- and p-tolyl)succinimide, which are chemically inert structural analogs of PDM. This inhibition suggests that PbK has binding sites for PDM and that PDM acts as an affinity cross-linker in binding to these sites prior to forming cross-linked conjugates. The largest effect on cross-linking in progressing from o- to p-PDM was on the αγγ trimer, which is preferentially formed by the p-isomer. Activation of the enzyme by either phosphorylation or the allosteric activators ADP and GDP resulted in large increases in the amount of αγγ formed, small increases in βγγ, and little change in αβ. When cross-linked in the presence of the reversibly activating nucleoside diphosphates, PbK remained activated after their removal, indicating that cross-linking had locked it in the active conformation. Our results provide direct evidence for perturbations in the interactions of the catalytic γ subunit with the regulatory α and β subunits upon activation of PbK.

Through allosteric and covalent modification sites on its three regulatory subunits, phosphorylase b kinase (PbK) integrates neural, hormonal, and metabolic signals to modulate glycolytic flux in skeletal muscle (for review, see Refs. 1 and 2). Although the α, β, and δ (calmodulin) regulatory subunits clearly control the activity of the catalytic γ subunit, little is known concerning the mechanisms through which they exert this control, including the extent to which their regulatory influence on γ is direct versus indirect; this is especially the case for the larger regulatory subunits, α and β. Phosphorylation (3) or proteolysis (4) of α causes increased activity of γ within the (αβγδ)4 holoenzyme, but evidence for a direct α-γ interaction that is altered by this activation has not been observed. Likewise, multiple means of activating the holoenzyme cause common conformational changes in the β subunit (5–7); but again, no evidence for alteration of a direct β-γ interaction has been observed. In two previous studies, cross-linking was used successfully to detect changes in the cross-linking patterns of both the α (8) and β (5) subunits upon activation of the holoenzyme; but with the cross-linkers used, the observed changes involved only a second α or δ subunit to form homodimers and did not involve the catalytic γ subunit. Even though cross-linking has been shown to be a potentially promising approach to probing structural changes associated with activation of the hexadecameric PbK holoenzyme, it has not been explored widely because of the intrinsic difficulty in identifying unambiguously the subunit composition of cross-linked complexes (techniques used previously with PbK to analyze such complexes have been limited to approximate mass, relative susceptibility to proteolysis, and specific radioactivity of individual subunits (5, 9, 10)).

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1 The abbreviations used are: PbK, phosphorylase b kinase; PDM, phenylenedimaleimide; mAb, monoclonal antibody; TNB, free thiol of DTNB; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PAGE, polyacrylamide gel electrophoresis; NTS, N-(o- or p-tolyl)succinimide.
Enzymes and Proteins—PhK was isolated from fast-twitch skeletal muscle of New Zealand White rabbits (15), diazylated against a solution of Hepes buffer (50 mM, pH 6.8), sucrose (10%), EDTA (0.2 mM), and either used immediately or stored frozen at −60 °C. All experiments described in this study were repeated a minimum of three times using three different PhK preparations. When autophosphorylated PhK was required for cross-linking studies, the phosphorylation was carried out at pH 8.2 in Hepes buffer for 1 min using the methodology of King et al. (16). The extent of phosphate incorporation with different kinase preparations ranged from 1.2 to 2.1 mol per a and β subunit and 0.8–1.0 β/g subunit. The enzyme was also phosphorylated by the catalytic subunit of protein kinase A, as described previously (12), to the extent of 1 mol of phosphate per a and β subunit. Prior to cross-linking, PhK phosphorylated through either mechanism was purified by gel filtration over a Sepharose 6B (column, 1.5 × 112 cm) developed with Hepes buffer (50 mM, pH 6.8), 0.2 mM EDTA, and 10% sucrose. Fractions that eluted at the position of native holoenzyme were collected, buffer was exchanged, and the enzyme was concentrated to 4.5 mg/ml by ultrafiltration using a Centricon-30 concentrator (Amicon). Nonphosphorylated enzyme used as the control to determine the effects of phosphorylation was subjected to the same incubation, gel filtration, and concentration protocols. Phosphorylase b was isolated from rabbit skeletal muscle (17), 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 (18, 19).

