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The function of free sulfhydryl groups in the catalytic subunit of phosphorylase kinase

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The function of free sulfhydryl groups in the catalytic subunit of phosphorylase kinase

by

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ABBREVIATIONS

HPLC	High-performance liquid chromatography
DTT	Dithiothreitol
HEPES	N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid
PIPES	Piperazine-N,N'-[2-ethanesulfonic acid]
EGTA	[Ethylenebis(oxyethylene-nitrilo)]tetraacetic acid
EDTA	[Ethylenedinitrilo]tetraacetic acid
MAP	Mitogen-activated protein
DABIA	N,N-Dimethylaminoazobenzene iodoacetamide
DTNB	5,5'-Dithio-bis(2-nitrobenzoic acid)
TFA	Trifluoroacetic acid

GENERAL INTRODUCTION

Phosphorylase b kinase (ATP: phosphorylase b phosphotransferase, EC 2.7.1.38) plays a crucial role in the regulation of glycogenolysis. It catalyzes the conversion of phosphorylase b to an active a form by transferring the γ phosphate of ATP to the alcoholic group of Ser-14 in phosphorylase. The phosphorylase a then facilitates the breakdown of glycogen to glucose-1-phosphate.

Phosphorylase kinase is mainly found in skeletal muscle (1), constituting about 0.5% of the soluble proteins, but is also found in many other tissues including smooth muscle (2), heart (3,4), liver (5-7), adipose tissue (8), platelets (9) and brain (10). The enzyme was first isolated by Krebs et al. (1) from rabbit skeletal muscle and later purified to a homogeneous form by Cohen (11) and Hayakawa et al. (12). Enzymes purified from all sources show similar characteristics, e.g., physical properties, enzymatic activities, and mode of regulation. By far the most results are from the studies of the skeletal muscle enzyme and these results constitute the main body of this Introduction.

General Properties of Phosphorylase kinase

The homogeneous rabbit skeletal muscle phosphorylase kinase is a huge and complex protein with a molecular mass of approximately 1300 kDa (11,12). The holoenzyme is a hexadecamer and composed of four different subunits, $\alpha, \beta, \gamma,$ and δ, with a stoichiometry of $(\alpha\beta\gamma\delta)_4$ (11). Molecular weights of subunits, based on their sequences, are α = 138,422 (1237 amino acid residues)(13); β = 125,205 (1092 amino acid residues)(14); γ = 44,673 (386 amino acid residues)(15); and δ = 16,680 (148 amino acid residues) (16). Two isozymic forms of phosphorylase kinase have been identified and isolated from white (fast-twitch) and red (slow-twitch) muscles (17,18) They differ in the size of their largest subunits, which are designated α and α'. The difference in physiological functions between the two isozymes is not clear. The sedimentation coefficient of phosphorylase kinase is 23S, as determined by Cohen (11) or 26.1S by Hayakawa et al. (12). The frictional coefficient (f/f_0) of the holoenzyme is 1.17 and the isoelectric point is 5.77 as determined by sucrose density gradient isoelectric focusing (12).

Roles of Subunits

The catalytic subunit

The role of the γ subunit in the phosphotransferase activity of phosphorylase kinase was first proposed by Hayakawa et al. (12) in 1973. This proposal was based on the observation that partial proteolysis by trypsin activates the kinase activity of holoenzyme. The trypsin digestion dissected α and β but not the γ subunit and generated some active complexes containing mainly the γ subunit (19). The γ subunit contains the catalytic activity of phosphorylase kinase and was proved by the observations of Skuster et al. (20), Chan and Graves (21-23), Kee and Graves (24,25) in the early and mid 80's. Two active complexes, αγδ and γδ, were isolated and purified by treating the holoenzyme of phosphorylase kinase with a strong chaotropic salt, LiBr, at low temperature (21). A further characterization suggested that both complexes exhibit almost the full kinase activity of holoenzyme (22). Additionally, autophosphorylation could occur in αγδ. Although γδ could not phosphorylate itself it did phosphorylate and activate phosphorylase kinase. More direct evidence about the role of γ came from the studies of Kee and Graves (24,25). Their studies showed that the renatured HPLC-purified γ subunit also contained

phosphorylase kinase activity and phosphorylated phosphorylase. Kinetic studies suggests that the reactivated γ subunit has similar K_m values as those of holoenzyme against both substrates (98 μM for MgATP and 80 μM for phosphorylase b (25)). The Vm, however, is about 10-fold lower.

Primary sequence alignment in the catalytic domain of various kinases (26) provides additional evidence for the role of γ in phosphotransferase activity of phosphorylase kinase. According to the sequence alignment, the N-terminal 276 amino acid residues of γ contains a catalytic domain with a homology of around 40% with that of cAMP-dependent protein kinase and other kinases. The homology of conserved residues in γ which are important for kinase activity is even higher. The C-terminus (301-386) of γ is believed to be the calmodulin binding domain according to the studies of Dasgupta et al. (27) and James et al. (28).

The expression of the full length γ subunit (1-386) in *E. coli*. (29) and other systems (30,31) has been performed by our and other laboratories. However, purification of the recombinant γ subunit to homogeneity is difficult. Recently our lab (32) and another laboratory (33) showed that the expression of the N-terminal catalytic domain of γ(1-300), denoted as a truncated form of γ or TR-γ, was feasible and the enzyme could be purified to homogeneity. There are

several advantages of using TR-γ over full length γ; (i) TR-γ has higher catalytic activity than γ, (ii) TR-γ can be purified to homogeneity, and (iii) TR-γ, missing the inhibitory C-terminal calmodulin binding domain (34), can be used to probe more directly the catalytic function of phosphorylase kinase. TR-γ subunit is used in this thesis for the studies of tyrosine kinase activity of phosphorylase kinase and the role of free sulfhydryl groups in the catalytic domain of γ.

The δ subunit and its function

The δ subunit was not found until 1978 (35), largely because of its small size and poor staining after gel electrophoresis with typical protein stains. It was found essentially identical to bovine brain calmodulin in its primary sequence and in its ability to activate calmodulin-dependent enzymes (35). The amino acid sequence of the δ subunit differs only at two residues from that of bovine brain calmodulin (36). Calmodulin is a member of a group of Ca^{2+} binding proteins mediating the control of a variety of enzymes in response to physiological fluxes of Ca^{2+}. Thus, the δ subunit acts as a built-in sensor for Ca^{2+} and confers Ca^{2+}-sensitivity to phosphorylase kinase. Muscle phosphorylase kinase is a Ca^{2+}-dependent enzyme and requires Ca^{2+} for its activity. The comparison of the Ca^{2+} binding

properties of holoenzyme and the isolated δ subunit by Heilmeyer et al. (37) and Kohse and Heilmeyer (38) indicates that the holoenzyme binds Ca^{2+} exclusively through the δ subunit. Different from other calmodulin-dependent enzyme systems, the δ subunit is an integral part of phosphorylase kinase and associates with the holoenzyme tightly even in the presence of EGTA (15,35,39).

The regulatory subunits: α and β

The α and β subunits are partly homologous in primary sequence except for an N-terminal sequence that is unique to β and two sequences in the middle of α not seen in β (14). They serve as the regulatory subunits in phosphorylase kinase. The activation of the holoenzyme is due to the phosphorylation of the α and β subunits by either cAMP-dependent protein kinase (11,40) or by Ca^{2+}-dependent autophosphorylation (1,41). A total of 10 phosphorylation sites on α and β are known, i.e., 7 on α and 3 on β. Nine of ten known phosphorylation sites are located (13,14). The activation of phosphorylase kinase catalyzed by cAMP-dependent protein kinase is initiated by the phosphorylation of the β subunit. The phosphorylation of α does not occur until at least 1 mole phosphate/$(\alpha\beta\gamma\delta)_4$ has been incorporated into the β subunit (11). The phosphorylation in β is essential for activation and independent of the

level of phosphorylation in α (42). Modification of α may be important in sustaining an activation level of the holoenzyme (12,42-44). The phosphorylation of the α subunit alone does not affect kinase activity (11,42,45,46). In the presence of Ca^{2+}, Mg^{2+}, and ATP, phosphorylase kinase undergoes autophosphorylation and autoactivation by the incorporation of phosphates into multiple sites in the α and β subunits (41,47,48). The autophosphorylation is an intrinsic activity and intramolecular process of phosphorylase kinase (49,50). The observation that the same site in the β subunit can be phosphorylated by either cAMP-dependent protein kinase or by autophosphorylation (51) indicates the similarity in the mechanism of the activation of holoenzyme by two different processes.

Beside the phosphorylation sites, the α and β subunits also contain calmodulin binding sites. The binding of calmodulin • Ca^{2+} complex to the α and β subunits (52) activates phosphorylase kinase (53-55). The interaction between exogenous calmodulin and α and β is Ca^{2+}-dependent and can be inhibited by trifluoperazine, phosphodiesterase, calcineurin, and calmodulin-binding peptides, e.g., melittin and mastoparan. It has been demonstrated that calmodulin and cAMP-dependent protein kinase regulate the activity of phosphorylase kinase in a complementary manner (56). The binding of calmodulin can prevent β from phosphorylation by

cAMP-dependent protein kinase and *vice versa*. Several possible calmodulin binding sequences in α and β have been proposed (13,14) based on their primary sequences with those of other calmodulin-binding peptides. A possible calmodulin binding site near the N-terminus of the β subunit rich in basic, hydrophobic and aromatic residues is postulated to play an important role in a complementary regulation of holoenzyme, because it serves also as the substrate for phosphorylation (14).

The α and β subunits in holoenzyme may act as the negative regulators to exert an inhibitory effect to the catalytic γ subunit of phosphorylase kinase (15,57-59). The phosphorylase kinase can be activated by treating with 1.8 M LiBr which dissociates holoenzyme into small active complexes (15). Paudel and Carlson (57) found that isolated renatured α/β mixture inhibits the activity of reactivated γ. Peptides isolated from proteolyzed β subunit (58) or synthesized upon the sequence of postulated calmodulin binding sites in β (59) are found to inhibit the activity of phosphorylase kinase. The inhibitory effect of α/β and inhibitory peptides can be relieved by phosphorylation and calmodulin binding. These results suggests that the α and/or β subunits may be intimately involved in the regulation of phosphotransferase activity of phosphorylase

kinase by directing an inhibitory domain to the active site region in γ.

Catalytic Properties

Nature of phosphotransferase activity of phosphorylase kinase

Phosphorylase kinase requires Mg^{2+} to form an active substrate with ATP, $MgATP^{2-}$, for its phosphotransferase activity. However, Mg^{2+} added in excess of that required to form the substrate can further stimulate the activity of phosphorylase kinase (60,61). The stimulatory effect of free Mg^{2+} was seen as an increase in V_m and a decrease in K_m for both substrates, MgATP and phosphorylase b (62). The reactivated HPLC-γ subunit can also be stimulated by free Mg^{2+}(25). The level of stimulation by Mg^{2+} in γ is the same as that of stimulation in holoenzyme. Accordingly, an additional metal ion binding site in γ was proposed (25). In contrast, Mn^{2+} was found to activate at lower and inhibit at higher concentrations the activity of both the reactivated γ subunit (25) and holoenzyme (61).

Nonactivated phosphorylase kinase has little activity at physiological pH (around 7.0), but exhibits a considerable activity at higher pH (1). The pH dependency of phosphorylase kinase is changed by the activation via

phosphorylation, calmodulin binding, and proteolysis. The ratio of activity at pH 6.8 to at 8.2 is 0.04-0.08 for nonactivated enzyme and 0.3-1.0 for activated forms, respectively (63). The difference in pH-dependency among different enzyme forms suggests that a functional group in the active site region important for catalysis may be affected by the state of activation. The idea of a catalytic base helping to pull a proton from a reactive seryl residue in substrates during catalysis was proposed by Yoon and Cook (64), and Taylor et al. (65) to explain the high turnover rate of serine kinases. The "proton pulling" ability of this catalytic base could be affected by its pKa and the pH value of the surrounding environment. In phosphorylase kinase, the pH-dependency change that occurs upon activation may reflect a change in the microenvironment of the catalytic base in the active site region and, hence, its pKa value. If the active site region becomes more hydrophilic during activation, the putative catalytic base, a carboxyl group of an aspartate residue, could become more ionized at physiological pH. In this ionized form the carboxylate could serve to H-bond or pull a proton away from the phosphorylatable serine.

Substrate specificity

Phosphorylase b is known to be an *in vivo* substrate of phosphorylase kinase. In the cell it associates with a glycogen particle along with phosphorylase kinase (66). Phosphorylase kinase catalyzes the formation of phosphorylase a by phosphorylation of a serine residue at position 14 in glycogen phosphorylase (67). A series of studies were done using synthetic peptide derivatives according to the phosphorylation site sequence (^{1}Ser-Arg-Pro-Leu-Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu^{18}) (68-70). The results suggest that (i) basic amino acid residue is required on both N- (P-3) and C-terminal (P+2) sides of the phosphorylatable serine, (ii) threonine cannot replace serine as a phosphate group acceptor, (iii) the first eight amino acid residues may not be important.

Besides phosphorylase b, glycogen synthase (53,71,72), troponin I (73), troponin T (74,75), casein (76), myosin light chain (77),and several other proteins can be phosphorylated *in vitro* by phosphorylase kinase. These proteins are not phosphorylated, however, as effectively as phosphorylase b. The proteins are also phosphorylated on serine except for troponin I which is phosphorylated on threonine (73).

