Zero Length Conformation-dependent Cross-linking of Phosphorylase Kinase Subunits by Transglutaminase*

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Transglutaminase, a zero length cross-linker that catalyzes the formation of isopeptide bonds between proximal Gln and Lys side chains, was used as a structural and conformational probe of the hexadecameric phosphorylase kinase molecule (αβγδ). Brief cross-linking of nonactivated kinase caused formation of αβ dimers, with no cross-linking involving the γ and δ-subunits. When the kinase was first activated by autophosphorylation, significant amounts of α-δ dimers were also observed in addition to the α-β, demonstrating the occurrence of a conformational change in the α-subunits concomitant with activation. Both dimers resulted from intramolecular cross-linking. Because the COOH-terminal regions of the α-subunits are at the lobe tips of this bilobal kinase (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), the formation of zero length cross-linked α-δ dimers indicates that the polypeptide backbones of these subunits must stretch from the lobe tips to a more central location where they abut each other. Excess putrescine, as the amine substrate in place of endogenous Lys, was incorporated by transglutaminase predominately into the α-subunits of the kinase, with only slight modification of the β- and γ-subunits. Exogenous calmodulin (β'), an activator of the kinase with a binding site on the α-subunits (James, P., Cohen, P., and Carafoli, E. (1991) J. Biol. Chem. 266, 7097-7091), was a potent inhibitor of cross-linking. It also inhibited incorporation of putrescine into the α-subunits but stimulated incorporation into the β- and γ-subunits. Heparin, another activator of the kinase, had the same effects as exogenous calmodulin on cross-linking and putrescine incorporation, suggesting a commonality in the mechanism through which these two effectors activate the holoenzyme, including promoting a conformational change that increases the surface accessibility of target Gln residues on the catalytic γ-subunit.

Phosphorylase kinase, the first protein kinase discovered (1), is a structurally complex enzyme whose Ca2+-dependent phosphorylation of glycogen phosphorylase b couples glycogenolysis with contraction in skeletal muscle. Although its catalytic and regulatory properties have been extensively studied (reviewed in Refs. 2 and 3), much less is known about the structure of this large oligomer. The enzyme is a hexadecamer composed of four different subunits in the stoichiometry (αβγδ)4, with a total mass of 1.3 × 106 Da. The γ-subunit, which is catalytic (4), and the δ-subunit, which is regulatory (5), have masses of 45.7 and 16.7 kDa, respectively, as derived from their sequences (6, 7). The sequences of the regulatory, homologous α- and β-subunits have been deduced from their cDNA clones, with their respective masses calculated to be 138.4 and 125.2 kDa (8, 9).

With the ultimate goal of determining specific regions of individual subunits that are adjacent in the enzyme’s quaternary structure, we have initiated studies to characterize the actions of short and zero length cross-linkers on the hexadecameric holoenzyme. One such zero length cross-linker in transglutaminase from guinea pig liver, a Ca2+-dependent enzyme that catalyzes the formation of isopeptide bonds between proximal Gln and Lys side chains (reviewed in Ref. 10). This enzyme efficiently cross-linked the α- and β-subunits of phosphorylase kinase. The shortest cross-linker previously used to analyze the quaternary structure of phosphorylase kinase was 1,5-difluoro-2,4-dinitrobenzene, which spans a distance of 3-5 Å (11). This reagent acted as a conformational probe by preferentially forming cross-linked homodimers of β-subunits with the activated form of the enzyme. Consequently, we also compared the actions of transglutaminase on nonactivated and activated enzyme and observed that with this cross-linker, homodimers of α-subunits were observed in significant amounts only with the activated form of the kinase. All previously published studies have detected conformational changes associated with activation in only the β-subunits (11-13), although concomitant conformational changes in other subunits certainly must have occurred, undetected by the probe utilized. The occurrence of zero length cross-linked α-δ dimers, considered together with the known location of the COOH-terminal region of these subunits in the holoenzyme (14), also provides valuable information concerning the arrangement of the α-subunit polypeptide backbones within the individual lobes of the bilobal hexadecamer (15). Transglutaminase was also employed as a conformational probe by exploiting its ability to incorporate exogenous amines (16), in this case [3H]putrescine, into the Gln donor sites of the individual kinase subunits. Exogenous calmodulin, an activator of the kinase (17) with a known binding site on the α-subunit (18), altered incorporation of putrescine into three different subunits in a manner similar to that of another activator (19), but with unknown subunit binding site(s). A preliminary account of this work has been published (20).