Bovine serum albumin (A-9647) was from Sigma, and the catalytic subunit of cAMP-dependent protein kinase was from Promega. Melittin was from Sigma, with its concentration determined as described previously (20). Monoclonal antibodies against the α (mAbs) against the α, β, and γ subunits of phosphorylase kinase were generated in mice against the native enzyme on Sepharose 6B (data not shown), this cross-linking was quenched by dilution of an aliquot of the reaction mixture into an equal 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, the samples (14 μg/ lane) were run on SDS-polyacylamide gradient gels (4–15%) (23) and stained with Coomassie Blue. All gels were destained in 40% methanol, 10% acetic acid (2 h) and 7% acetic acid, 4% methanol (15 h). All conclusions regarding the relative amounts of cross-linked species formed were based on the integrated optical density of the protein bands determined using a BioImage whole band analyzer.

The N-(tolyl)succinimidyl-

RESULTS

Time- and Concentration-dependent Cross-linking of Nonactivated PhK by α-, m-, and p-PDM followed by SDS-PAGE. Panel A, PhK was cross-linked with the indicated concentrations of p-PDM (lane 1), m-PDM (lane 2), and p-PDM (lane 3) for 2 min. Panel B, PhK was incubated with the isomers of PDM in the stoichiometry (10 PDM:1 αβγδ) for the times indicated. The reactions were carried out as described under “Experimental Procedures,” with the cross-linking concentration of PhK being 1.73 μM in both panels. The position of the putative αβ dimer is indicated by ♦.
Either possibility indicates more than one region of cross-linking by PDM on the \( \alpha \) and/or \( \beta \) subunits. Similarly, the cross-linking of \( \beta \) subunits to form \( \beta \beta \) dimers with different migrations has been observed previously when activated forms of the kinase are treated with 1,5-difluoro-2,4-dinitrobenzene (5), indicating that more than one region of the \( \beta \) subunit is also subject to chemical modification by this bifunctional reagent.

Based on the densities of the bands in Fig. 1, greater than 90% of the \( \alpha \) and \( \beta \) subunits lost formed \( \alpha \beta \) dimers when the nonactivated holoenzyme was treated with the three PDM isomers under those conditions (low concentrations of cross-linkers or short incubation times). Not only was \( \alpha \beta \) formation relatively specific, it was also an efficient process, given that 15% of the \( \alpha \) and \( \beta \) subunits were cross-linked during a short period of cross-linking (2 min) at the low ratio of 1 PDM:2\( \alpha \beta \gamma \) (Fig. 1A). This high yield of cross-linking under these conditions indicates minimal competition from monosubstitution reactions or hydrolysis of the active maleimide, a significant reaction at pH 8.2 (27), our standard pH of cross-linking. The specificity, rapidity, and extent of cross-linking are consistent with the presence of binding sites for PDM on the \( \alpha \) and/or \( \beta \) subunits, which have significant sequence similarity (28).

