Effector-sensitive cross-linking of phosphorylase *b* kinase by the novel cross-linker 4-phenyl-1,2,4-triazoline-3,5-dione

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The dienophile 4-phenyl-1,2,4-triazoline-3,5-dione (PTD) was identified as a novel protein cross-linker, and utilized as a conformational probe of phosphorylase *b* kinase (PhK), a hexadecameric enzyme with the subunit composition ($\alpha\beta\gamma\delta$)₄. In its reaction with this enzyme, PTD produced five major cross-linked conjugates as resolved by denaturing gel electrophoresis: $\alpha\beta$, $\beta\gamma\gamma$, $\alpha\gamma$ and a doublet of differently migrating homodimers, $\beta\beta_1$ and $\beta\beta_2$. Cross-linking in the presence of six different activators of the kinase targeted to its various subunits caused substantial changes in the amounts of three of the conjugates. The formation of $\alpha\gamma$ was increased by all of the activators but the largest enhancement was caused by exogenous Ca²⁺/calmodulin. All except one of the activators decreased the amount of $\beta\gamma\gamma$

formed, with Mg²⁺ having the greatest effect, and all except two increased the amount of $\beta\beta_1$, with Mg²⁺ again having the largest influence. From the overall similarity of the changes in cross-linking by PTD induced by the various activators, we conclude that, even though they are targeted to different sites and subunits, they induce activated conformations of PhK that have certain structural features in common. Regarding the mechanism of cross-linking by PTD, its reaction with a model nucleophile suggests that its initial reaction with a side chain nucleophile of PhK involves a 1,4-conjugate addition to form a urazole adduct, with the secondary cross-linking reaction occurring through an as yet unknown pathway.

INTRODUCTION

Phosphorylase *b* kinase (PhK) is a regulatory enzyme in the cascade activation of glycogenolysis that integrates neural, hormonal and metabolic stimuli through allosteric and covalent modification sites on its three regulatory subunits, which account for 86% of its mass (reviewed in [1,2]). PhK from skeletal muscle is a hexadecamer of subunit composition ($\alpha\beta\gamma\delta$)₄ [3]. The activity of its catalytic γ subunit is tightly controlled by its regulatory α , β and δ subunits, with the last being intrinsic calmodulin (CaM) [3]. Although it is clear that the alteration of quaternary interactions is crucial in controlling the activity of the catalytic subunit of PhK, little is known concerning the actual changes in those interactions that are induced by activators of the enzyme.

Because of its large molecular mass, 1.3 MDa, many common techniques used to examine protein structure are ineffective or not applicable to PhK. Chemical cross-linking as a tool for probing subunit interactions has been a powerful technique with other systems, but with PhK its four copies of four different subunits yields such a large number of theoretical permutations that an analysis of subunit composition and stoichiometry of cross-linked conjugates is problematic, at least when based predominantly on molecular mass. The recent availability, however, of a panel of subunit-specific monoclonal antibodies (mAbs) against PhK's four different subunits [4–6] now allows an unequivocal analysis of most cross-linked conjugates of intermediate molecular mass. As a result, we have initiated a search for cross-linkers capable of detecting changes in the quaternary structure of PhK that are induced by its activators.

Here we identify a compound, 4-phenyl-1,2,4-triazoline-3,5-dione (PTD; compound 1 of Scheme 1 below), that, although not

previously used as a chemical cross-linker of proteins, cross-links PhK in a manner that is sensitive to activators. PTD is a commonly used dienophile in Diels–Alder reactions, although it has been employed in the oxidation of alcohols to carbonyl compounds [7] and in the incorporation of C–H bonds in conjugated dienes [8]. With PhK, PTD forms five major cross-linked conjugates: $\alpha\beta$, $\beta\gamma\gamma$, $\alpha\gamma$ and a doublet of differently migrating homodimers of the β subunit ($\beta\beta_1$ and $\beta\beta_2$). The formation of three of these conjugates is influenced similarly by various activators of PhK. We have found that the reaction of PTD with a primary alkyl amine occurs with nucleophilic attack at a double-bonded nitrogen within the triazoline ring, although the exact cross-linking mechanism is as yet unknown.