Phosphorylase kinase may play a different role in addition to the regulation of phosphorylase. First, it has

been found that the glycogen particle is not the only place that phosphorylase kinase is located. About 20-30% of the kinase is glycogen bound (67), whereas, phosphorylase b, when isolated, is totally associated with the glycogen particle. Second, a membrane-bound form of phosphorylase kinase is reported (78) and may be anchored through the C-terminus of the α and β subunits of phosphorylase kinase which contain an isoprenoid group. The isoprenylation is largely seen in G-proteins and oncoproteins (79) as a membrane anchor (80). Third, it was found that phosphorylase kinase exhibited phosphatidyl-inositol kinase activity and catalyzed the formation of phosphatidylinositol-4-phosphate (81). Fourth, it may exist with different active site conformations from that for phosphorylase b and accommodate a different substrate. This speculation is supported by the finding that both phosphorylase kinase and HPLC-isolated γ-calmodulin complex exhibit different substrate specificity and, hence, pH-dependency to syntide-2 and syntide-3 in the presence of different ligands, Ca^{2+} and heparin (82). All of these data suggest that phosphorylase kinase may contribute to different signal transduction pathways other than just being involved in the regulation of glycogen metabolism. Many studies remain to be done before a full understanding of the role of phosphorylase kinase in the cell can be appreciated.

Explanation of Dissertation Format

This dissertation consists of three papers after the general introduction. The first paper describes the tyrosine kinase activity of phosphorylase kinase. It was published in J. Biol. Chem. 268, 17683-17686 (1993). The second and third papers deal with the characterizations of the free sulfhydryl groups in the catalytic subunit of phosphorylase kinase by using biochemical and molecular biological methods. These two papers will be submitted to the J. Biol. Chem. for publication. Following the third paper is the general summary and discussion. The references cited in the general introduction are listed following the general summary and discussion.

PAPER 1. PHOSPHORYLASE KINASE: A METAL ION-DEPENDENT DUAL SPECIFICITY KINASE

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SUMMARY

Phosphorylase kinase is shown to be a dual specificity kinase. The specificity of phosphorylation is determined by divalent cation. Mg^{2+} causes seryl phosphorylation of phosphorylase b, but Mn^{2+} activates tyrosine phosphorylation of angiotensin II. In contrast to seryl phosphorylation, the tyrosine kinase activity of holoenzyme is not regulated by Ca^{2+}. Preincubation of the holoenzyme with Ca^{2+}, Mg^{2+} and ATP which causes autophosphorylation activates tyrosine kinase activity. The tyrosyl kinase activity is a property of the g subunit. Addition of varying amounts of Mn^{2+} to a truncated form of the g subunit of phosphorylase kinase containing MgATP inhibits serine kinase, but activates tyrosine kinase activity. This result along with an oxidative reaction caused by Cu^{2+} and site-directed mutagenesis of the putative catalytic base inhibiting both serine and tyrosine kinase activity suggest that one active site is involved in both activities. Kinetic studies with Mn^{2+} and ATP show that K_m for nucleotide is not changed with a seryl or tyrosyl substrate. The V_m values are different and the value for tyrosyl phosphorylation is similar to other tyrosyl kinases. We propose two conformations for the active site, one favors seryl phosphorylation and the second tyrosyl phosphorylation caused by the binding of divalent cation at a second metal ion binding site.

INTRODUCTION

Protein kinases modulate diverse physiologic processes by catalyzing the incorporation of a phosphate group into the side chain of various amino acid residues of proteins. Several amino acids can serve as acceptors of phosphate, but the alcoholic group of serine and threonine, and the phenolic group of tyrosine are the major targets for protein kinases (1,2). Recently, it has been shown that the imidazole group of histidine can be phosphorylated by histidine protein kinase (3) . Most protein kinases show selectivity and phosphorylate the side chain of only one of the functional groups, but some protein kinases have been found to phosphorylate more than one type of amino acid residues, and are termed dual-specificity kinases (4). MAP kinase kinase, a member of this family, can autophosphorylate and phosphorylate MAP kinase on alcoholic and phenolic groups (5-7). These dual specificity kinases are not very different in primary structure of their catalytic cores from that of the tyrosine kinases and the serine/threonine kinases (4). Little is known about what structural features and factors are important for dual specificity.

Protein kinases require a divalent cation along with a nucleoside triphosphate for effective phosphorylation. Serine/threonine kinases use Mg^{2+} more effectively than Mn^{2+}

but tyrosyl kinases differ because Mn^{2+} stimulates phosphorylation equal to or better than that caused by Mg^{2+} (8). Several serine/threonine protein kinases, including cAMP-dependent protein kinase (9) and phosphorylase kinase (10,11), have been shown to bind more than one metal ion. The binding of the additional metal ion may either activate or inhibit kinase activity. Results obtained from x-ray crystallography of the catalytic subunit of cAMP-dependent protein kinase (12,13), a serine kinase, show that two metal ion binding sites exist and these sites are in close proximity. Occupancy of the first site activates phosphotransfer activity but binding at the second site inhibits the reaction. Phosphorylase kinase, a calcium dependent protein kinase which regulates glycogen metabolism (14,15) by phosphorylating a single seryl residue per monomeric unit of phosphorylase b (841 amino acid residues), also contains two metal ion binding sites associated with its catalytic subunit. In this instance, binding of divalent cation, i.e., Mg^{2+}, at the second site can activate the reaction with phosphorylase b but the activity is inhibited by the binding of Mn^{+2} (10). Phosphorylase kinase acts on few other substrates, but it can phosphorylate a threonine residue in troponin I (16) and even inositol in phosphatidylinositol (17).

We report in this communication that the binding of second divalent cation in the recombinant catalytic subunit of phosphorylase kinase determines enzyme specificity. With Mg^{2+}, seryl phosphorylation occurs, but with Mn^{2+} tyrosine phosphorylation is favored. Similar results are seen with the holoenzyme, i.e., phosphorylase kinase has tyrosine kinase activity in the presence of Mn^{2+}.

EXPERIMENTAL PROCEDURES

Materials

Highly purified rabbit skeletal muscle phosphorylase kinase was a kind gift from Dr. G. M. Carlson of the University of Tennesee. Recombinant truncated γ subunit (1-300) of phosphorylase kinase is expressed, purified and renatured as described by Huang et al. (18). Phosphorylase b is prepared as described (19). Angiotensin II (AspArgValTyrIleHisProPhe) is from Sigma. Phosphocellulose paper and ET-31 filter paper are from Whatman. [γ-^{32}P]-ATP is the product of ICN. Other reagents used in experiments are reagent grade.

Protein concentration determination

The concentration of phosphorylase kinase and phosphorylase b is determined spectrophotometrically (20,21) Angiotensin II solution is prepared based on its dry weight. The concentration of truncated γ subunit is determined by reaction with Bradford reagent (Bio-Rad).

Activity assay

The seryl kinase activity of phosphorylase kinase is determined by measuring the incorporation of ^{32}P into phosphorylase b as described with some modification (18).

The standard assay contained 50 mM Tris, 50 mM PIPES, pH 8.2, 10 mM Mg^{+2}, 10 mg/ml phosphorylase b, and 1 mM [g-^{32}P]-ATP. The final concentration of truncated γ is adjusted to around 0.3 nM. The reaction is carried out at 30°C and initiated by adding enzyme solution. After incubation a portion of reaction mixture is spotted on a square ET-31 filter paper, washed with trichloroacetic acid and analyzed by liquid scintillation counting (22). The tyrosine kinase activity is assayed using angiotensin II as a substrate. The reaction is performed in an assay mixture containing 10% glycerol, 50 mM Tris, 50 mM PIPES, pH 7.9, 3 mM $MnCl_2$, 2.5 mM angiotensin II, and 1 mM [γ-^{32}P]-ATP. The final concentrations of the truncated γ subunit and phosphorylase kinase were 10 and 100 μg/ml, respectively. The tyrosine kinase assay is allowed to proceed at 30°C for 30 min. After incubation, a portion of reaction mixture is spotted on the phosphocellulose paper and washed with cold 0.5% phosphoric acid. When using poly(Glu,Tyr) as substrate, the reaction mixture is handled as the serine kinase assay.

Oxidation

The truncated γ subunit is oxidized as essentially described by Landgraf et al. (23). To remove DTT, the truncated γ subunit is dialyzed against R buffer (10% glycerol, 50 mM Tris, 50 mM HEPES, 50 mM NaCl, pH 7.8) on

Microdialyzer 500 (Bio-Rad) in the cold room for at least two buffer changes. The oxidation is carried out at room temperature in R buffer containing 0.1 mg/ml truncated g and an appropriate amount of Cu^{2+} (0.5 to 16 μM) for 20 min. After incubation, the oxidized truncated γ subunits are subjected to seryl and tyrosyl kinases assay.

Oligonucleotide-directed site-specific mutagenesis

Mutagenesis is performed using an Amersham commercial kit as described previously (18). Mutation is identified by restriction enzyme analysis and verified by sequencing.

Data analysis

The kinetic data are analyzed with computer software Enzfitter (Elsevier Science Publishers).

RESULTS AND DISCUSSION

Phosphorylase kinase holoenzyme purified from rabbit skeletal muscle shows significant tyrosine kinase activity with angiotensin II in the presence of Mn^{2+} but not with Mg^{2+} (Figure 1). Poly(Glu,Tyr) and Raytide, two other common tyrosine protein kinase substrates with poly-negative charge, can be phosphorylated at a very low level. The tyrosine kinase activity of holoenzyme is affected little by 2 mM EGTA, a concentration that completely inhibits seryl phosphorylation, (Figure 1) suggesting that Ca^{2+} was not essential for tyrosyl phosphorylation.

Autophosphorylation on α and β subunits of phosphorylase kinase is known to activate the enzyme (24-26). To determine whether preincubation of the enzyme with Ca^{2+}, Mg^{2+}, and ATP, a condition which causes autophosphorylation, can also activate its tyrosine kinase activity, the holoenzyme is first treated with the above reagents and then assayed for tyrosine kinase activity. Note that if the enzyme is not preincubated, there is a lag in the reaction (Figure 2). After preincubation, the lag disappears and activity increases about 8- to 20-fold in the early stage of time course (5-20 min). Phosphorylation of α and β subunits occurs as assessed by SDS-PAGE under these conditions

Figure 1. Tyrosine kinase activity of phosphorylase kinase holoenzyme in the presence or absence of calcium. The tyrosine kinase activity is determined in the presence of Mg^{2+} (filled bars) or Mn^{2+} (dashed bars) and in the presence or absence of Ca^{2+}. The tyrosine kinase activity assay is essentially as described in Experimental procedures. Phosphorylase kinase holoenzyme 100 µg/ml is incubated with 50 mM Tris, 50 mM PIPES, pH 7.9, 10% glycerol, 3 mM $MnCl_2$ or $MgCl_2$, 1 mM [γ-^{32}P]-ATP, 2 mM angiotensin II, in the presence of 0.1 mM Ca^{2+} or 2 mM EGTA, at 30°C for 30 min.