EXPERIMENTAL PROCEDURES

Enzymes and Proteins—Phosphorylase kinase was isolated from fast twitch skeletal muscle of New Zealand White rabbits (21), dialyzed against Hepes (50 mM, pH 6.8), sucrose (10%), EDTA (0.2 mM), and stored frozen at -80 °C. All experiments described in this study were repeated a minimum of three times using three different phosphorylase kinase preparations. When autophosphorylated phosphorylase kinase was required for cross-linking studies, the phosphorylation was carried out at pH 7.5 in Hepes buffer for 30 min using...
the methodology of King et al. (22). The extent of phosphate incorporation with different kinase preparations ranged from 0.6–1.0 per β-subunit and 1.2–2.3 per α-subunit. Prior to its cross-linking, the auto- 
phosphorylated enzyme was purified by gel filtration over a Bio-Rad A-5m column (0.5 x 24 cm) developed with Hepes buffer (50 mM, pH 6.8), 0.2 mM EDTA, and 10% sucrose. Fractions that eluted at the void volume were pooled, buffer exchanged, and the enzyme was concentrated to 4.5 mg/ml by ultrafiltration at 3000 x g. Monoclonal antibodies (mAbs) against the individual subunits of phospho-
ylase kinase were generated in mice against the holoenzyme as antigen (14, 23). Detection conjugates for immunoblots were from Southern Biotechnology. Phosphorylase b was isolated from rabbit skel-
etal muscle (24), and residual AMP was adsorbed with activated char-
coal (Sigma, C-4386). Bovine brain calmodulin (P-2277), α-casein (C-8032), and bovine serum albumin (A-9647) were from Sigma. Guinea pig liver transglutaminase was purchased from Sigma (T-5398) and was either used directly or affinity purified using the procedure of Lee et al. (25).

Assays—The activity of phosphorylase kinase was determined following the incorporation of 32P from γ-32PATP into phosphoribosylase b at 30 °C using phosphocellulose strips (26). Final concentrations in the assay mixture were as follows: phosphorylase kinase, 0.7 μg/ml at pH 6.8 or 0.07 μg/ml at pH 8.2; buffer, 50 mM Tris, 50 mM β-glyceropha-
phate, pH 6.8 or pH 8.2; phosphorylase b, 6.0 mg/ml; EDTA, 0.1 mM; CaCl2, 0.2 mM; β-mercaptoethanol, 13 mM; γ-32P-ATP (Dupont NEN), 1.5 μM, 0.17 Ci/mM; MgCl2, 0.1 M; and sucrose, 2–3%.

The activity of transglutaminase was determined at 30 °C using the incorporation of [14C]putrescine (DuPont NEN) either into α-casein (2.7 mg/ml) using the filter paper assay described by Lorand et al. (27) or into phosphorylase kinase (0.54 mg/ml), whose subunits were subsequently resolved by SDS-PAGE (22). The incorporation of [14C]pu-
trescine into the kinase subunits was quenched by dilution of an aliquot of the assay mixture into an equivalent volume of SDS buffer (0.135 × 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 were run on SDS-polyacrylamide gradient gels (2–20%) or 6% gels (28) and stained with Coomassie Blue. All gels were destained with 250 ml of 30% H2O2 for 80 °C. The samples and blanks, which contained equivalent amounts of polyacryl-
amide and H2O2, were diluted with 7 ml of Ecoscint scintillation mixture (ICN), and the 14C content was determined. In addition to the protein targets, the assay mixtures for incorporation of putrescine included transglutaminase (30 μg/ml) when used directly from Sigma or 5 units/ml of affinity-purified enzyme, where 1 unit is defined as the amount of transglutaminase necessary to catalyze the incorporation of 1 n mole of [14C]putrescine into 1 mol of protein per min. [14C]putrescine (360 μCi at 221 μCi/mmol), buffer (41.5 mM Tris, pH 7.5), CaCl2 (1.25 mM), and EDTA (1.0 mM).

The cross-linking of phosphorylase kinase was carried out at 30 °C with a free Ca2+ concentration of 0.17 mM; and sucrose, 2–3%.