EXPERIMENTAL

Enzymes and proteins

PhK was isolated from fast-twitch skeletal muscle of New Zealand White rabbits [9], dialysed against 50 mM Hepes (pH 6.8)/10% (w/v) sucrose/0.2 mM EDTA and either used immediately or stored frozen at –80 °C. Bovine brain CaM was isolated as previously described [10]. The concentrations of PhK and CaM were determined spectrophotometrically from their respective absorbance indices [11,12]. The mAbs against the α , β and γ subunits of PhK were produced in mice by using the holoenzyme as antigen and have been characterized previously [5,6]. Anti-CaM mAb, which recognizes an epitope C-terminal to metal-binding site IV [4], was purchased from Signal Transduction Laboratories. All other detection conjugates were purchased from Southern Biotechnology.

Abbreviations used: CaM, calmodulin; mAb, monoclonal antibody; PhK, phosphorylase *b* kinase; PTD, 4-phenyl-1,2,4-triazoline-3,5-dione.

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Cross-linking

All experiments described in this study were repeated a minimum of three times with at least two different PhK preparations. Before cross-linking, PhK was preincubated for 2 min at 30 °C and the cross-linking reactions were initiated with PTD and performed at 30 °C for 2.5 min, which was found to be the optimal time for maximal formation of intermediate-sized conjugates. The final concentrations in the standard cross-linking reaction were: 1.4–1.73 μ M $\alpha\beta\gamma\delta$ protomer of PhK, 50 mM Hepes, pH 8.2, 1.0 mM EDTA and 2 mM PTD (stock dissolved in acetone). Under these conditions, varying the concentration of PTD from 250 μ M to 5 mM caused little change in the cross-linking pattern (results not shown). Reactions were quenched by dilution with an equal volume of SDS buffer [0.125 M Tris (pH 6.8)/20% (v/v) glycerol/5% (v/v) 2-mercaptoethanol/4% (w/v) SDS]. After being heated for 5 min at 90 °C, samples (18 μ g per lane) were run on SDS/4–15% (w/v) polyacrylamide gradient gels [13], stained with Coomassie Blue and destained in solutions of 40% (v/v) methanol/10% (v/v) acetic acid (2 h) and 7% (v/v) acetic acid/4% (v/v) methanol (15 h). Integrated optical densities of the protein bands were determined on a Bio Image whole-band analyser. Under the standard cross-linking conditions, the final concentration of acetone carried along with the PTD was 1% (v/v); in separate assays, this amount of acetone caused no change in the activity of PhK, as determined by 32 P incorporation during autophosphorylation (results not shown).

Although in this study we have focused on PhK, we have found in preliminary experiments that other oligomeric proteins are cross-linked by PTD under the standard conditions above.

Identification of cross-linked species

Apparent molecular masses of the cross-linked conjugates were determined by comparison with the migration of protein standards [myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), fructose-6-phosphate kinase (84 kDa), bovine plasma albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa) and glyceraldehyde-3-phosphate dehydrogenase (36 kDa), all purchased from Sigma] on SDS/4–15% (w/v, linear gradient) polyacrylamide gels. The subunit composition of each cross-linked species was determined by Western blotting with subunit-specific mAbs as previously described [5,6]. For determination of the subunit stoichiometries of cross-linked conjugates, the following masses were used: α , 138.4 kDa [14]; β , 125.2 kDa [15]; γ , 44.7 kDa [16]; δ , 16.7 kDa [17].