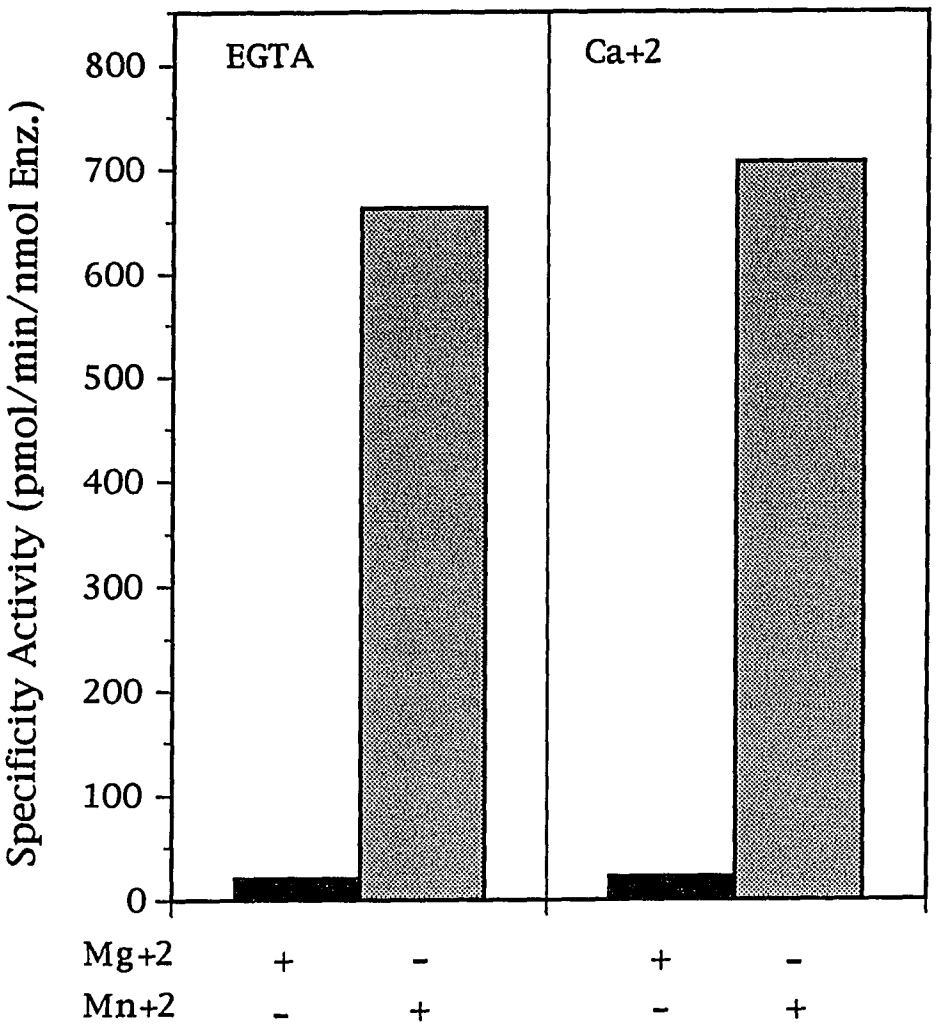
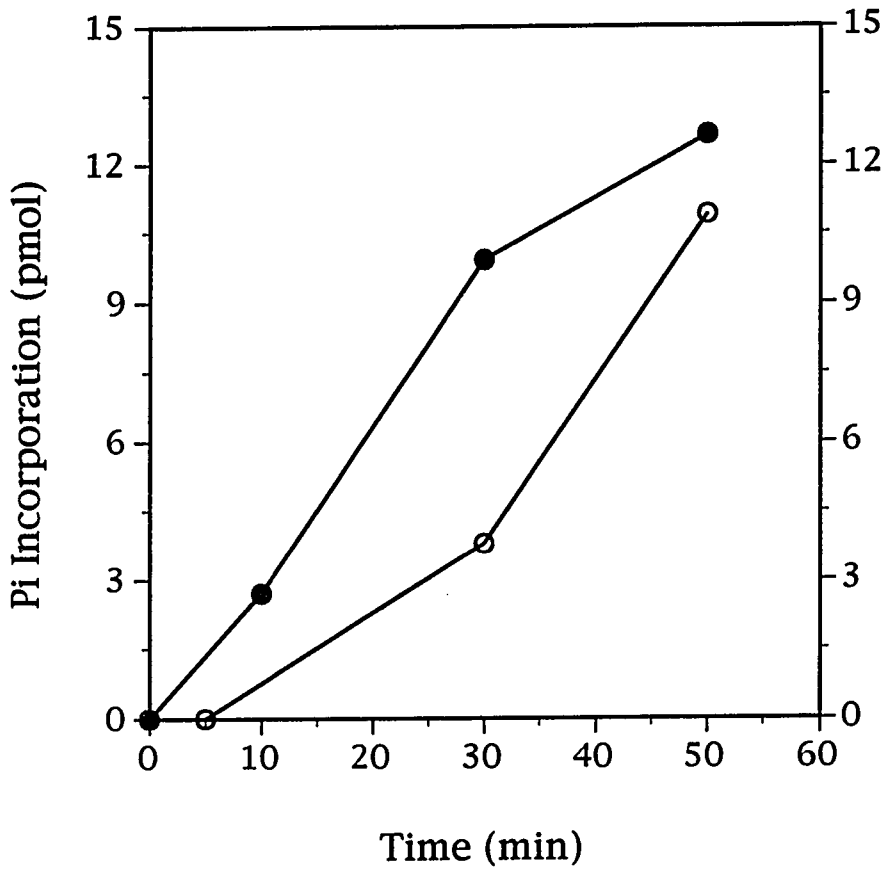

EGTA
Ca+2
800
700
600
500
400
300
200
100
0
Specificity Activity (pmol/min/nmol Enz.)
Mg+2
Mn+2
+
-
-
+
+
-
-
+

Figure 2. Effect of preincubation on tyrosine kinase activity. The tyrosine kinase activity of holoenzyme is determined with (-●-) or without (-O-) preincubation. Prior to tyrosine kinase assay, phosphorylase kinase 1 mg/ml in R Buffer is preincubated with 0.1 mM Ca^{2+}, 3 mM $MgCl_2$, and 1 mM ATP, at 30°C for 30 min. After preincubation, one part of enzyme solution is mixed with nine parts of substrate mixture containing 56 mM Tris, 56 mM PIPES, pH 7.9, 10 % glycerol, 3.3 mM $MnCl_2$, 2.8 mM angiotensin II, and 1.1 mM [γ-^{32}P]-ATP and incubated at 30°C for 5, 20, and 50 min. Phosphorylase kinase preincubates at 30°C without MgATP is used as the control. The substrate mixture for control experiment is about the same as above except 0.3 mM Mg^{2+}, 0.1 mM ATP, and 10 mM Ca^{2+} were added along with 3.3 mM Mn^{2+} and 1.1 mM [γ-^{32}P]-ATP.


Pi Incorporation (pmol)
Time (min)
0
3
6
9
12
15
0
10
20
30
40
50
60

(result not shown). After approximately 50 min the tyrosine kinase activities are similar with or without preincubation.

To investigate the tyrosine kinase activity of phosphorylase kinase more fully, a recombinant truncated form of catalytic subunit (1-300) of phosphorylase kinase was used. Any contamination of a bacterial tyrosine protein kinase in truncated γ preparation is excluded by the fact that recombinant protein is renatured and purified to homogeneity as judged by SDS-PAGE (18). The recombinant truncated γ subunit also phosphorylates angiotensin II. Mn^{2+} is required for the tyrosine kinase activity, although Co^{2+} can also stimulate a moderate (about 25% of tyrosine kinase activity with Mn^{2+}) activity. In contrast, a little tyrosine kinase activity (1-5%) can be seen by using Mg^{2+} alone. These results are consistent with the observation that most tyrosine kinases prefer Mn^{+2} more than Mg^{2+} for their activity (8). Poly(Glu,Tyr) and Raytide are poor substrates for truncated γ. It is reasonable to suggest that both angiotensin II and phosphorylase can be recognized by a common set of determinants and hence a common active site because angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) shows some similarity in primary sequence to that of phosphorylase (Arg^{10}-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu^{18}).

It has been demonstrated that the serine/threonine kinase activity of the truncated γ subunit can be inhibited by the

oxidation of Cu^{2+} (27). The oxidation causes the formation of two pairs of disulfide bonds in or near the active site region and inactivated the conversion of phosphorylase b to a by interfering with the binding of substrates. If both serine/threonine and tyrosine kinase activities of phosphorylase kinase share the same active site in the γ subunit, oxidation should interfere with the binding of both substrates. As shown in Figure 3, both serine/threonine and tyrosine kinase activities of the truncated γ subunit are inhibited identically with increasing Cu^{2+}. The idea of two kinase activities sharing one active site is further supported by result of site-directed mutagenesis at an invariant residue of the catalytic loop, Asp^{150} corresponding to Asp^{166}, the putative catalytic base, of cAMP-dependent protein kinase (28). A similar but uncharged residure, Asn, was used to replace Asp^{150} in γ. The mutant is constructed, expressed, and purified to homogeneity. Both serine/threonine and tyrosine kinase activities are abolished by this mutagenesis.

The influence of metal ions on the substrate specificity of the truncated γ subunit of phosphorylase kinase is shown in Figure 4. Inhibition of serine/ threonine kinase activity and activation of tyrosine kinase activity of truncated γ show parallel changes by increasing Mn^{2+} in the presence of equivalent amount of Mg^{2+} and ATP. The two activity curves

Figure 3. Oxidation inhibits both serine/threonine and tyrosine kinase activities. The inhibition curves for serine/threonine kinase (-●-) and tyrosine kinase (-O-) are presented. Oxidation of truncated γ is carried out as described in Experimental Procedures. After oxidation the truncated γ subunit is diluted to an appropriate concentration with R buffer and subjected to serine kinase and tyrosine kinase assay.

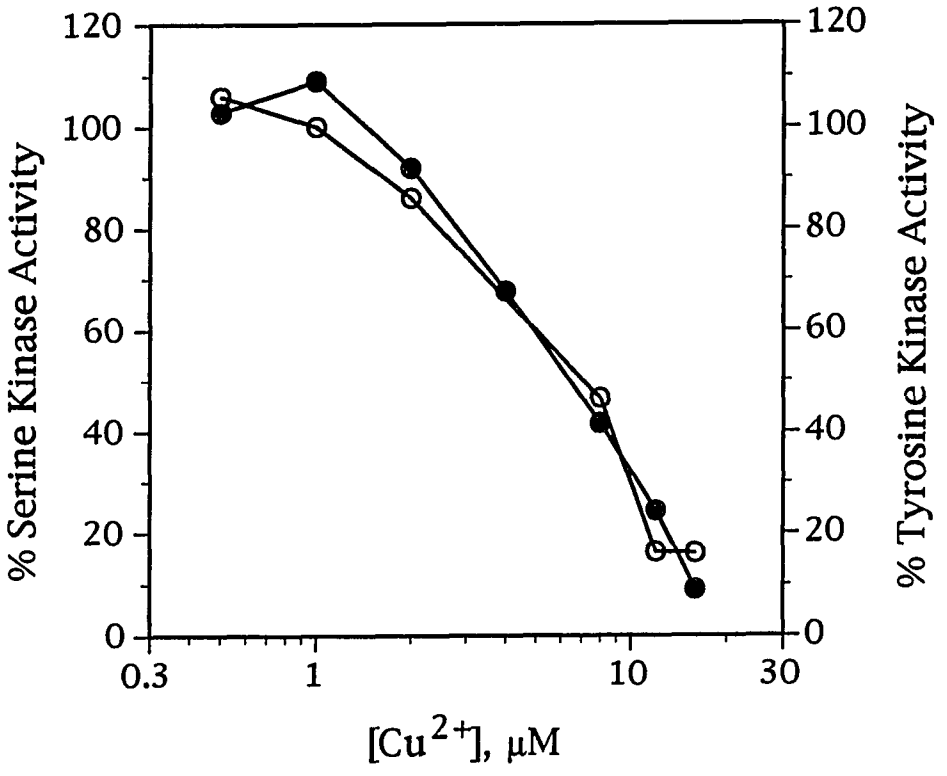
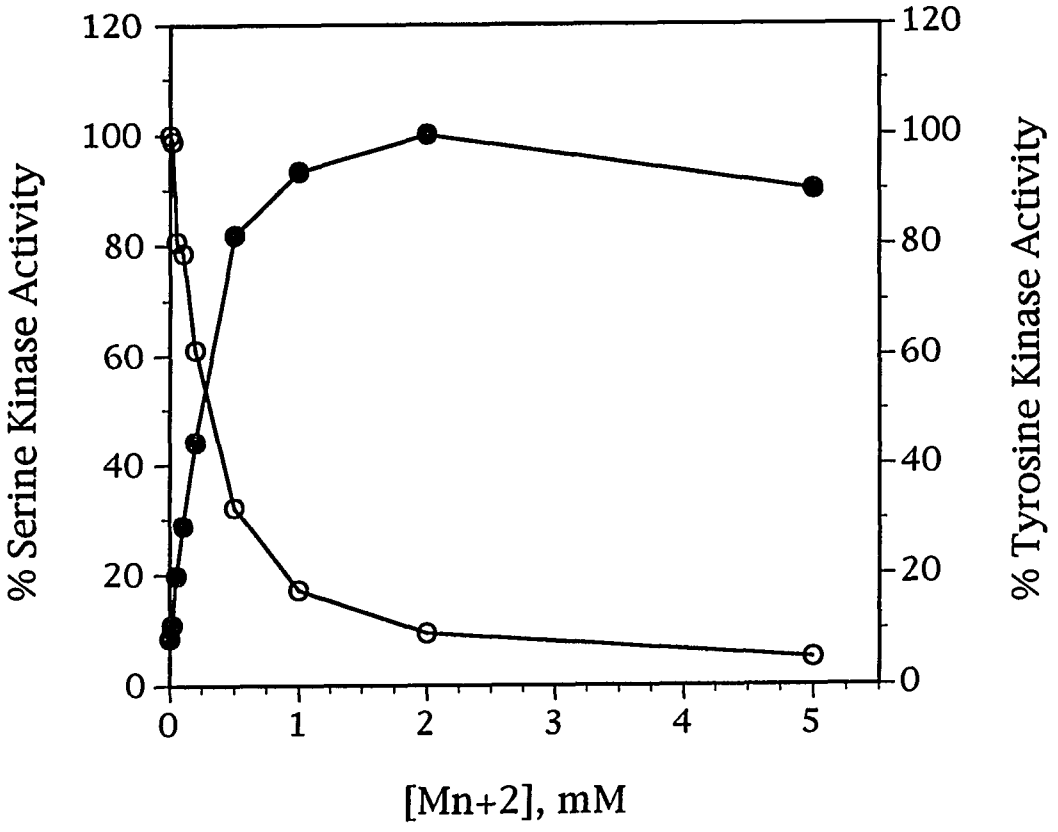

120
100
80
60
40
20
0
% Serine Kinase Activity
120
100
80
60
40
20
0
% Tyrosine Kinase Activity
0.3
1
10
30
[Cu2+], μM

Figure 4. Manganese induces parallel changes in serine/threonine and tyrosine kinase activities of the truncated γ subunit. The serine/threonine kinase activity (-O-) and tyrosine kinase activity (-●-) is measured with increasing Mn^{2+}. The assays are done as essentially described in Experimental Procedures except that 3 mM Mg^{2+} is added in all assay mixtures along with various amounts of Mn^{2+} as indicated.