Identification of Cross-linked Species—Apparent molecular weights of the cross-linked species were determined from comparison with the migration of cross-linked phosphorylase b (Sigma; monomer Mr = 97,400) on 6% continuous polyacrylamide slab gels (with a 4% stacking gel) using the buffer system of Davies and Stark (29). Lower molecular mass species were evaluated using the following protein standards: bovine a-casein, 29,000; p-agarase, 116,000; phosphorylase b, 97,400; bovine plasma albumin, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 29 kDa (all from Sigma) on 2–20% linear gradient PAGE (28).

Subunit composition of cross-linked species was analyzed by Western blotting as previously described using subunit-specific antibodies (14, 23) and by the time-dependent disappearance of individual subunits

1. The abbreviations used are: mAb, monoclonal antibody; PAGE, poly-
acrylamide gel electrophoresis.
Western blots of the cross-linked species. A, native phosphorylase kinase was incubated with transglutaminase as described under "Experimental Procedures." At the indicated times, aliquots of the solution were removed, subjected to SDS-PAGE (6% polyacrylamide), and stained for protein to determine the extent of cross-linking. At the indicated times, aliquots (1.5 μg of protein) were subjected to SDS-PAGE, electroblotted onto nitrocellulose, and cross-reacted with anti-α, anti-β, and anti-γ mAbs. Band 4, α-β heterodimer; bands 1 and 2, more extensively cross-linked species containing α- and β-subunits in indeterminate stoichiometry.

FIG. 1. Time-dependent cross-linking of phosphorylase kinase and Western blots of the cross-linked species. A, native phosphorylase kinase was incubated with transglutaminase as described under "Experimental Procedures." At the indicated times, aliquots of the solution were removed, subjected to SDS-PAGE (6% polyacrylamide), and stained for protein to determine the extent of cross-linking. At the indicated times, aliquots (1.5 μg of protein) were subjected to SDS-PAGE, electroblotted onto nitrocellulose, and cross-reacted with anti-α, anti-β, and anti-γ mAbs. Band 4, α-β heterodimer; bands 1 and 2, more extensively cross-linked species containing α- and β-subunits in indeterminate stoichiometry.

FIG. 2. Time-dependent cross-linking of autophosphorylated phosphorylase kinase and Western blots of the cross-linked species. Autophosphorylation of phosphorylase kinase was carried out as described under "Experimental Procedures." The extent of phosphorylation was 2.3 mol of P/mol of α and 0.97 mol of P/mol of β. The observed phosphate content of bands 3 and 4 (α-α and α-β dimers, respectively) were 2.9 mol of P/mol of dimer and 1.5 mol of P/mol of dimer, respectively. Panels A (6% polyacrylamide gel stained for protein) and B (Western blot) were generated as described under the legend for Fig. 1. Bands 1 and 2 are as described under Fig. 1.

measured at 260 kDa, or 1.4% below the calculated mass of an α-β dimer. No attempt was made to estimate the masses of the very heavy bands 1 and 2 because of their limited migration into the gel; however, as is discussed below, they appear to contain only α- and β-subunits.

The subunit composition of the cross-linked complexes was further investigated by immunoblotting with subunit-specific monoclonal antibodies against the α-, β-, and γ-subunits. Band 3, the putative α-α dimer (based on mass), cross-reacted only with the anti-α mAB (Fig. 2B), thus confirming its identity. Using this mAB, the α-α dimer could also be observed in trace amounts with the nonactivated enzyme (Fig. 1B). The mass of band 4 also accurately predicted its composition as an α-β dimer in that it cross-reacted equally well with both the anti-α and anti-β mAbs (Figs. 1B and 2B). Bands 1 and 2 also contained only α- and β-subunits (Figs. 1B and 2B) but in an indeterminate stoichiometry. Although a mAB specific for the δ-subunit (calmodulin) was not used in these studies, we nevertheless conclude that cross-linked complexes containing significant amounts of the δ-subunit were not formed. This conclusion is based in part on the constant amount of δ-subunit throughout cross-linking as measured by densitometry. In addition, incorporation experiments of the type described below indicated that isolated calmodulin is not a substrate for transglutaminase under the conditions used herein (data not shown).