RESULTS

Characterization of cross-linking of PhK by PTD

A slightly alkaline pH brings about conformational changes in PhK [6], greatly stimulates its activity [18] and could potentially cause the deprotonation of nucleophilic side chains capable of reacting with PTD. Consequently, we first studied the pH dependence of cross-linking in the pH range 6.45–8.2 (Figure 1). The subunit composition and stoichiometry of each cross-linked conjugate was determined from its apparent molecular mass and cross-reactivity against subunit-specific mAbs (Figure 2). At the lowest pH values, the predominant conjugates formed were an $\alpha\beta$ dimer (theoretical 264 kDa, 3.0% error) and a $\beta\gamma\gamma$ trimer (theoretical 215 kDa, –3.2% error). At higher pH values, additional conjugates were observed and there were increased

Figure 1 pH dependence of PhK cross-linking by PTD

PhK was cross-linked at the indicated pH values as described in the Experimental section. Conjugates of PhK are labelled as determined by apparent molecular mass and by Western blots with subunit-specific mAbs (Figure 2).

amounts of all conjugates (Figure 1). At the highest pH value tested (pH 8.2), five major conjugates were formed. Besides the previously mentioned $\alpha\beta$ and $\beta\gamma\gamma$ complexes, there was an $\alpha\gamma$ dimer (theoretical 183 kDa, –1.6% error) and a doublet of $\beta\beta$ dimers, $\beta\beta_1$ and $\beta\beta_2$, with the faster-migrating $\beta\beta_2$ (theoretical 250 kDa, 1.2% error) being predominant. Previously, 1,5-difluoro-2,4-dinitrobenzene had been noted to form $\beta\beta$ dimers with different mobilities in SDS/PAGE [19], presumably resulting from combinations of intra- and inter-subunit cross-linking. In amount formed, the conjugate least influenced by changes in pH was the $\alpha\beta$ dimer, whereas that most influenced was the $\alpha\gamma$

Figure 2 Identification of cross-linked species by Western blots

PhK was cross-linked by PTD before immunoblotting with the indicated anti-subunit mAbs as described in the Experimental section. In each panel, C is control uncross-linked PhK; PTD refers to cross-linked PhK. Conjugates are labelled that correspond to the most prominent bands identified by SDS/PAGE analysis. A small amount of a non-cross-linked α -cross-reactive band corresponding to a frequently observed degradation product of α resulting from storage [11,35] is indicated by an asterisk. In the anti- β blot the putative $\beta\delta$ dimer is marked with +. Additionally, as seen in the appropriate control lanes, there was a slight, non-specific, reciprocal cross-reactivity of the anti- β and anti- γ mAbs against those two subunits.

Figure 3 Effector-induced changes in the cross-linking of PhK by PTD

Under otherwise standard conditions, PhK was cross-linked in the presence of the following effectors: 1.2 mM Ca^{2+} (200 μM Ca^{2+} in excess of chelator), 1.2 mM $\text{Ca}^{2+}/1.4 \mu\text{M}$ CaM (1 CaM/1 $\alpha\beta\gamma\delta$ protomer), 10 mM magnesium acetate, 100 μM ADP, 100 μM GDP or 0.5 mg/ml glycogen. After their separation by SDS/PAGE, integrated optical densities were determined for each conjugate with a Bio Image whole-band analyser. Bars represent the percentage change in conjugate formation in the presence of the indicated effector compared with its absence, where the absence of bars indicates no change. No significant change was observed in $\alpha\beta$ or $\beta\beta_2$ formation in the presence of any of the effectors.

dimer. Because it gave the most conjugates and in the largest amounts, pH 8.2 was chosen as standard for cross-linking.

In addition to the above five conjugates observed in both SDS/PAGE gels and Western blots, a band that cross-reacted with anti- β and had a mass corresponding to a $\beta\delta$ dimer (theoretical 142 kDa, 8.3 % error) was observed in Western blots (denoted by † in Figure 2). Although this band did not cross-react with the anti-CaM mAb, no other combination of subunits except $\beta\delta$ could account for its observed mass. A complex of the same mass and cross-reactivity, and identified as $\beta\delta$, has also been observed during the cross-linking of PhK by *m*-maleimido-benzoyl-*N*-hydroxysuccinimide ester, where it was assumed that the epitope on the small δ subunit recognized by the anti-CaM mAb was masked by cross-linking [20].