**Formation of PDM Conjugates of Intermediate Mass**—Increasing the concentration of PDM or the time of cross-linking resulted in increased formation of two additional conjugates with apparent masses of 225 and 205 kDa, corresponding to the masses of \( \alpha \gamma \) (mass\(_{\text{theor}} = 228 \text{ kDa} \); -1.3% error) and \( \beta \gamma \) trimers (mass\(_{\text{theor}} = 215 \text{ kDa} \); -4.7% error), respectively (Fig. 1). The subunit composition of these new heteromers was verified further by cross-reactivity with subunit-specific mAbs (Fig. 2). As was the case with the \( \alpha \beta \) dimer, the \( \beta \gamma \) trimer was also present as a doublet. In contrast, however, to the \( \alpha \beta \) dimer, the rate of formation of the trimers, especially of \( \alpha \gamma \gamma \), varied with the cross-linker used: the \( p \)-isomer caused the greatest formation of \( \alpha \gamma \gamma \), although the amount of it formed using this isomer and nonactivated enzyme was still less than that of \( \beta \gamma \gamma \) (Fig. 1). Also, the extent of \( \alpha \gamma \gamma \) and \( \beta \gamma \gamma \) formation increased over a 10-min period at excess PDM concentrations (10 PDM:1 \( \alpha \beta \gamma \)) (Fig. 1B), whereas the amount of discrete \( \alpha \beta \) dimer reached a maximum within 0.5 min and then slowly decreased upon further cross-linking. Because only \( \alpha \beta \) dimers are observed when the enzyme is cross-linked below pH 7.5 (data not shown), the formation at higher pH values of \( \alpha \gamma \gamma \) and \( \beta \gamma \gamma \) trimers presumably results either from the cross-linking of different types of side chains or from a conformational change induced by alkaline pH, which is known to stimulate the activity of phosphorylase kinase greatly and to induce a conformational change (13).

**Formation of a Low Mass PDM Conjugate**—A minor cross-linked species corresponding to the known mass of an \( \alpha \delta \) dimer (mass\(_{\text{theor}} = 155 \text{ kDa} \), 0.6% error) was observed under conditions identical to those under which \( \alpha \gamma \gamma \) and \( \beta \gamma \gamma \) trimers formed (Fig. 1). As was observed for \( \alpha \gamma \gamma \), the rate of formation of this putative \( \alpha \delta \) dimer increased in order of cross-linking by \( o \)-, \( m \)-, and \( p \)-PDM. In blots of enzyme cross-linked by either \( o \)- or \( p \)-PDM, the conjugate interacted with the anti-\( \alpha \) mAb but not with the anti-calmodulin mAb (the \( \delta \) subunit is endogenous calmodulin) (Fig. 2, \( \bullet \)). Although the anti-calmodulin mAb, which targets the COOH-terminal region of calmodulin (11), has been used successfully with other cross-linkers of PhK to detect trace amounts of calmodulin-containing conjugates (29), it is possible that the calmodulin epitope in this particular conjugate is masked as a result of the cross-linking by PDM.

**Inhibition of \( o \)- and \( p \)-PDM Cross-linking by Their Structural Analogs \( N \)-(o- and p-Tolyl)succinimide**—The rapid and rela-
monosuccinimide isomer suggests that these four compounds nearly identical inhibition of both PDM cross-linkers by either reactive succinimide structural analogs indicates the presence linking by the respective PDM isomers by 86 and 87%. The pd and from a comparison of the total combined optical density for the cross-linked as above (lane 2, respectively). The percent cross-linking was determined o and p-NTS (the more potent inhibitor) over PDM inhibited cross-linking by 95 and 91%, respectively; under identical conditions p-NTS inhibited cross-linking by the respective PDM isomers by 86 and 87%. The potential inhibition of o- and p-PDM cross-linking by their non-reactive succinimide structural analogs indicates the presence of binding sites on PhK for the PDM isomers. Furthermore, the nearly identical inhibition of both PDM cross-linkers by either monosuccinimide isomer suggests that these four compounds bind to a common site or sites.

Activators of PhK Perturb Its Cross-linking Pattern—Because activation of PhK is associated with conformational changes, some of which can be detected by cross-linkers (5, 7, 8, 29), we wished to determine whether PDM could also distinguish the activated conformation(s) of the holoenzyme. The allosteric activators ADP and GDP caused a large increase over controls in the formation of αγγ, especially by m- and p-PDM, and a small increase in βγγ (Fig. 4A). Likewise, activation by autophosphorylation also caused a large increase in the formation of αγγ and a small increase in βγγ (Fig. 4B); activation via phosphorylation by the catalytic subunit of protein kinase A caused similar effects (data not shown). The isomer selectivity for trimer formation with these activated conformers of PhK induced by either nucleoside diphosphates or phosphorylation changed from predominantly βγγ with o-PDM, to approximately equivalent amounts of αγγ and βγγ with m-PDM, to predominantly αγγ with p-PDM. Thus, the relative selectivity for increased αγγ formation in progressing from o- to p-PDM is the same as was described previously for the nonactivated enzyme (Fig. 1); of course, it is possible that the basal formation of these trimers with the nonactivated enzyme may simply reflect the fact that cross-linking is carried out at the stimulatory pH of 8.2. Unlike the very large increase in αγγ and the small increase in βγγ, there were only modest increases in the αβ and putative αδ dimers upon activation.