To further examine the subunit composition of the cross-linked species, we compared the specific radioactivities of bands 3 and 4 with those of the α- and β-subunits after the cross-linking of enzyme that had been previously autophosphorylated using [32P]ATP. In the non-cross-linked activated control enzyme, the 32P content of the α-subunit was 2.3 times that of the β-subunit. The specific radioactivity of band 4, the α-β dimer, was found to be within 6.2% of the calculated value for this dimer. The specific radioactivity of band 3, the α-α dimer, was 21% greater than calculated. However, because the specific radioactivity of the α-subunit was 2.3 times greater than that of the β-subunit and because neither the γ- nor δ-subunits are phosphorylated, any cross-linked complex that contained β-, γ-, or δ-subunits would have a specific radioactivity substantially less than that of the α-subunit and certainly not greater. Either the protein content of band 3 measured by densitometry is a slight underestimate of the actual value or formation of cross-linked α-α dimers preferentially occurs with those subunits that are phosphorylated to the greatest extent. The specific radioactivities of bands 1 and 2 were inconclusive regarding the stoichiometry of the α- and β-subunits in those bands.

In summary, based on subunit disappearance, apparent molecular weights of cross-linked species, their cross-reactivity with subunit-specific mAbs, and their specific radioactivities, we conclude that the major species that result from the cross-linking of activated phosphorylase kinase by transglutaminase are α-β and α-α dimers. In contrast, with nonactivated enzyme the major cross-linked species is the α-β dimer, with only trace amounts of the α-α dimer being generated.

Molecularity of Cross-linking—To fully interpret the zero length cross-linked complexes in terms of the subunit topography of the holoenzyme, it was first necessary to establish that
the cross-linking was intramolecular, i.e. within a kinase hexadecamer. Nonactivated cross-linked enzyme was passed over a Sepharose 6B gel filtration column, and its elution profile was compared with that of non-cross-linked control enzyme (Fig. 3A). Two protein peaks were observed in the elution profile of the nonactivated cross-linked enzyme: a minor peak (fractions 23–25 containing 20% of the total protein) that eluted in the void volume and a major peak (fractions 27–36 containing the remaining 80% of the protein) that coeluted with non-cross-linked control enzyme. Fractions from both elution peaks of the cross-linked enzyme were analyzed for covalently cross-linked species and were present throughout both peaks. Moreover, the densitometric ratio of band 4 to the major cross-linked species and was present throughout the major peak, that which coeluted with the native enzyme (data not shown). Because at high concentrations phosphorylase kinase has a strong tendency to aggregate (32), the major breakthrough peak in Fig. 3B could represent aggregated enzyme, intermolecularly cross-linked enzyme, or a combination of both. However, analysis of the protein band patterns in Fig. 3B suggests that it predominantly represents aggregated enzyme. These results indicate that transglutaminase catalyzes the zero length cross-linking of α- and β-subunits within a single molecule of the hexadecameric, nonactivated holoenzyme.

The molecularity of cross-linking of the autophosphorylated, activated form of the enzyme was analyzed in a similar fashion. Because autophosphorylation requires Ca²⁺ and Mg²⁺ ions, which synergistically promote aggregation (33), the autophosphorylated enzyme was first purified by gel filtration, and only those fractions that coeluted with native, nonactivated enzyme were subjected to subsequent cross-linking. As was observed with the nonactivated enzyme, two protein peaks resulted from gel filtration of the cross-linked autophosphorylated enzyme (Fig. 3B): a peak that eluted in the void volume (fractions 20–24 containing 52% of the protein) and a second peak (fractions 25–32 containing 48% of the protein) that coeluted with non-cross-linked native control enzyme. Bands 3 and 4 (the α-α and α-β dimers, respectively) were the major cross-linked species and were present throughout both peaks (Fig. 3C). An additional band (band 5, Fig. 3C) with an apparent molecular mass of 205 kDa was also observed, but only after passage of the cross-linked enzyme over the final Sepharose column. Thus, it is likely a proteolytic fragment of an originally heavier cross-linked species. Both aggregation and proteolysis presented greater technical difficulties with the autophosphorylated enzyme, presumably due in part to the necessary preincubation with divalent cations and the extra manipulations of desalting and concentrating. Nevertheless, from analysis of the bands in Fig. 3C, it is clear that the α-α dimer, like α-β, also results from intramolecular cross-linking within a hexadecamer.