Activator-induced perturbations in cross-linking

There are numerous known activators of PhK that exert their effects through allosteric sites, virtually all of which are thought to be on the enzyme's three different regulatory subunits. We examined whether cross-linking by PTD could be used as a probe to detect similarities and differences in the activated conformations induced by various activators targeted to different sites on the $(\alpha\beta\gamma\delta)_4$ holoenzyme. Of the five cross-linked conjugates produced by PTD under standard conditions, the formation of three ($\beta\beta_1$, $\beta\gamma\gamma$ and $\alpha\gamma$) was influenced by most or all of the activators tested; none of the activators significantly altered the formation of either $\alpha\beta$ or $\beta\beta_2$.

Ca^{2+} is arguably the most fundamental activator of PhK, in that the conformations induced by all other activators still retain Ca^{2+} -dependent catalytic activity. The effects of Ca^{2+} are mediated through the endogenous CaM of PhK [21,22], its δ subunit [3]. The inclusion of saturating Ca^{2+} ion concentrations in the cross-linking produced a large increase in $\alpha\gamma$ formation (+56 %) and a slight concomitant decrease in $\beta\gamma\gamma$ (−18 %) (Figure 3). That

Scheme 1 Reaction of PTD (compound 1) with isopropylamine

the two conjugates influenced by Ca^{2+} both contained the γ subunit is not surprising, considering that the δ subunit has been shown to interact directly with γ [22–24].

Besides the effect of Ca^{2+} through endogenous CaM (δ), PhK activity is further stimulated by the Ca^{2+} -dependent binding of exogenous CaM, which is termed δ' . The binding site for δ'/Ca^{2+} is distinct, apparently being composed of residues from both the α and β subunits [24–26]. The effects of δ'/Ca^{2+} on $\alpha\gamma$ and $\beta\gamma\gamma$ formation were similar to those caused by Ca^{2+} alone, but nearly double in size (+107 % in $\alpha\gamma$ and −28 % in $\beta\gamma\gamma$; Figure 3). In addition, δ'/Ca^{2+} caused a slight increase in the formation of $\beta\beta_1$, which was not observed with Ca^{2+} alone (Figure 3). It should be noted that the effects of δ'/Ca^{2+} are in addition to those of Ca^{2+} alone, in that Ca^{2+} is present in sufficient excess to saturate its binding sites on the δ subunit.

The activity of PhK is stimulated by Mg^{2+} ions in excess of nucleotide; this effect is mediated, at least in part, by the binding of this cation to the γ subunit [27,28]. With respect to its reciprocal effects on the formation of $\alpha\gamma$ (+51 %) and $\beta\gamma\gamma$ (−56 %), the influence of Mg^{2+} was qualitatively similar to that of Ca^{2+} and δ'/Ca^{2+} , but was considerably more pronounced with $\beta\gamma\gamma$ (Figure 3). Even more pronounced was the 128 % increase in $\beta\beta_1$ formation caused by Mg^{2+} (Figure 3); this was the largest relative change in any complex brought about by any of the effectors.

The purine nucleoside diphosphates ADP and GDP are allosteric activators of PhK that most probably exert their effects through binding to the β subunit [29,30]. Their greatest effect on cross-linking was to increase modestly the formation of $\alpha\gamma$ (ADP by +32 % and GDP by +33 %; Figure 3). The smaller effects of the two nucleotides were somewhat different in that ADP caused a slight increase in $\beta\gamma\gamma$ and GDP in $\beta\beta_1$ (Figure 3).

Glycogen is among the least well characterized of PhK's activators; however, from indirect evidence, it has been suggested to bind to the enzyme's α subunit [21,31]. As with all other activators, glycogen increased the formation of $\alpha\gamma$ (+37 %). It also caused the second largest increase, after Mg^{2+} , in $\beta\beta_1$ (+46 %); also, as observed with all but one of the other activators, glycogen also inhibited formation of the $\beta\gamma\gamma$ trimer (−17 %).