120
100
80
60
40
20
0
% Serine Kinase Activity
% Tyrosine Kinase Activity
0
1
2
3
4
5
[Mn+2], mM

intersect at a concentration of approximately 250 mM Mn^{2+}, which is also the EC_{50} for both curves, suggesting this effect is due to the binding of Mn^{2+} at a common site. The crystal structure of cAMP-dependent protein kinase (12) shows that the nucleotide binding site is located at the base of the small lobe and the second metal ion and peptide substrate are mainly situated on the large lobe. The binding of a second metal ion, Mg^{2+} or Mn^{2+}, may hold the nucleotide in the active site and cause inhibition (9,29). For phosphorylase kinase, the second metal ion, especially Mn^{2+}, may have a different role, modulating substrate specificity. Bollen et al. (30) suggested that the catalytic subunit of phosphorylase kinase might have two active conformations to recognize different seryl substrates. Olah et al. (31) demonstrated that the large lobe of the catalytic subunit of cAMP-dependent protein kinase can rotate about 35 degree around a hinge residue, Gly 125, a residue which is invariant in protein kinases (32). Lindberg, et al. (4) suggested a flexible active site region to explain the dual specificity of some kinases. The two metal ions, Mn^{2+} and Mg^{2+}, could stabilize different conformations because of differences in their chemical properties and ionic radii. These two metal ions interact differently in the nucleotide binding region of a mutant nitrogenase (33).

Table I

Comparison of Kinetic Parameters of the Truncated γ Subunit in the presence of Mg^{2+} or Mn^{2+} and with Different Substrates

Substrates	Mg^{2+}#		Mn^{2+}	
	Km	Vm (nmol/min/nmol)	Km	Vm (nmol/min/nmol)
ATP (μM)	79 ± 10	4100 ± 30	54 ± 3	163 ± 8@
Phos b (μM)	18 ± 1		4.0 ± 0.2@	
Angiotensin (mM)	ND	ND	4.4 ± 0.2	0.47 ± 0.02

see ref 18.
@ Apparent Km and Vm were obtained from the mean value of three different experiments.
ND= not determined.

Kinetic results (Table I) show that in the presence of Mn^{2+} the Km for ATP (54 ± 3 μM) is about the same as that measured in the presence of Mg^{2+} (79 μM) (18). The apparent Km for phosphorylase b (4.0 ± 0.2 μM), however, is about 5-fold lower. In the presence of Mg^{2+} the tyrosine kinase activity is almost non-detectable. The Vm and Km are 0.47 ± 0.02 nmol/min/nmol and 4.4 ± 0.2 mM, respectively, using angiotensin II as substrate in the presence of Mn^{2+}. This result suggests that a conformational change at active site region caused by the binding of Mn^{2+} may further expose the binding site to its substrate and gives more room for a phenolic group. It is known that the binding of second metal ion can increase the affinity of enzyme for its substrate with the insulin receptor tyrosine kinase (34). A conformational change in g induced by Mn^{2+} may turn the alcoholic group of Ser^{14} of phosphorylase b away from the putative catalytic base, Asp^{150}, and reduce the rate of phosphoryl transfer (apparent Vm=163 ± 8 nmol/min/nmol)(Table 1). The tyrosine kinase activity of truncated γ is similar to that of most tyrosine kinases. When compared with its serine kinase , the tyrosine kinase activity is about 1/350 of its serine kinase activity using MnATP as substrate (Table I). This result is consistent with the general observation that turnover rates of tyrosine protein kinases are usually several orders lower than that of serine/threonine kinases.

So far, at least 11 dual specificity kinases have been reported (4). Most of these enzymes, however, have been identified from autophosphorylation reactions targeting tyrosine and serine/ threonine residues or from reactivity with anti-phosphotyrosine antibodies after expression in bacteria. Some dual-specificity kinases have been reported to phosphorylate exogenous substrates (5-7,35, 36) and have a role in cell development. The results presented herein show that phosphorylase kinase has dual specificity but whether this new activity is physiologically significant remains to be determined.

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PAPER 2. REVERSIBLE INACTIVATION OF PHOSPHORYLASE KINASE BY THE OXIDATION OF VICINAL THIOLS

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ABSTRACT

The truncated form of the γ subunit of phosphorylase kinase is found to be inactivated by μM amounts of Cu^{2+}. The inactivation of truncated γ can be readily reversed by DTT but not by the metal chelating agents, EDTA or EGTA. This result suggests that inactivation is caused by disulfide bond formation. Maximally two intramolecular disulfide bonds are formed by the oxidation of Cu^{2+} as determined by DTNB titration and non-reducing SDS-polyacrylamide gel electrophoresis. The formation of the first disulfide bond, denoted as the semi-oxidized form, results in 50% loss of specific activity. Kinetic study of the semi-oxidized form of truncated γ shows that Vm (3.4 ± 0.4 μmol/min/nmol) and K_m for phosphorylase b (18.0 ± 0.2 μM) are similar to those of the reduced form. However, the K_m for MgATP (780 ± 50 μM) is about 10-fold higher. This result suggests that the first disulfide bond may be in or near by the nucleotide binding domain of the truncated γ. This speculation is further supported by the localization of the first disulfide bond (between Cys-36 and Cys-172) by peptide mapping and amino acid sequencing.

The truncated γ subunit can also be inactivated by the disulfide form of a peptide analog of the phosphorylation site in glycogen phosphorylase, containing cysteine instead

of serine, through thiol/disulfide exchange. This is proved by the fact that inactivation can be reversed by DTT. The kinetic study suggests that inactivation is not due to blocking of the active site because K_m values of Cys-peptide coupled truncated γ for ATP (143 ± 16 μM) and phosphorylase b (39 ± 2 μM) are only about 2-fold higher than those of reduced form. This result is consistent with the finding that Cys-138 is the site cross-linked to the Cys-peptide.

INTRODUCTION

Phosphorylase kinase plays a crucial role in the regulatory cascade of glycogen metabolism (1-3). It activates phosphorylase and facilitates the breakdown of glycogen by transferring the γ-phosphate of ATP to the alcoholic group of serine-14 of phosphorylase. Phosphorylase kinase contains four different subunits, α, β, γ, and δ with a stoichiometry of $(\alpha\beta\gamma\delta)_4$ (4). The α and β subunits are regulatory subunits (5-7), and δ is almost identical to bovine brain calmodulin (8). The γ subunit has been demonstrated to be the catalytic subunit (9,10) containing a kinase domain (11) and a calmodulin (CaM) or the δ subunit binding domain (12).

The crystal structure of the γ subunit of phosphorylase kinase is not known so far. However, the primary sequence of the catalytic domain of γ is highly homologous to cAPK and other kinases (11). Accordingly, the catalytic domain of γ likely shares some structural features with those in cAPK (13-16). Several conserved sequences in kinases have been speculated to be either directly contacting substrates or important for the active site configuration (16). Among these conserved sequences none are cysteine residues. However, several pieces of evidence have suggested that sulfhydryl groups may be directly or indirectly involved in

the regulation and/or substrate recognition in the kinase family. Cys-199 in the C subunit of cAPK is at the edge of the active site region (13,14) and provides a general hydrophobic environment to the P+1 site of the substrate. In addition, this residue has been shown to cross-link with Cys-97 of the R_{II} subunit *in vitro* through a disulfide bond (17). Cys-518, corresponding to Cys-199 of cAPK, in the catalytic domain of cGMP-dependent protein kinase (cGPK) was found to form a disulfide bridge with Cys-312 and induced a cGMP-independent kinase activity (18). A similar result was obtained with protein kinase C when it was oxidized with H_2O_2 (19). A Ca^{2+}/phospholipid-independent activity was induced after oxidation. In the case of CaM-dependent protein kinase II, the modification of cysteine residues by N-ethylmaleimide and DTNB inactivated the kinase activity (20). The inactivation, however, could be eliminated by ADP but not by peptide substrate, Ca^{2+}-CaM, or phosphorylation.

The γ subunit of phosphorylase kinase has seven sulfhydryl groups, one in the CaM binding domain and six in the catalytic domain. According to the crystal structure of cAPK (13,14) four out of seven cysteine residues, 36, 172, 184, and 197, are suggested to be in or near the active site region of γ. The Cys-184 is conserved among several kinases including cAPK (Cys-199), cGPK (Cys-512), and protein kinase C (11). Recently, a truncated γ subunit (1-300) without the

C-terminal CaM binding domain, denoted as TR-γ, was constructed (21) in our laboratory. The usage of TR-γ allows us to characterize the functions of sulfhydryl groups more fully without the influence of the CaM binding domain and to gain information about the proximity of the SH groups of the enzyme in aqueous solution. In this paper we present a series of experiments showing that the catalytic subunit of phosphorylase kinase can be inactivated reversibly by the formation of a disulfide bond in the active site region.

EXPERIMENTAL PROCEDURES

Materials

The recombinant truncated γ subunit (1-300) of phosphorylase kinase was expressed, renatured, and purified as described previously (21). Renatured TR-γ was stored at 0°C in R Buffer (10% glycerol, 50 mM Tris-HCl, 50 mM HEPES, pH 7.8, 50 mM NaCl) containing 5 mM DTT. Phosphorylase b was prepared as described (22). A Cys-peptide (LysArgLysGlnIle-CysValArgGlyLeu) was synthesized by the Protein facility at Iowa State University with an Applied Biosystems 430A peptide synthesizer. [γ-^{32}P]ATP was obtained from ICN. Dimethyl-aminoazobenzene iodoacetamide (DABIA) was from Fluka Chemical. Econo Pac-10DG gel filtration column and Bio-Gel P-6 resin (50-100 mesh) were from Bio Rad. Iodoacetamide and glutathione disulfide were obtained from Sigma. DTNB was from Pierce. All other reagents were reagent grade.

Oxidation of the truncated γ subunit by copper ion

The oxidation of the truncated γ subunit of phosphorylase kinase is essentially as described by Yuan, et al. (23) with some modification. The renatured, purified TR-γ was first passed through an Econo Pac-10DG gel filtration column equilibrated with R Buffer to remove DTT. The enzyme (0.2 mg/ml, at pH 7.8) was then oxidized by incubation with

various amounts of $CuCl_2$ (0.5 to 20 μM) at room temperature for around 20 min. After incubation, the oxidized TR-γ was diluted with R Buffer if necessary and assayed for enzymatic activity. The oxidized TR-γ subunit could be reactivated by treating with DTT. After incubating with Cu^{2+}, 10 μl of the protein mixture was mixed with an equal volume of R Buffer containing 50 mM DTT, and 1 mM EDTA. The whole mixture was then incubated on ice for 9 h after which enzymatic activity was measured.

DTNB titration

Sulfhydryl groups of the reduced and oxidized TR-γ subunit were determined by DTNB titration (24). The titration was carried out by dissolving solid urea in 190 μl of protein solution (0.2 mg/ml) with or without Cu^{2+} treatment and 0.4 M Na_2HPO_4, pH 8.0 to give a total volume of 400 μl. The final concentration of urea and phosphate was 6 M and 100 mM, respectively. The reaction was initiated by adding 15 μl of freshly prepared 20 mM DTNB. The mixture was incubated at room temperature for 15 min before measuring the absorbance at 414 nm. The concentration of sulfhydryl groups was calculated using ε_{414} = 13,600 $M^{-1}cm^{-1}$ (24).

Coupling of Cys-peptide to the truncated γ subunit

The Cys-peptide dimer, a peptide analog to the phosphorylation site sequence of glycogen phosphorylase, was prepared by incubating the peptide solution at 30 °C in the absence of reducing agent for 24 h or longer. The dimerization of the Cys-peptide was checked by DTNB titration. The TR-γ solution, free of DTT, (0.2 mg/ml) was incubated with appropriate amounts of Cys-peptide dimer in R Buffer at room temperature for 20 min.

Activity assay

The serine kinase activity of reduced and oxidized TR-γ was determined by the incorporation of ^{32}P into phosphorylase b. The assay was performed at pH 8.2 in the presence of 60 mM Tris-HCl, 60 mM HEPES, 10 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, and 100 μM phosphorylase b. The final concentration of the reduced and oxidized TR-γ subunit was adjusted to about 0.6 nM. After incubating the reaction mixture at 30°C for 5 min an aliquot was removed, spotted onto a square ET-31 paper (Whatman), and washed with trichloroacetic acid and analyzed by liquid scintillation counting (25).

Labeling of sulfhydryl groups with DABIA

After DTT was removed, the TR-γ subunit (around 1 mg) was oxidized with 4 μM Cu^{2+} (semi-oxidized) or 0.5 mM Cys-peptide

dimer as described previously. The labeling of the remaining free sulfhydryl groups follows and is based on modified procedures of Lewis et al. (26) and Landgraf et al. (18). The protein treated with Cu^{2+} or Cys-peptide was first mixed with solid iodoacetamide to 100 mM followed by the addition of solid urea to 6 M. The sample was allowed to incubate at 37 °C in the dark for 1 h before precipitating protein with 20% TCA. The precipitate was spun down and pellet was washed twice with cold acetone. The pellet was air dried and dissolved in 150 µl 5 M guanidine-HCl, 50 mM Tris, 5 mM EDTA, pH 8.0, and 1 mM DTT. The mixture was incubated under N_2 in the dark at 37 °C for 2 h. This cleaves the disulfide bonds formed in the reaction with Cu^{2+} or the Cys-peptide dimer. After incubation, the free thiols generated from the disulfides were labeled with DABIA (18,27). The reaction was carried out by mixing the above solution with one volume of 5 M guanidine-HCl, containing 50 mM Tris, pH 8.0, and 5 mM EDTA and two volumes of DABIA (1 mg/ml in dimethylformamide). The samples were incubated at room temperature in the dark for 2 h with stirring. The mixture was acidified by adding 10 µl of TFA and loaded onto a Bio-Gel P-6 column (1.2 x 15 cm) pre-equilibrated with 50% acetic acid. The protein was eluted with 50% acetic acid. Labeled protein containing DABIA was identified by following the absorbance at 540 nm of

the collected fractions. The pooled fractions were then dried using a SpeedVac (Savant).