Inhibition of Cross-linking of the Nonactivated Holoenzyme—Transglutaminase brings about the cross-linking of proteins by catalyzing acyl transfer between Gln and Lys side chains; competitive inhibition of this cross-linking occurs in the presence of exogenous primary amines (such as putrescine), which are incorporated in the place of Lys into Gln substrate sites on the target protein (16). Although putrescine did inhibit the cross-linking of the α- and β-subunits of phosphorylase kinase (Fig. 4), total inhibition required a concentration of approximately 50 mM. This high concentration required for inhibition is consistent with an intramolecular mechanism of cross-linking in which the exogenous putrescine does not effectively compete with the endogenous lysine substrate(s), presumably due to steric protection provided by the bound transglutaminase. When dansylcadaverine was used as the exogenous amine in place of putrescine, similarly high concentrations were re-

![Fig. 3. Gel filtration of cross-linked and native phosphorylase kinase and SDS-PAGE of the fractionates. A. Coomassie-stained gel of fractions of cross-linked nonactivated enzyme from the breakthrough and major absorbance peaks (panel B) analyzed by SDS-PAGE (2–20% polyacrylamide). Band 4 is the α-β cross-linked dimer. Listed from top to bottom, the far right lane in the gel contains 5 μg each of 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) as mass standards. B, native (●), cross-linked nonactivated (○) (1-ml sample containing 4 mg), and cross-linked autophosphorylated phosphorylase kinase (■) (1-ml sample containing 2 mg) passed over a Sepharose 6B column developed with Hapes buffer (50 mM, pH 6.8, 0.2 mM EDTA, and 10% sucrose). C. Coomassie-stained gel of fractions of cross-linked autophosphorylated enzyme (■) from the breakthrough peak and the peak that comigrated with native enzyme (panel B) analyzed by SDS-PAGE (4–15% polyacrylamide). Prior to cross-linking, native kinase was autophosphorylated (2.17 mol of P/mol of α and 0.72 mol of P/mol of β) as described under "Experimental Procedures," which resulted in a 10.5-fold activation over the nonactivated control enzyme at pH 6.8. The autophosphorylated enzyme was purified by gel filtration, cross-linked, and passed over the Sepharose 6B column as described under B. Band 3 (α-α dimer, Mₙ = 270,000) cross-reacted only with α-subunit-specific mAb 157 (data not shown). Band 4 (α-β dimer, Mₙ = 260,000) cross-reacted with both α- and β-subunit-specific mAbs. Band 5 is a proteolytic fragment (Mₙ = 205,000) that was not present after cross-linking but only after gel filtration chromatography. The far right lane contains equivalent amounts of the Mₙ standards described under A.](image-url)
Zero Length Cross-linking of Phosphorylase Kinase

**FIG. 4. Inhibition of cross-linking.** Native phosphorylase kinase was cross-linked with transglutaminase at 0 min (lanes 1, 2, 3, 5, and 7) and 20 min (lanes 2, 4, 6, and 8) in the absence of inhibitors (lanes 1 and 2) or in the presence of putrescine (4.0 mM) (lanes 3 and 4), heparin (0.5 mg/ml) (lanes 5 and 6), and calmodulin (equimolar with αβγδ isotopes) (lanes 7 and 8). Denaturation of the Coomassie-stained 2-20% polyacrylamide gel showed that the 4 mol wt putrescine to cause a 30% decrease in the amount of band 4 (α-β dimer) after 20 min of cross-linking. The δ-subunit, which is absent in the lanes containing heparin, could be observed only if the SDS-denaturation buffer was added directly to the cross-linking mixture rather than after cross-linking aliquots being removed and added to the denaturation buffer.

required to inhibit cross-linking (data not shown).