Mechanism of reaction with PTD

Because PTD has not previously been used as a chemical cross-linker of proteins, we wished to determine the probable reaction mechanism for its cross-linking of PhK. With isopropylamine as a model nucleophile, we found that it reacted with PTD, in either ethanol or methylene chloride at room temperature, to give the urazole derivative (compound 3 of Scheme 1) in 15–20 % yield

after purification on silica gel. The ^1H and ^{13}C NMR chemical shift values for compound **3** of Scheme 1 were: ^1H , (270 MHz, CDCl_3) 1.1 (d, 6H, J 6.6 Hz, 2 CH_3); 3.9 (m, 1H, CHMe_2); 5.05 (broad d, 1H, J 7.25 Hz, CHNH); 6.9 (m, 2H, ArH and CONH); 7.15 (m, 4H, ArH); and ^{13}C , (75 MHz, CDCl_3) 23.21, 42.08, 120.59, 123.35, 129.14, 138.90, 155.48. A similar urazole adduct has been reported previously for the reaction of PTD with 2-hydroxypyridine [32]. This result suggests that the mechanism for the first reaction of the protein with PTD during cross-linking involves a 1,4-conjugate addition of the side chain nucleophile as illustrated in Scheme 1. It is important to note that although we used a model for lysine in this study, other potential nucleophiles are available within the protein, which is pertinent considering that some cross-linking of $\alpha\beta$ and $\beta\gamma\gamma$ was observed even at pH 6.45 (Figure 1), where lysine side chains would usually be protonated.

DISCUSSION

The results of this study indicate that the novel cross-linker PTD is an effective structural probe for conformers of PhK that are induced by its activators. For example, with the $\alpha\gamma$ dimer, every single activator tested, despite being targeted to different sites on different subunits, caused a significant increase in the amount of $\alpha\gamma$ that was formed, implying that the different activator-induced conformers share the structural feature that allows increased cross-linking of these two subunits by PTD. The $\alpha\gamma$ dimer was also the conjugate whose formation was most enhanced by increasing the pH of cross-linking to 8.2; this same change in pH markedly stimulates the activity of the highly regulated oligomeric PhK holoenzyme [18], but not that of its free catalytic γ subunit [22]. We think that the effect of pH on $\alpha\gamma$ formation is due to the induction of an activated conformer by the slightly alkaline pH, as opposed to the simple deprotonation of a side chain that subsequently becomes cross-linked, because it was at pH 8.2 that all of the various activators were observed to enhance the formation of $\alpha\gamma$ (Figure 3). The activator-induced perturbation in the interaction of the α and γ subunits detected by PTD is consistent with the observations that phosphorylation [33] or proteolysis [11] of α causes increased activity of γ within the $(\alpha\beta\gamma\delta)_4$ holoenzyme. Recently it has been noted that the activators Ca^{2+} and δ'/Ca^{2+} also cause increased formation of an $\alpha\gamma$ dimer by the cross-linker formaldehyde [34], although we do not yet know whether the same regions of α and γ are cross-linked by the two relatively short cross-linkers PTD and formaldehyde. The use of phenylenedimaleimides as structural probes for activated conformers of PhK has also yielded increased amounts of cross-linked conjugates comprising the α and γ subunits but in this case they were $\alpha\gamma\gamma$ trimers [20,35]. No $\alpha\gamma$ dimers were detected by using the phenylenedimaleimides, nor were $\alpha\gamma\gamma$ trimers observed with PTD. Because the activated conformers of the holoenzyme being probed by these two reagents were in some cases identical, the differences in their cross-linking patterns must reflect differences in the physical or chemical properties of the cross-linkers themselves.