Proteolysis, HPLC purification, and sequence analysis

The DABIA-labeled semi-oxidized (0.8 mg) and Cys-peptide coupled (0.8 mg) TR-γ subunits were suspended in 600 µl 0.1 M NH_4CO_3, pH 8.3. Labeled proteins were then digested with 2-4% TPCK-trypsin (w/w) at 37 °C for 20 h. An additional amount of trypsin was added after 4 h incubation. The peptide mapping of tryptic peptides was performed on a reverse-phase C_{18} column (4.6 x 250 mm, Vydac) with 0.1% TFA in H_2O as Buffer A, 0.08% TFA in acetonitrile as Buffer B, and at a flow rate of 1 ml/min. The DABIA-labeled peptides were isolated using a linear gradient from 20 to 40% Buffer B in 40 min. Modified peptides containing the DABIA group were detected by simultaneously monitoring the eluant at 215 and 540 nm. The DABIA-labeled peptides were pooled and re-injected onto a reverse-phase C_{18} column for further purification. The purified peptides were submitted to the Protein Facility at Iowa State University for sequencing with an Applied Biosystems 477A automated amino acid sequencer.

Data analysis

Data obtained from the kinetic studies were analyzed with the Enzfitter computer software program (Elsevier Science Publishers).

RESULTS

Reversible inactivation of truncated γ with Cu^{2+}

The divalent transition metal ion, Cu^{2+}, is used as a mild oxidizing reagent because of its positive redox potential (18). As shown in Figure 1 the TR-γ subunit of phosphorylase kinase can be inactivated by Cu^{2+} at pH 7.8 in a range of 1 to 20 μM with an IC_{50} of around 4 μM. The inactivation of TR-γ is partially protected by MgATP but not by protein substrate, Mg^{2+}, or ATP at low concentration of Cu^{2+} (data not shown). At a high concentration of Cu^{2+}, however, inactivation still occurs with or without ligands. The inactivation effect of Cu^{2+} on TR-γ is reversible when treated with DTT (Figure 2). In contrast, the chelating agents, EDTA or EGTA, do not reverse the inactivation (data not shown). These results suggest that the inactivation of the enzyme is likely caused by the formation of a disulfide bond but not by a thiol-metal linkage. Non-reducing SDS-polyacrylamide gel electrophoresis shows that no dimeric or heavier forms of TR-γ can be detected after reaction with Cu^{2+} (Figure 3). This suggests that if cross linking has occurred, as presumed, the reaction occurs through the formation of an intra- but not inter-molecular linkage.

Figure 1. Inactivation of truncated-γ with various amounts of Cu^{2+}. The oxidation of truncated-γ by Cu^{2+} (0.5 to 20 μM) is as described in Experimental Procedures. After oxidation the truncated γ was diluted for serine kinase assay.

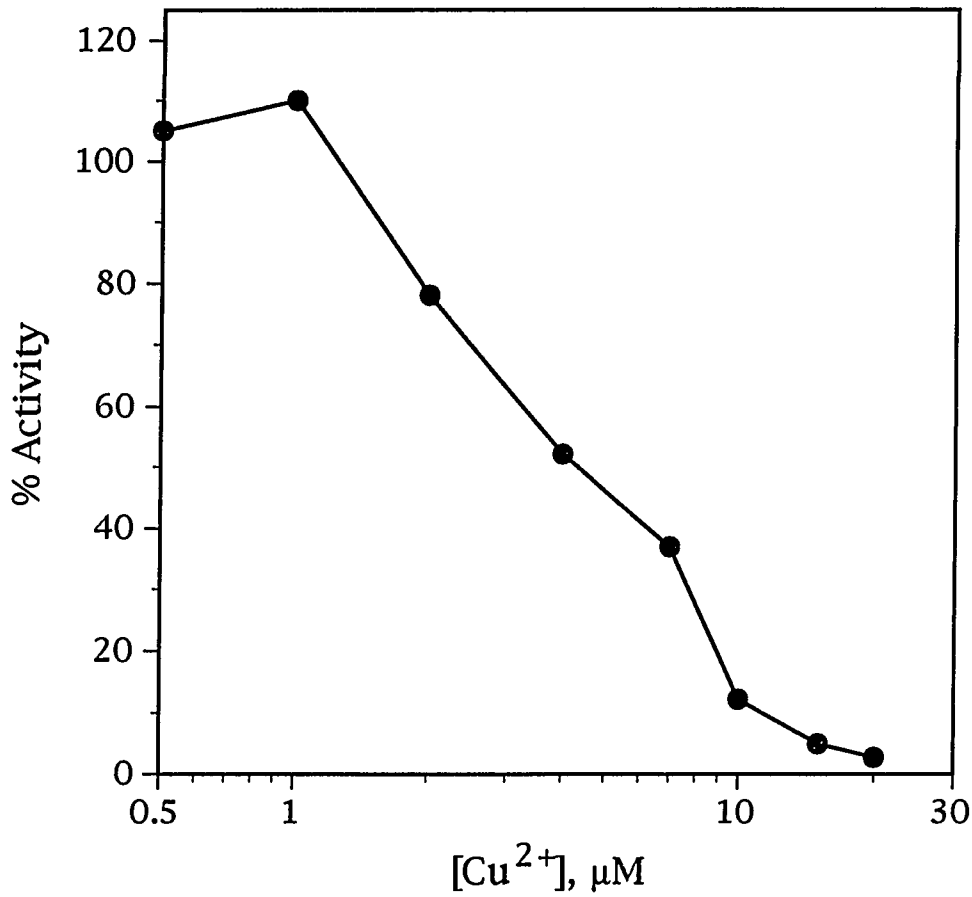

120
100
80
60
40
20
0
% Activity
0.5
1
10
30
[Cu2+], µM

Figure 2. Reversal of the inactivation of the truncated γ subunit by treatment with DTT. The oxidation and reactivation of the truncated γ subunit are as described in Experimental Procedures.

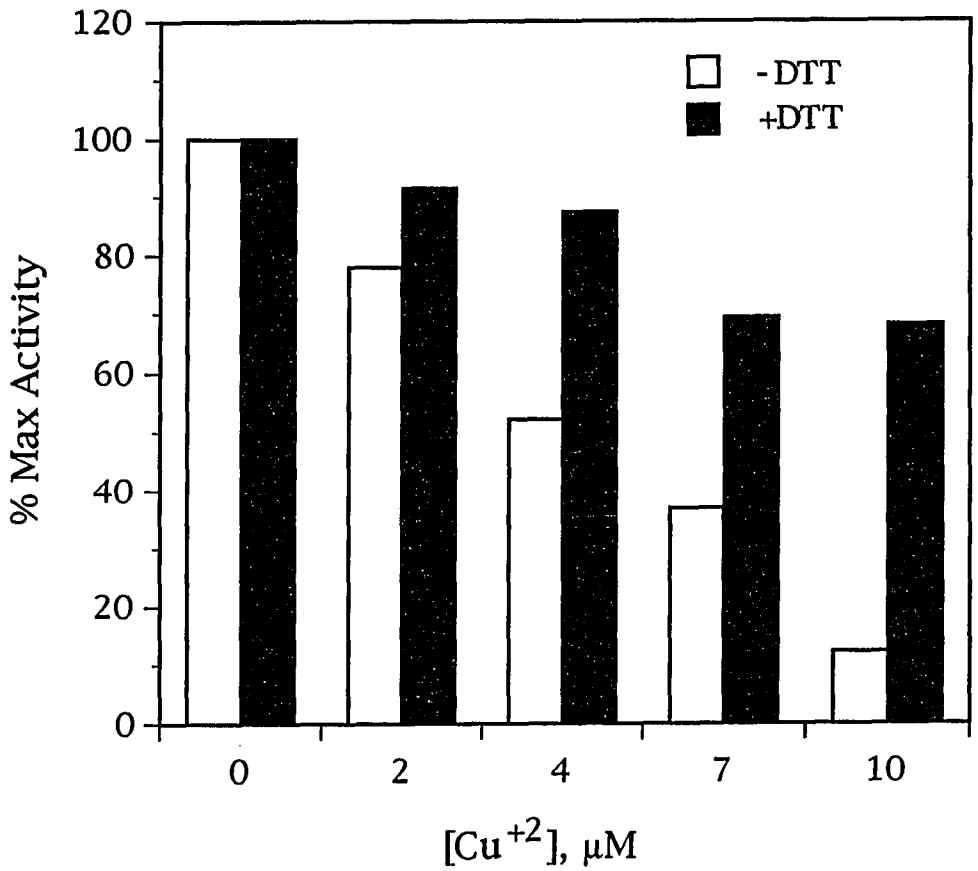
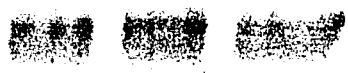

120
100
80
60
40
20
0
% Max Activity
-DTT
+DTT
0
2
4
7
10
[Cu+2], μM

Figure 3. Non-reducing SDS-polyacrylamide gel electrophoresis of the truncated γ subunit with or without the treatment of Cu^{2+}. Truncated γ was oxidized with the indicated amounts of Cu2+ at room temperature for 20 min. Iodoacetamide (50 mM) was added 20 min before electrophoresis to prevent any disulfide exchange. Samples were electrophorsed with a 12.5% non-reducing SDS-polyacrylamide gel. Approximately 5 μg protein was used per lane. Molecular weight markers used from the top are: phosphorylase b (97,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin soybean inhibitor (20,500), and lysozyme (14,500).

Cu^{+2} (μM)	20	8	0	M.W. Markers



Determination of free sulfhydryl groups

The TR-γ subunit of phosphorylase kinase contains 6 cysteines/molecule (11,28). Titration of -SH groups in the reduced TR-γ subunit with DTNB yields 5.8 ± 0.2 SH groups/ molecule in the presence of 6 M urea (Table I) and 3.9 ± 0.2 without urea (data not shown). This observation suggests that two cysteines are buried and inaccessible to DTNB. As shown in Table I the serine kinase activity of the enzyme decreases along with the decrease in SH groups/molecule. When TR-γ is incubated with low concentration of Cu^{2+} (2 and 4 μM) there is one disulfide bond formed as determined by the DTNB titration (3.9 ± 0.1 and 3.6 ± 0.4 SH groups/molecule, respectively, Table I). The serine kinase activity of the enzyme is also inactivated to around 78 ± 1 % and 52 ± 4 %, respectively, compared with that of reduced enzyme. At higher concentration of Cu^{2+} (7 and 10 μM) however, two disulfide bonds are formed as demonstrated by the yields of 2.3 ± 0.2 and 2.1 ± 0.3 SH groups/molecule, respectively. No further change in the number of titratable sulfhydryl groups is found with higher concentrations of Cu^{2+}. These results provided evidence for the view that the inactivation of phosphorylase kinase is caused by the formation of disulfide linkages. The TR-γ subunit oxidized by 4 μM Cu^{2+} is denoted as "semi-oxidized" and used for further characterization.

Table I

Inactivation of truncated γ and DTNB titration @

$[Cu^{2+}]$, μM	% Activity	-SH groups/molecule
0	100 ± 2	5.8 ± 0.2
2	78 ± 1	3.9 ± 0.1
4	52 ± 4	3.6 ± 0.4
7	30 ± 6	2.3 ± 0.2
10	10 ± 2	2.1 ± 0.3

@ The truncated γ subunit is preincubated with Cu^{2+} as indicated. All titrations are performed in the presence of 6 M urea as described in Experimental Procedures . The activity of reduced and oxidized kinase is assayed in the absence of DTT at pH 8.2. The values are means ± S.E. from two independent experiments.

Characterization of semi-oxidized truncated γ

It has been suggested that the inactivation of TR-γ subunit is caused by the formation of disulfide bond. However, why this causes inactivation is unclear. Because MgATP can protect the enzyme from oxidation with a low concentration of Cu^{2+}, the involvement of cysteine residues in the nucleotide binding region is possible. To learn whether Cys residues are involved, a semi-oxidized form of TR-γ was used for kinetic studies. As shown in Figure 4 the metal ion response of the serine kinase activity of the semi-oxidized form is about the same as that of the reduced form except that some inhibition is observed with 10 mM or higher Mg^{2+}. Because of this slight inhibition, 5 mM Mg^{2+} was used in the kinetic studies of semi-oxidized TR-γ. As shown in Table II the K_m with respect to phosphorylase b of the semi-oxidized form (18.0 ± 0.2 μM) is the same as that of the reduced form (18 ± 1 μM)(21). The K_m for ATP (780 ± 50 μM), however, is about 10-fold higher than that of the reduced one (79 ± 10 μM). The V_m of the semi-oxidized form (3.4 ± 0.4 μmol/min/nmol) is similar to that of reduced enzyme (4.10 ± 0.03 μmol/min/ nmol). This result suggests that there are two cysteines near by or in the nucleotide binding site which are needed in their free state for optimal binding.