In light of the preferential cross-linking of the α-subunit in enzyme activated via autophosphorylation (Fig. 2A), we asked whether exogenous calmodulin (8), an activator of the kinase with a known binding site on the α-subunit (18), had any effect on cross-linking. When nonactivated phosphorylase kinase was incubated with 0.25 mM free Ca2+ and calmodulin (equimolar with respect to αβγδ isotopes), cross-linking was inhibited by 90% (Fig. 4, lanes 5 and 6). Because there is a consensus sequence for the binding of heparin (34, 35) (another activator of phosphorylase kinase (19)) within the reported calmodulin binding site on the α-subunit of the holoenzyme (18), we asked whether heparin might also inhibit cross-linking. At a concentration of 0.5 mg/ml, heparin inhibited cross-linking by 80% (Fig. 4, lanes 7 and 8). Heparin also promoted dissociation of the δ-subunit, corroborating a previous report of this effect of the polyanion (36). To establish that calmodulin and heparin were exerting their inhibitory effects through the kinase and not through transglutaminase, the enzymatic activity of transglutaminase on alternative substrates was determined in the presence of the two effectors. Under the identical conditions at which they inhibited cross-linking, neither calmodulin nor heparin affected the incorporation of [3H]putrescine into α-casein by transglutaminase. These results indicate that calmodulin and heparin inhibit cross-linking by interacting with phosphorylase kinase.

**DISCUSSION**

Cross-linking of nonactivated phosphorylase kinase with transglutaminase resulted in the formation of an α-β dimer, whose identity was based on the equivalent time-dependent disappearance of both the α- and β-subunits, its apparent molecular mass, and its cross-reactivity with subunit-specific antibodies. Cross-linking of kinase that had been previously activated via autophosphorylation resulted in the preferential loss of the α-subunit, with formation of significant amounts of both α-α and α-β dimers. The identities of these dimers were established by the methods described above as well as by their specific radioactivities. Based on their presence in cross-linked enzyme that coeluted in gel filtration with non-cross-linked sample, both the α-β and α-α dimers were deemed to result from intramolecular cross-linking.

From the difference in cross-linking patterns between the nonactivated and autoactivated enzyme, we conclude that activation by autophosphorylation results in a conformational change characterized by either the translation of two α-subunits to abut each other or the unmasking of a Lys or Gln residue on one or both of two α-subunits that were already abutted. Although phosphorylation of the α-subunit has been unequivocally shown to regulate phosphorylase kinase activity (provided the β-subunit is first phosphorylated (37)), we are unaware of any previous reports in the literature of a conformational change in the α-subunit that could be associated with activation. In corroboration of such a conformational change, employing carboxymethylation or chymotrypsin as conformational probes, it has recently been observed in our laboratory that ADP, glycogen, and heparin, all activators of phosphorylase kinase, also cause significant changes in the rate at which the α-subunit is either carboxymethylated or proteolyzed (35). Thus, even though previous probes have detected conformational changes in only the β-subunit upon activation (11-13), these more recently used conformational probes, including transglutaminase, detect changes in the α-subunit as well.

The formation of zero length, cross-linked, intramolecular α-α dimers provides useful information pertaining to the location of the α-subunits within the overall structure of the hexadecameric holoenzyme. Phosphorylase kinase is composed of...
two equivalent (αβγδ), lobes bridged back-to-back (15), an arrangement in which the probable αβγδ-elongated protomers are packed head-to-head in a pseudo-tetrahedron (38). Immunoelectron microscopy has shown an epitope proximal to the carboxyl terminus of the α-subunit to be symmetrically distributed at the opposing tips of each lobe of the nonactivated enzyme (14). However, the results with activated enzyme in this current study indicate that the polypeptide backbones of the α-subunits must stretch from the lobe tips to a more central location where they abut each other, thus allowing cross-linking. This conclusion regarding the position of the polypeptide backbones assumed, of course, that the α-subunits do not undergo a large change in their location within the quaternary structure of the holoenzyme upon its activation. We presume that the cross-linked α-α dimers are intrasubunit, i.e. that they are formed from the two α-subunits that occur within a single lobe of the bilobal hexadecamer (14), where each lobe seems to represent a dimer of αβγδ protomers (Ref. 38 and references therein). This hypothesis is based on evidence that suggests that the α-subunits are not extensively involved in the interlocal isologous interactions of the tetrahedral oligomer. For instance, the preferential and extensive hydrolysis of the α-subunit of the holoenzyme by chymotrypsin results in no discernible change in the enzyme's overall quaternary structure (15), i.e. the α-subunits do not appear to be extensively involved in the association of the two octameric lobes with each other. In support of this notion, αβγδ trimers, which are 69% α by mass, have a shape that corresponds to the contours of the outer portions of the enzyme's lobes, the side opposite the interlocal contact regions (39). Furthermore, a relatively exposed surface location for the α-subunit is suggested by its extreme sensitivity to a large variety of proteases (12); certainly the regions of this subunit that interact with transglutaminase and exogenous calmodulin are surface exposed.