In contrast to the significant amounts of αγγ and βγγ conjugates induced by nucleoside diphosphates and by phosphorylation, only trace amounts of these trimers were formed when the enzyme was cross-linked in the presence of the activator heparin. Instead, heparin protected the γ subunit while promoting extensive cross-linking of the δ subunit (Fig. 4A). These results suggest that heparin, which also promotes dissociation of the δ subunit (30), activates the enzyme through a different mechanism than ADP, GDP, or phosphorylation.

Melittin, a model calmodulin-binding peptide that is an inhibitor of PhK (31), did not enhance, with respect to control, the formation of either αγγ or βγγ (Fig. 4A), which is consistent with the notion that formation of these trimers is character-
The cross-linking of PbK by PDM was conformation-depend-
ent in that stimulators of activity (ADP, GDP, and phosphorylation) caused a large increase in the formation of αγγ (especially with p-PDM) and a small increase in βγγ. Thus, activation of PbK is associated with changes in quaternary structure involving the α, β, and γ subunits and/or the unmasking of nucleophilic residue(s) on at least one of these subunits. Regardless, however, of the exact mechanism through which activation by nucleoside diphosphates and phosphorylation occurs, cross-linking with PDM provides direct structural evidence for alterations in the interactions of the catalytic γ subunit with the regulatory α and β subunits upon activation. The structural changes resulting in increased formation of αγγ and βγγ trimers caused by the above well characterized activators of the kinase were not observed with the polyanionic activator heparin, which has previously been shown to have different structural effects on PbK than other activators (32), including causing dissociation of the δ subunit (30). Melittin, an inhibitor of PbK (31), likewise did not cause increased formation of αγγ and βγγ trimers.

The ability of PDM to lock the enzyme in the active form(s) induced by ADP and GDP further indicates that these cross-linkers are effective reporters of active conformations of the holoenzyme. Intramolecular cross-linking has been shown to stabilize given forms of proteins (36), effectively locking them in specific conformations. For example, cross-linking by bis(3,5-dibromosalicyl)fumarate has been reported to lock hemoglobin in its T-state (37). In an oligomer the size and complexity of PbK, there is undoubtedly a large number of intersubunit contacts that define its active conformation(s) (38), and only a small fraction of these are likely to be targeted by PDM. Nevertheless, our results indicate that in the case of activation by ADP and GDP, PDM cross-links at least several sites in the activated enzyme that allow it to remain activated after removal of the activators. Of the PDM conjugates formed, αγγ, and to a lesser extent βγγ, are apparent indicators of the activated conformer(s), in that both trimers are preferentially formed with activated kinase. The extent of formation of the αβ dimer was essentially the same with both activated and non-activated forms of the kinase; moreover, when cross-linking was performed at a low pH so that the only conjugate formed to a significant extent was the αβ dimer, no activation was observed in subsequent assays. Similarly, zero length cross-linking with transglutaminase, which resulted in αβ dimers as the predominant conjugate, did not give rise to activation (8). Based on the time- and concentration-dependent cross-linking of PbK by PDM under different conditions, there are two major sets of PDM binding sites on the PbK holoenzyme: high efficacy site(s) involving the formation of αβ, which is generated in greater amounts than the remaining conjugates with all forms of the enzyme tested, and low efficacy site(s) involving αγγ and βγγ formation. It is the cross-linking between the catalytic γ subunits and the inhibitory α and β subunits (39–41) at the low efficacy site(s) that reflects the activation state of the kinase.

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