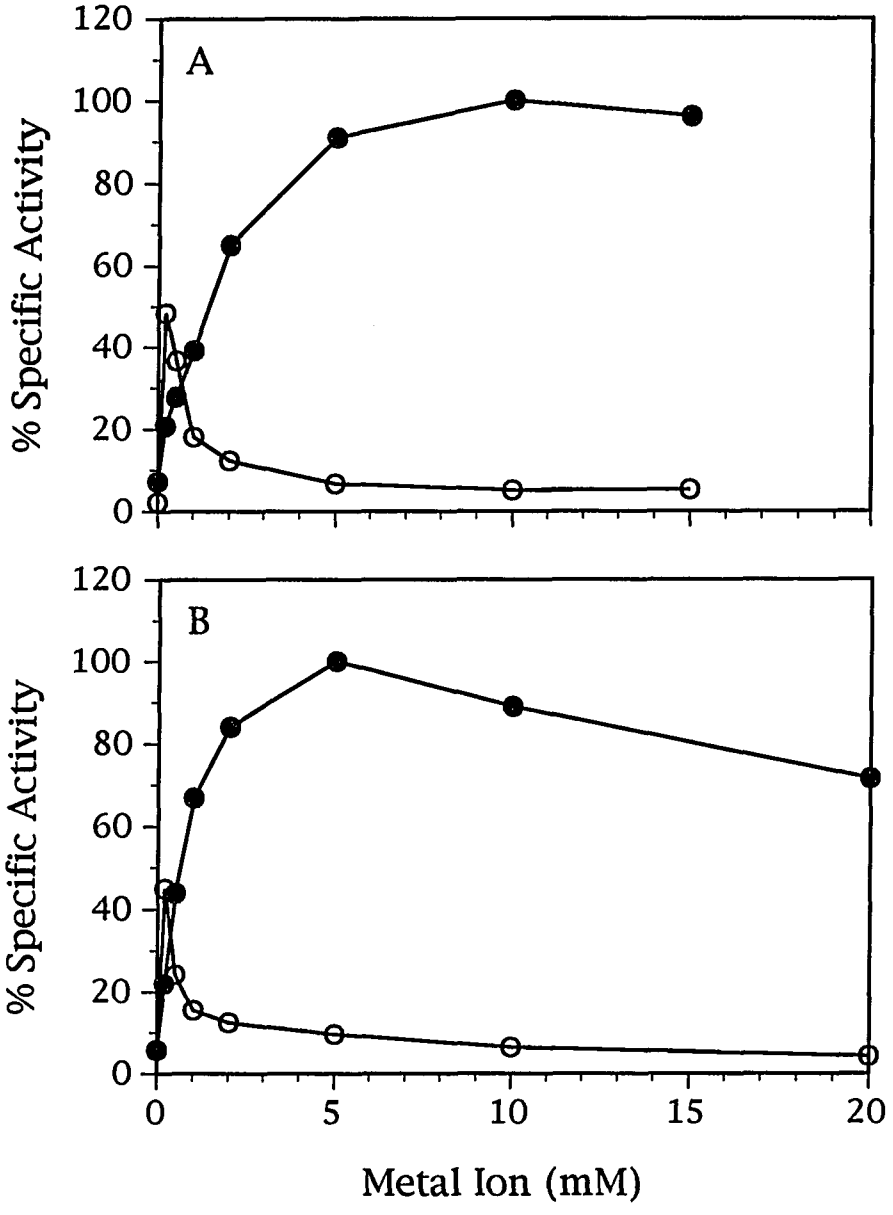
Table II

Comparison of Kinetic Parameters of Reduced and Modified Truncated γ Subunit of Phosphorylase Kinase

TR-γ	Phos b (μM)		ATP (μM)		V_m (μmol/min/nmol)
	K_a	K_{ia}	K_b	K_{ib}	
Reduced@	18 ± 1	24 ± 3	79 ± 10	105 ± 21	4.10 ± 0.03
Semi-Oxidized	18.0 ± 0.2	22.4 ± 0.1	780 ± 50	981 ± 25	3.4 ± 0.5
Cys-peptide Coupled	39 ± 2	37 ± 1	143 ± 16	135 ± 7	1.5 ± 0.2

@ See Huang, C.-Y. F. et al. (1993) Mol. Cell. Biochem. (in press).

Figure 4. Metal ion response of the reduced and semi-oxidized truncated γ subunit. (A) Reduced form of truncated-γ. (B) Semi-oxidized form of truncated γ was prepared by incubating with 4 μM of Cu^{2+} as described in Experimental Procedures. Metal ion response curves are done by determining serine kinase activity of truncated γ in the presence of 0.2 mM [γ-^{32}P]-ATP and various amount of Mg^{2+} (-●-) or Mn^{2+} (-O-) as indicated.


A
B
% Specific Activity
% Specific Activity
120
100
80
60
40
20
0
0
5
10
15
20
Metal Ion (mM)

Inactivation of truncated γ with Cys-peptide dimer

The Cys-peptide was synthesized according to the phosphorylation site sequence of phosphorylase b (^{9}LysArgLysGlnIle<u>Ser</u>ValArgGlyLeu^{18}) with cysteine instead of serine at the phosphorylation site. This peptide is not a substrate of phosphorylase kinase. The SH group of Cys-peptide can be easily oxidized and cross-linked to the thiol group of the second Cys-peptide, termed Cys-peptide dimer, in the absence of DTT. As shown in Figure 5 about 90% of serine kinase activity of the enzyme is lost upon the incubation of the enzyme with the Cys-peptide dimer. No inactivation occurred if DTT was present during incubation. The inactivation can easily be reversed by treatment with 50 mM DTT (Figure 6) suggesting a thiol/disulfide exchange has occurred between the Cys-peptide dimer and protein. To evaluate the mechanism of inactivation by the Cys-peptide, the kinetic parameters of the kinase reaction catalyzed by Cys-peptide-coupled TR-γ was studied (Table II). The K_a for phosphorylase b and K_b for ATP (39 ± 2 and 143 ± 16 μM, respectively) were around 2-fold higher than those of wild type TR-γ. The V_m, however, is about 7-fold lower (0.60 ± 0.07 μmol/min/nmol). These results suggest that the thiol group coupled with Cys-peptide is not crucial for binding of either substrate. The SH group might not even be in the active site. However, the coupling of Cys-peptide to TR-γ

Figure 5. The inactivation of the truncated γ subunit by a Cys-peptide dimer. Cys-peptide dimer is prepared by incubating peptide solution at 30 °C under air for around 24 h. The dimerization of Cys-peptide was confirmed by DTNB titration. The DTT-free truncated γ subunit (0.1 mg/ml) is incubated with 0.5 mM Cys-peptide dimer at pH 7.8 in the presence or absence of 1 mM DTT at room temperature for approximately 20 min. The serine kinase assay is then carried out as described in Experimental Procedures.

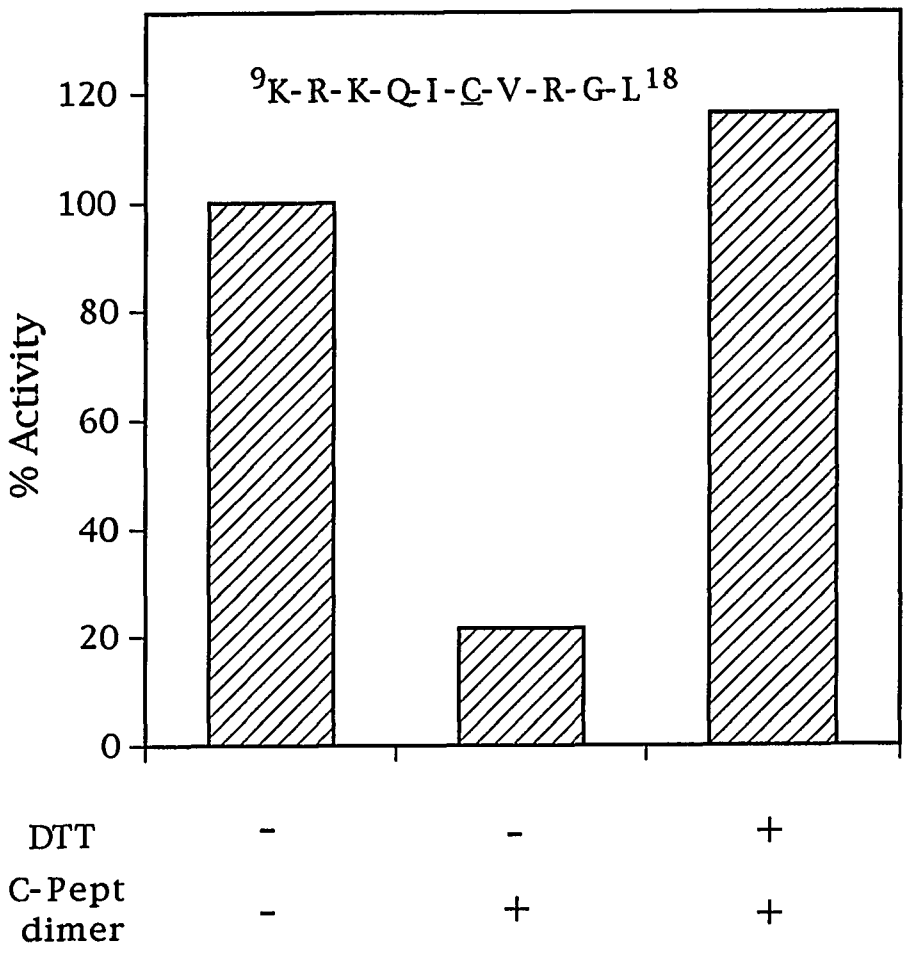

^{9}K-R-K-Q-I-C-V-R-G-L^{18}
% Activity
120
100
80
60
40
20
0
DTT
C-Pept dimer
-
-
+
-
+
+

Figure 6. Reactivation of Cys-peptide coupled truncated γ by DTT. The reactivation of Cys-peptide coupled truncated γ's is carried out by mixing equal volume of Cys-peptide coupled truncated γ with 10 mM DTT on ice for 1 hr. Activity is measured as described in Experimental Procedures.

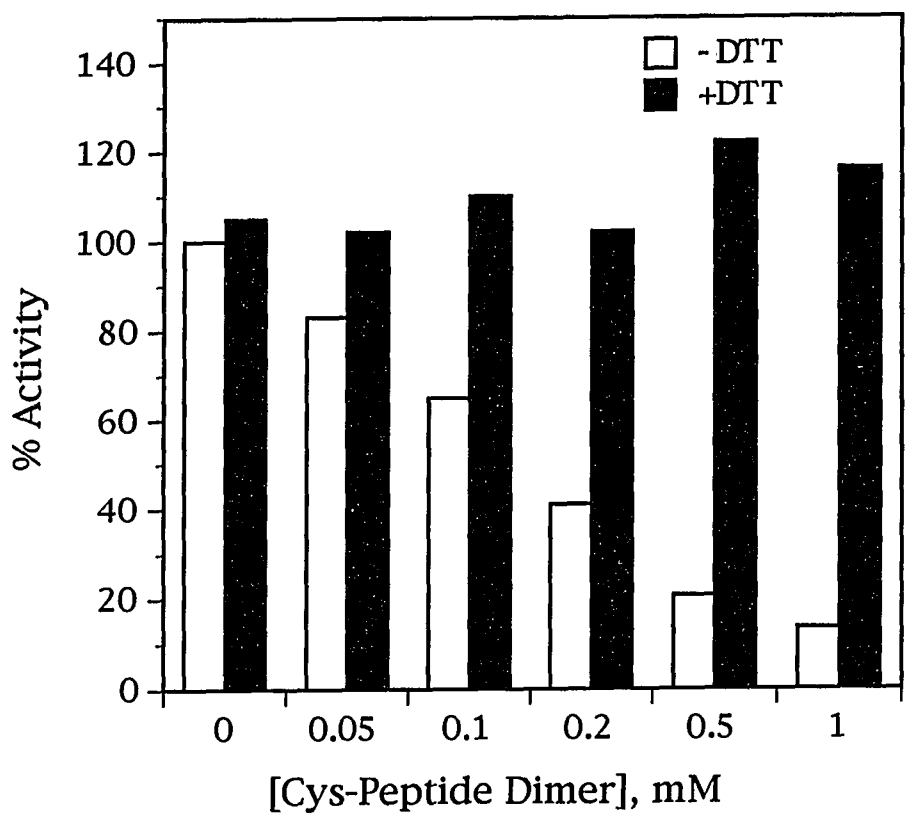
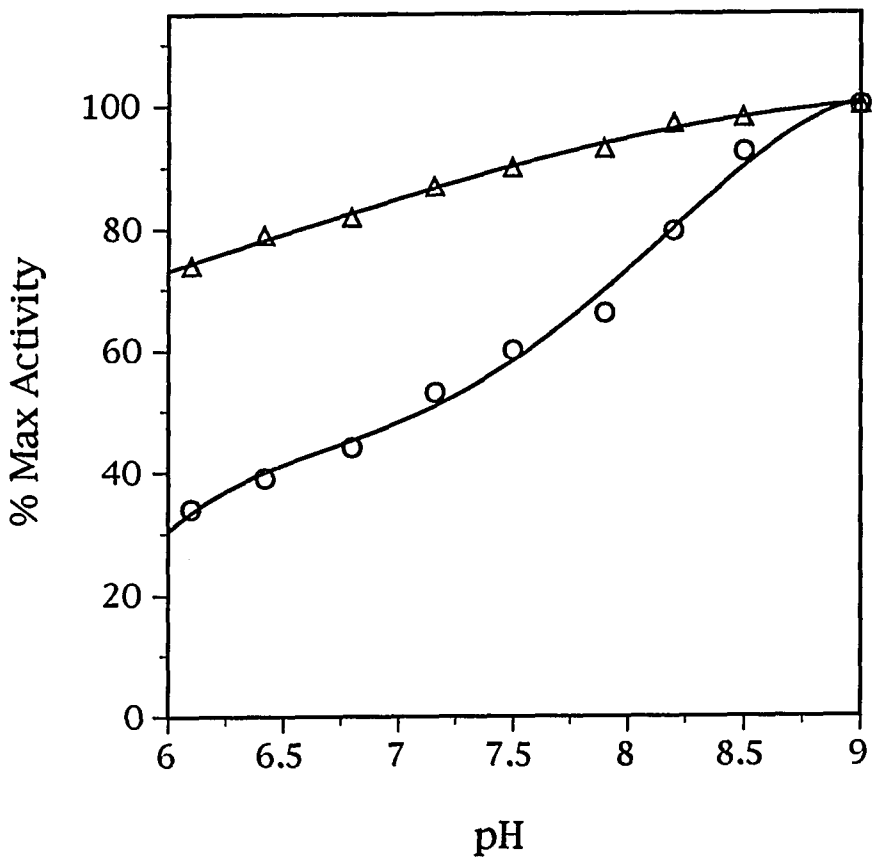

-DTT
+DTT
140
120
100
80
60
40
20
0
% Activity
0
0.05
0.1
0.2
0.5
1
[Cys-Peptide Dimer], mM

Figure 7. The pH curves of reduced and Cys-peptide coupled truncated γ. The pH-dependent curves of reduced (-Δ-) and Cys-peptide-coupled (-O-) truncated γ was done by assaying the serine kinase activity under various pH (6.1 to 9.0). The activity assay is performed as described in Experimental Procedures.