Cross-linking by dimethylsuberimidate of the holoenzyme with exogenous calmodulin (δ') implicated both the α- and β-subunits in the binding of δ', although the cross-linking yield was relatively low (3-4%) (40). More recently, an equimolar concentration of derivatized calmodulin was shown to modify only the α-subunit of the holoenzyme (18). The modification occurred within residues 1080-1114 of the α-subunit, which coincides with the existence of a known calmodulin-binding peptide containing residues 1070-1093 of that subunit (41). Other evidence implicating the α-subunit in the binding of exogenous calmodulin is the finding that selective proteolysis of the α-subunit blocks the binding of the enzyme to immobilized calmodulin (40). Moreover, exogenous calmodulin stimulates the activity of αγδ, but not γδ, complexes (42). Finally, the α'-containing isozyme of phosphorylase kinase, which is found in cardiac, uterine, and slow twitch skeletal muscle (43), interacts quite differently with calmodulin than does the α-containing isozyme; the activity of the α' holoenzyme is neither stimulated by exogenous calmodulin nor does the enzyme bind to immobilized calmodulin at standard Ca2+ concentrations (44). The α' isoform arises from alternative RNA splicing resulting in an internal deletion of 59 residues, which, paradoxically, does not correspond to the region modified by the derivatized calmodulin described above but to residues 654-712 (43). However, with the free α-subunit (18) and with proteolytic digests (45), portions of this region of α deleted in α' have also been shown to interact with calmodulin. In summary, even though participation of the β-subunit cannot be ruled out and identification of the involved regions of the α-subunit may be incomplete, there is nevertheless a seemingly overwhelming body of evidence indicating a direct involvement of the α-subunit in the binding of exogenous calmodulin by the phosphorylase kinase holoenzyme. That exogenous calmodulin inhibits transglutaminase-catalyzed cross-linking and incorporation of exogenous amines into only the α-subunit is certainly consistent with a binding site for δ' being on the α-subunit. If this inhibition by calmodulin is due to a steric effect, there are 5 Glu residues (1065, 1067, 1069, 1087, and 1094) within or near the known calmodulin-binding region of the α-subunit that could be considered as potential substrates for transglutaminase. The distribution of charged side chains adjacent to accessible glutamines appears to govern the substrate specificity of transglutaminase (46) and would apparently eliminate Glu-1067 and Glu-1087 as potential substrates in this region. Unlike the case with the α-subunit, calmodulin actually stimulates incorporation of putrescine into the catalytic γ-subunit. These opposite effects of calmodulin are consistent with a conformational change in the γ-subunit induced indirectly by the binding of calmodulin to the α-subunit. An increased accessibility of a region of the γ-subunit in response to the binding of exogenous calmodulin to the holoenzyme has also been observed through utilization of a monoclonal antibody (mAb 79) against the γ-subunit (23). Mapping of the γ-subunit labeled by putrescine in the presence and absence of calmodulin should allow identification of the location at which the activator-induced conformational change modulates the accessible solvent interface of the catalytic subunit.

There were noteworthy similarities between the influence of heparin and calmodulin on the interaction of transglutaminase with phosphorylase kinase. Both effectors of the kinase inhibited cross-linking and the incorporation of putrescine into the α-subunit while stimulating incorporation into the β- and γ-subunits. These similarities suggest a commonality in the mechanism through which the two activators interact with the holoenzyme. In this regard, it has been noted (35) that a consensus sequence for heparin-binding (34) occurs at the amino terminus of the known calmodulin-binding region of the α-subunit at residues 1070-1073. Heparin also protects the α-subunit from being preferentially hydrolyzed by chymotrypsin (35). Thus, calmodulin and heparin may exert their similar effects through interacting with the same region of the regulatory α-subunit. The cumulative effects of heparin on phosphorylase kinase are, however, more complex than those of calmodulin, e.g. stimulation of the γ-calmodulin complex (47) and dissociation of the δ-subunit (Ref. 36 and this study). The latter effects of heparin are consistent with the existence of additional binding sites for the effector, especially on the γ-subunit. In support of this idea, a monoclonal antibody generated against the free γ-subunit (mAb 79) shows decreased binding to the isolated γ-subunit and to the holoenzyme in the presence of heparin.2

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