In addition to enhancing formation of the $\alpha\gamma$ dimer by PTD, all of the activators except ADP caused a concomitant inhibition of $\beta\gamma\gamma$ formation. ADP might be the exception because, at the concentrations tested, it, unlike GDP, can simultaneously bind to the purine nucleoside diphosphate allosteric site(s) as an activator and to the catalytic site as an inhibitor [36], presumably inducing a distinct conformation in which $\beta\gamma\gamma$ formation is now slightly increased rather than decreased. A slight increase in formation of a $\beta\gamma\gamma$ trimer by phenylenedimaleimides in response to several activators, including ADP, has also recently been

noted [35]. The largest influence on $\beta\gamma\gamma$ formation by PTD was that of Mg^{2+} , which is consistent with the recent finding that this activator causes parallel increases in the accessibility of regions of the β and γ subunits lying on PhK's interior lobe face that are structurally coupled to each other and with enzyme activation [6]. The altered interactions, detected by PTD, between the β and γ subunits in response to activators are not surprising given that multiple means of activating the catalytic γ subunit within the holoenzyme cause common conformational changes in the β subunit [6,19,37,38].

The third conjugate whose formation was influenced by activators, but not by all of them, was $\beta\beta_1$ (Figure 3). Those activators that affected its formation caused an increase that was greatest with Mg^{2+} , which more than doubled the amount formed. It was the formation of $\beta\beta$ dimers by the cross-linker 1,5-difluoro-2,4-dinitrobenzene that provided the first direct evidence of a conformational change in PhK associated with its activation [19], and among the activators that induced this particular change were Mg^{2+} ions [39]. Partial proteolysis as a conformational probe has also shown that Mg^{2+} affects the structure of the β subunit [37]. With regard to $\beta\beta_1$ formation by PTD, glycogen was the next most potent stimulator of cross-linking, behind Mg^{2+} (Figure 3). In fact, comparing the relative changes caused by activators in the amounts of the three different conjugates ($\beta\beta_1$, $\beta\gamma\gamma$ and $\alpha\gamma$), Mg^{2+} and glycogen were the most similar to each other. This likeness could indicate a close similarity in the conformations induced by these two activators. It might be pertinent that Mg^{2+} is also known to enhance the interaction of PhK with glycogen [40].

When one compares the influence of all six different activators on the formation of all five different major conjugates cross-linked by PTD, their effects are found to be remarkably similar. None of the activators caused detectable changes in the amount of $\alpha\beta$ or $\beta\beta_2$ that was formed, whereas all increased the amount of $\alpha\gamma$. All decreased the formation of $\beta\gamma\gamma$, except ADP, which caused a slight increase but also has dual binding sites. All except two activators caused an increased formation of $\beta\beta_1$; the exceptions, ADP and Ca^{2+} , had no effect. Thus, of the 30 possibilities represented by the potential influence of six different activators on the formation of five different conjugates, only three did not fit the same general pattern (Ca^{2+} and ADP with respect to $\beta\beta_1$, and ADP with respect to $\beta\gamma\gamma$). From this we infer that the various activators of PhK, despite being targeted to different sites and subunits, induce conformations that have certain structural features in common. Moreover, PTD is an excellent probe for those common structural features associated with activation, which apparently alter the quaternary interactions that are detected by this cross-linker.

The reaction of isopropylamine with PTD in organic solvent, as analysed by NMR, gives an indication of how cross-linking might occur. The 1,4-conjugate addition of the amine to the double-bonded nitrogen of the triazoline ring of PTD suggests that a nucleophilic amino acid side chain of PhK might carry out an analogous 1,4-conjugate addition reaction to give a urazole adduct similar to compound **3** of Scheme 1. The mono-derivatized (non-cross-linked) complex must then undergo further reaction to form a cross-linked conjugate. Although the mechanism for the second reaction resulting in the cross-linked product remains unknown, it could involve a nucleophilic acyl substitution at one of the triazolinedione carbonyl carbon atoms, with ring opening of the heterocycle. Although this mechanism seems probable and appropriate to extrapolate to PhK, it ignores the potential role of aqueous solvent in the reaction. It is conceivable that the hydrolysis of PTD could result in the formation of an unknown active intermediate that is the actual cross-linking

species. Regardless, however, of the mechanism of action of the novel cross-linker PTD, it is an effective, conformation-dependent cross-linker of PhK that should also find use in the cross-linking of other proteins.

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