% Max Activity
100
80
60
40
20
0
6
6.5
7
7.5
8
8.5
9
pH

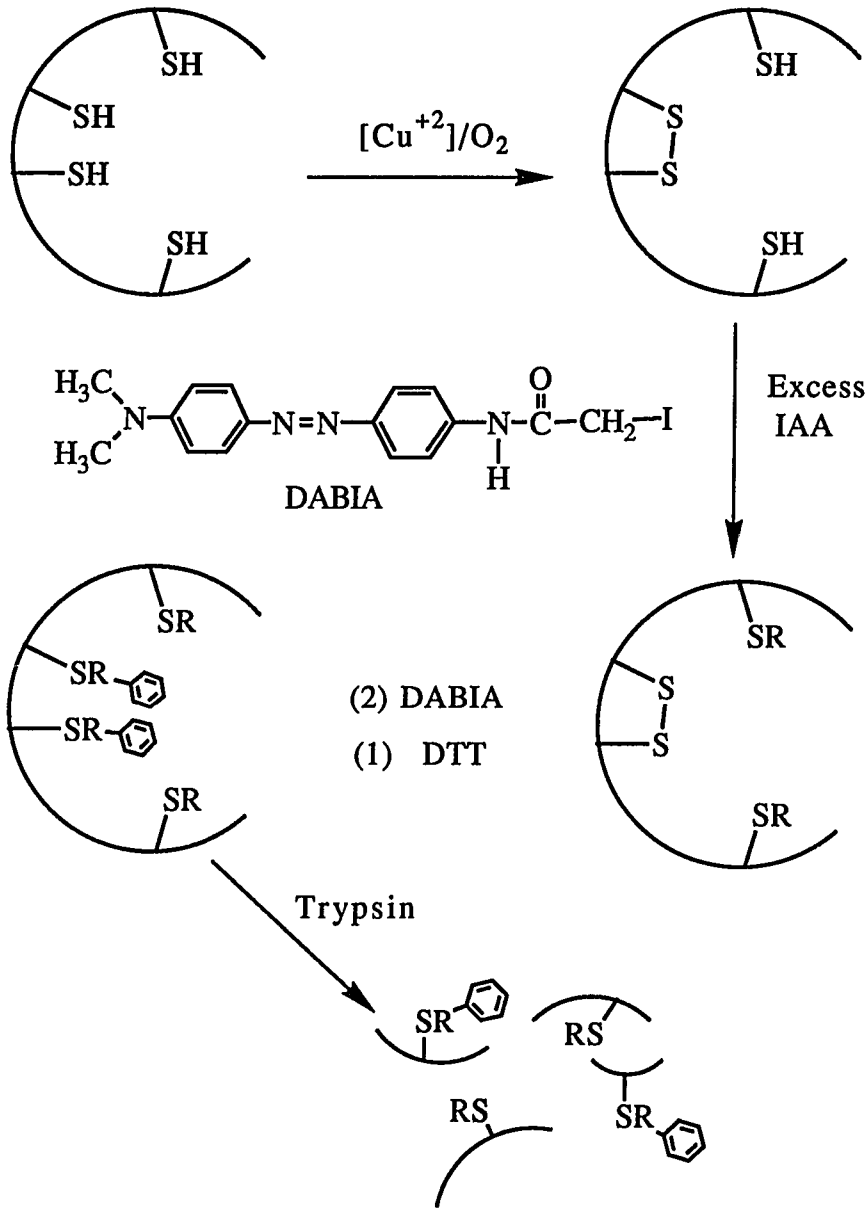
could restrict the conformational change of TR-γ to its optimal form. This speculation is suggested by results of the pH activity curves of the reduced and Cys-peptide coupled TR-γ subunit (Figure 7). The Cys-peptide-coupled TR-γ subunit shows a distinct pH curve from that of reduced TR-γ. The change of the pH curve in Cys-peptide-coupled TR-γ suggests that the environment of its catalytic base is different from that of the reduced form. To evaluate whether the effect is specific for the phosphorylase b sequence, the TR-γ was incubated with another disulfide peptide, glutathione disulfide. In contrast, glutathione disulfide did not inhibit the enzymatic activity of DTT-free TR-γ (data not shown).

Identification of -SH groups in truncated γ involved in oxidation and Cys-peptide coupling

The previous results suggest that the formation of intramolecular disulfide bonds in or near the active site region inhibits kinase activity. The location of these sulfhydryl groups involved in cross-linking reactions may help us to understand the conformation of the active site region of γ and facilitate the comparison with the 3D structure of cAPK (13-16). To identify the cysteinyl residues involved in disulfide bond formation, DABIA was used. DABIA has been demonstrated to be useful in labeling

and identification of cysteinyl containing peptides (18,27). However, to trap faithfully the original disulfides it is important to prevent thiol/disulfide exchange reactions, especially in the presence of strong denaturants, urea and guanidine HCl (29). Scheme I outlines the procedures we used to identify the vicinal sulfhydryl groups in TR-γ. After incubating with Cu^{2+} or Cys-peptide dimer the remaining free -SH groups in TR-γ are quenched by alkylation with excess amounts of iodoacetamide to eliminate any thiol/disulfide exchange. The alkylation is terminated by precipitating modified protein with 20% TCA. The protein pellet is, then, re-dissolved and treated with DTT at pH 8.0. The disulfide bond is reduced in this stage and newly formed free SH groups are ready for labeling with DABIA .

The isolation of DABIA-labeled peptide can easily be followed by monitoring the absorbance at 540 nm as shown in Figure 8. A tryptic digestion of both Cys-peptide coupled TR-γand the semi-oxidized TR-γ subunit yield two major peaks (C-1, C-2, S-1 and, S-2; Figure 8), respectively. The corresponding peptide sequences are shown in Table III. The Cys-138 forms a disulfide bond with Cys-peptide. This result along with the kinetic results suggests that a cysteinyl outside of active site region might be involved in cross-linking with Cys-peptide.


SH
SH
SH
SH
[Cu+2]/O2
S
S
SH
SH
H3C
N
H3C
N=N
N-C-CH2-I
O
H
DABIA
Excess
IAA
SR
SR
SR
SR
(2) DABIA
(1) DTT
S
S
SR
SR
Trypsin
SR
RS
RS
SR

Scheme I

Figure 8. Peptide mapping of the tryptic DABIA-labeled truncated γ subunit. (A) Cys-peptide coupled truncated γ. (B) Semi-oxidized form of truncated γ. Separation of DABIA-labeled peptides is performed as described in Experimental Procedures. The elution profile is monitored at 540 nm.

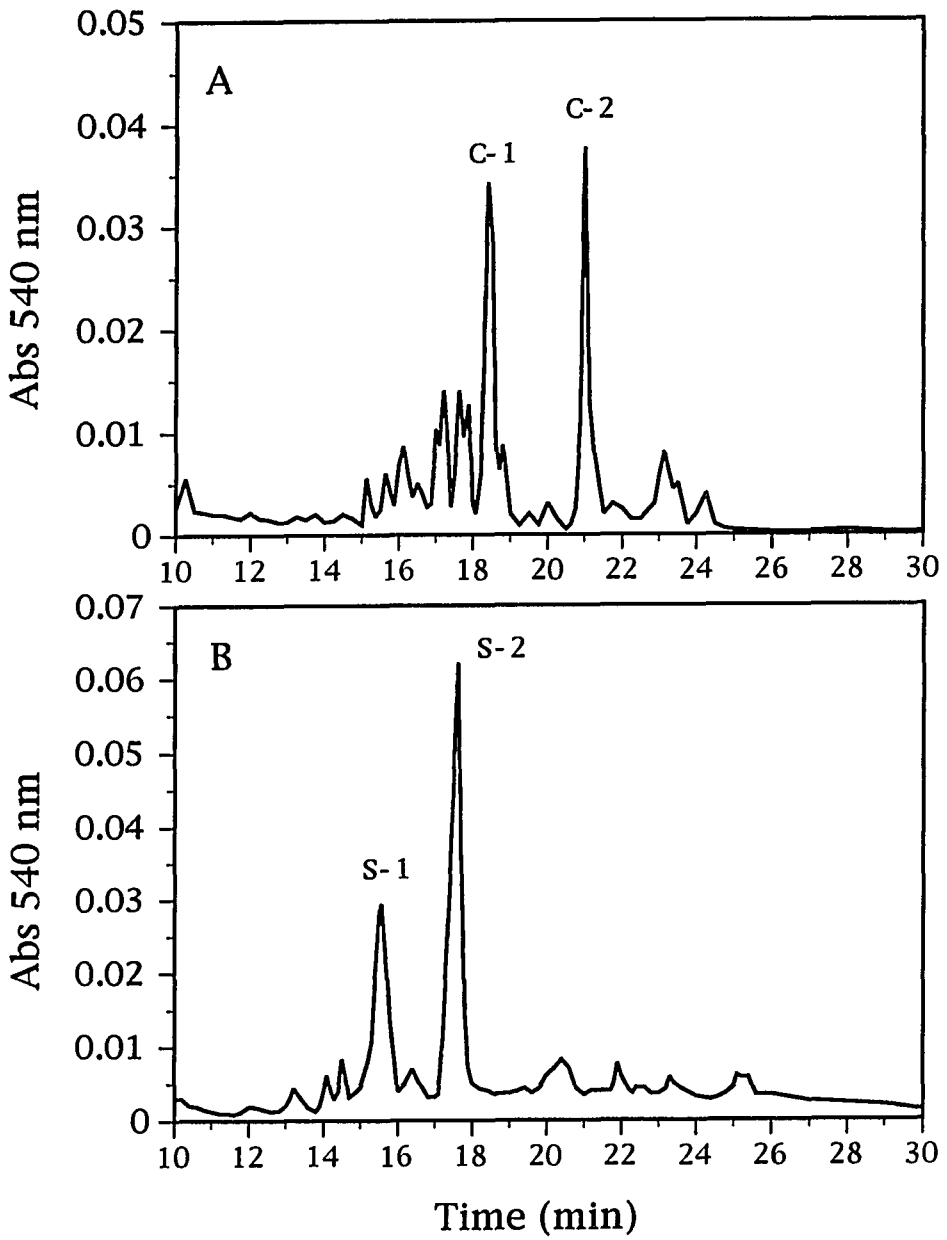

A
C-1
C-2
B
S-1
S-2
Abs 540 nm
Abs 540 nm
Time (min)
0.05
0.04
0.03
0.02
0.01
0
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
10 12 14 16 18 20 22 24 26 28 30

Table III

Sequences of DABIA-labeled Cys containing peptides from semi-oxidized and C-peptide coupled TR-γ

Peptide	Sequence
C-1@	^{12}Q-I-C-V-R^{16}
C-2	^{132}A-L-L-E-V-I-C-A-L-H-K^{142}
S-1	^{36}C-I-H-K^{39}
S-2	^{165}L-T-D-F-G-F-S-C-Q-L-D-P-G-E-K^{179}

@ Peptide C-1 is from Cys-peptide.(^{9}K-R-K-Q-I-C-V-R-G-L^{18}) of phosphorylase.

The first disulfide bond is formed between Cys-36 and Cys-172 (Table III). Both thiols are in the active site region of TR-γ and close to the nucleotide binding domain as predicted by the 3D structure of cAPK (13-16). This result is consistent with the kinetic study of the semi-oxidized TR-γ subunit (Table I). The nucleotide binding site could be shielded by the formation of a disulfide bond between Cys-36 and Cys-172. This result may explain the high K_m value of the semi-oxidized TR-γ for ATP. However, the possibility that both or one of these two thiols are involved in the recognition and binding of the nucleotide substrate can not be ruled out.

DISCUSSION

The transition metal ion, Cu^{2+}, an oxidizing agent, has been demonstrated by this work and other studies (18) to be useful for promoting disulfide reactions between neighboring thiol groups. The oxidation by Cu^{2+} results in the loss of activity of TR-γ. The reducing reagent, DTT, but not metal chelating reagents, EDTA and EGTA, reverse the inactivation effect of Cu^{2+} on the TR-γ subunit suggesting that inactivation is caused by the formation of -S-S- bridge(s). DTNB titrations of reduced and Cu^{2+} oxidized TR-γ indicates that four out of six free sulfhydryl groups are involved in the disulfide bonds formation (Table I). These four free thiols formed two intra- but not inter-molecular disulfide bonds demonstrated by the fact that the reduced and oxidized truncated γ subunit migrate the same distance on a non-reducing SDS-polyacrylamide gel. The oxidation inhibits the activity of TR-γ suggesting that these free thiols may be in or near the active site region of the catalytic subunit of phosphorylase kinase and must be spatially close.

According to the crystal structure of cAPK (13,14) and primary sequence alignment (11) four free sulfhydryl groups, Cys-36, Cys-172, Cys-184 and Cys-197, which are likely for Cu^{2+} oxidation and disulfide formation. Among these free thiols Cys-36 and Cys-172 are close to the nucleotide binding

site and have been demonstrated to be one vicinal thiols pair. Although the second disulfide bond has not been solved in this study, Cys-184 and Cys-197 are expected to be the candidates based on studies of the Cys-mutants (30). No other SH groups could be expected to be spatially close based on the 3D structure of cAPK (13-16).

Recently, a flexible active site region of cAPK has been demonstrated by several studies (31-33). The small lobe of cAPK can rotate 15-39° relative to the large lobe upon the binding of substrates or inhibitors. Without the binding of substrate the active site cleft is wide open, termed the open structure, and allows substrates free access (31-33). Once substrate binds a sort of "induced fit" may occur in the active site region and bring those conserved residues in the small lobe back to an optimal position, termed the closed structure, for interaction with the substrate. This conformational change may alter the local structure of the catalytic base and, hence, the catalysis of the kinase. The γ subunit could have a similar flexible structure in the absence of substrates. However, the small lobe of γ may have a larger movement toward the large lobe if a disulfide bond is formed between Cys-36 and Cys-172. Accordingly, the active site cleft of γ between small and large lobes may be narrower than that in the crystal structure of cAPK ternary complex (8-10), because Cys-36 is located on the small lobe

and Cys-172 on the large one. The flexible active site cleft can be fixed by the binding of substrates. This speculation is supported by the observation that MgATP can partially protect TR-γ from oxidation by Cu^{2+}.

Cys-138 in the TR-γ subunit must be relatively exposed as indicated by the fact that this residue but not others, e.g., Cys-184, can be easily cross-linked to Cys-peptide through thiol/disulfide exchange. Cys-199 of cAPK, corresponding to Cys-184 of γ, has been demonstrated to be the primary target of various thiol specific agents and peptide analog (34,35). This result is consistent with the observation that Glu-155 in the E-helix of cAPK, corresponding to Cys-138 of γ, is on the side facing the acyl-binding pocket and plays a role in the interaction with the myristyl group (33,36). This acyl-binding pocket is surrounded by the A-helix, E-Helix, a loop connecting C-helix and β-sheet 4, and part of C-terminal tail. In TR-γ, however, there is no A-helix and C-terminal tail in this region and could make Cys-138 wide open and easily accessible for a reaction with the Cys-peptide. The myristyl group in cAPK has been suggested to stabilize protein structure (33). Meanwhile, the cross-linking of Cys-peptide to Cys-138 of TR-γ may provide a steric hindrance and restrict the conformational change in the active site region. The flexibility in the active site region of the catalytic subunit of phosphorylase kinase seems important for its

phosphotransferase activity. This speculation is suggested by the observation that Cys-peptide-coupled TR-γ shows a sigmoid pH-activity curve while that for the wild type kinase is relatively flat (Figure 7). A higher pK_a value for the catalytic base in the Cys-peptide coupled TR-γ subunit is suggested by this result. Accordingly, the environment of the catalytic base in Cys-peptide coupled TR-γ could become more hydrophobic because of the coupling of Cys-peptide. Without the restriction provided by the coupled Cys-peptide TR-γ may adjust its active site region more effectively to an optimal conformation to expose its catalytic base as well as to accommodate the incoming substrates.

It is also possible that Cys-138 in γ may play a similar role by interacting with either regulatory subunit, α or β, or with its C-terminal calmodulin binding domain. The interaction of the regulatory subunits or C-terminal calmodulin binding domain to this region may regulate the phosphotransferase activity of the γ subunit. This speculation is partly suggested by the results that TR-γ has higher V_m and lower K_m values for both substrates (21) than those of various forms of phosphorylase kinase (10,37-39). However, further characterizations are needed to determine whether this region is involved in the interaction with other subunits.

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PAPER 3 CHARACTERIZATION OF CYS-MUTANTS OF THE CATALYTIC SUBUNIT OF PHOSPHORYLASE KINASE

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ABSTRACT

To determine the function of each free sulfhydryl group in truncated γ subunit of phosphorylase kinase all six thiols were replaced with serine by site-directed mutagenesis, i.e., C36S, C42S, C138S, C172S, C184S, and C197S. In addition, C36A mutant was also prepared. After Cu^{2+} oxidation, DTNB titration indicated that the number of free sulfhydryl groups of all but C42S and C138S mutants was decreasing by two. These results suggest that Cys-36, -172, -184, and -197 are involved in the formation of disulfide bonds in wild type truncated γ by the oxidation with Cu^{2+}. All mutants except C36S and C184S show normal serine and tyrosine kinase activities. The kinetic studies show that C36S has a similar K_m for phosphorylase b but its K_m for ATP is about 2-fold higher. The V_m, however, is about 8-fold lower than that of wild type truncated γ. These results suggest that Cys-36 may not be directly involved in the binding of ATP or phosphorylase b. This mutation also reduces tyrosine kinase activity greatly. The mutant C184S is particular interesting because tyrosine kinase activity is affected much more by this mutation than is the serine kinase activity. The tyrosine kinase activity of C184S is about 20-fold lower by comparison with wild type truncated γ. C184S and wild type truncated γ have similar V_m values and K_m's for ATP. The K_m

with respect to phosphorylase b of C184S, however, increased about 3-fold compared to that of wild type truncated γ. In the presence of Mn^{2+} angiotensin II inhibits C184S in a competitive manner with respect to peptide substrate, containing residues 9-18 of phosphorylase b. In the presence of Mg^{2+}, however, angiotensin II inhibits both wild type and C184S truncated γ as a non-competitive inhibitor. These studies showed that angiotensin II bound differently to the truncated γ subunit. Further studies suggest that C184S has a different preference for peptide substrates for tyrosine kinase activity, i.e., CDC2 peptide, RR-Src peptide, and Y-peptide, from that of wild type truncated γ. These results suggest that Cys-184 may play roles in the determination of substrate specificity of tyrosine kinase activity.

INTRODUCTION

Phosphorylase kinase is a complex enzyme composed of four subunits, α, β, γ, and δ, with a stoichiometry of $(\alpha\beta\gamma\delta)_4$ (1-3). It has been demonstrated that the γ subunit contains the active site and catalyzes the transfer of the γ-phosphoryl group from ATP to its acceptor, Ser-14 of phosphorylase b. The N-terminal region is partly homologous with the catalytic domain of cAMP-dependent protein kinase (cAPK) and other kinases (4). The C-terminal region, on the other hand, is thought to contain the calmodulin binding domain (5,6). There are seven cysteine residues in the γ subunit (7) with six out of the seven free thiols in the catalytic domain (Figure 1). Four of them (Cys-36,-172,-184, and -197) are located in or near the active site region, predicted from the sequence alignment (4) and the crystal structure of cAMP-dependent protein kinase (cAPK)(8-10). This is presumed to be the case if the structure of truncated γ is closely related to cAPK.

Previous studies (11) showed that the oxidation of the free sulfhydryl groups resulted in the formation of two intramolecular disulfide bonds. One of these disulfide bridges seems close to the nucleotide binding site and could insert a hindrance to the nucleotide binding. However, whether one or both cysteine residues are involved in the

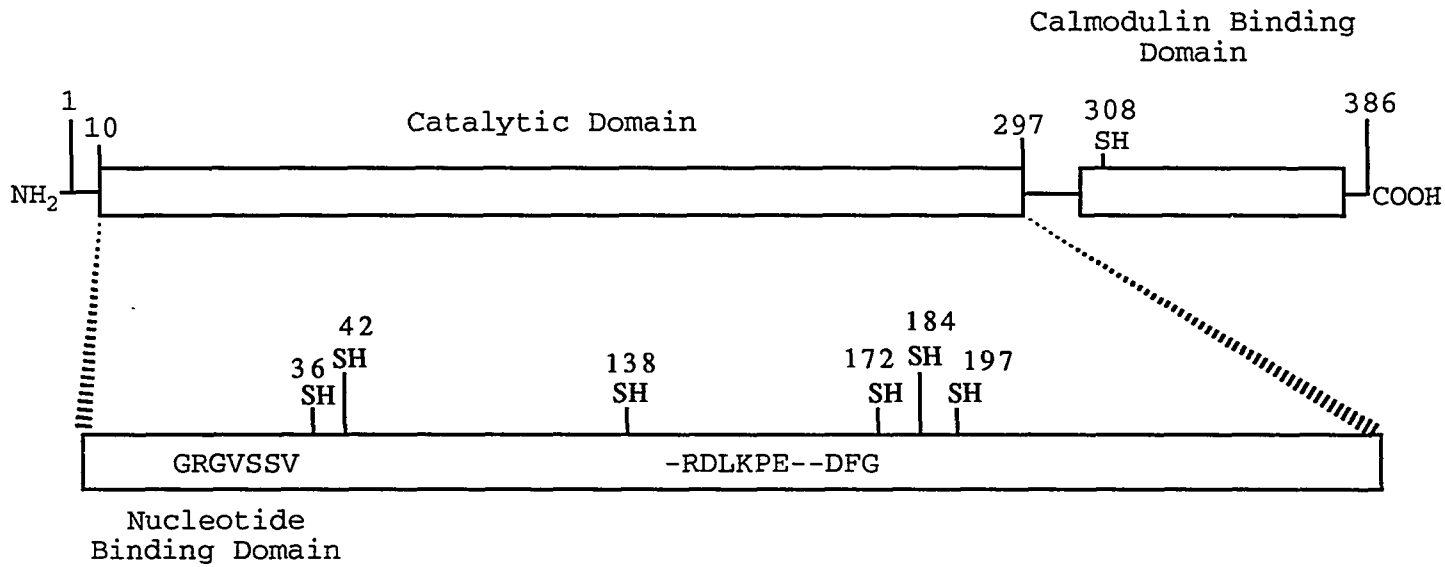

Figure 1. Location of Cys residues in the γ subunit of phosphorylase kinase

nucleotide binding and/or recognition is not clear. In CaM-dependent protein kinase II, Cys-30, which corresponds to Cys-36 of γ, is suggested to be involved in the binding of ATP (12). This speculation was based on the observation that MgADP could protect CaM-protein kinase II from inactivation by DTNB and N-ethylmaleimide.

The truncated form of γ subunit (1-300), denoted as TR-γ, was constructed and expressed in our laboratory (13). Previous studies suggest that TR-γ has properties, such as metal ion response, pH-dependency, and K_m values with respect to both substrates, similar to those of full length γ but with a higher turnover rate (13). Most importantly, TR-γ allows us to characterize the catalytic properties without the influence of the C-terminal calmodulin binding domain. Therefore, all of Cys-mutants were made in truncated γ (1-300) form. Recently, phosphorylase kinase was found to contain tyrosine kinase activity and phosphorylate angiotensin II (14). The tyrosine kinase activity of Cys-mutants was also tested to learn whether substitution influences this newly identified activity of phosphorylase kinase. The first intra-molecular disulfide bond formed by oxidization has been localized by peptide mapping and sequencing (11). The location of the second dithiol bridge can help us to learn

more about the structure of γ. To identify the second disulfide bond, Cys-mutants were oxidized with Cu^{2+} and the number of remaining free sulfhydryl groups were determined by DTNB titration.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes were products of New England Biolabs or Promega. Angiotensin II, [Sar^1,Ala^8]-angiotensin II, and [Asn^1,Val^5]-angiotensin II were purchased from Sigma. CDC2 and RR-Src peptides were kind gifts of Dr. Lee Graves. A peptide of residues 9-18 of phosphorylase *b*, phos 9-18 peptide, and its tyrosine analog, Y-peptide, were synthesized by the Protein Facility at Iowa State University. Phosphorylase *b* was prepared as described (15). [γ-^{32}P]ATP was obtained from ICN. Econo Pac-10DG gel filtration column was from Bio Rad. Phosphocellulose and ET-31 papers were from Whatman. DTNB was purchased from Pierce. All other reagents were reagent grade.

Oligonucleotide-directed site-specific mutagenesis

Olignucleotides used in site-directed mutagenesis were synthesized in the Nucleic Acid Facility at Iowa State University. Mutagenesis was performed using an Amersham commercial kit as described previously (13). Mutations were identified by restriction enzyme analysis and verified by sequencing. The Cys-mutants prepared in this study are C36S, C36A, C42S, C138S, C172S, C184S, and C197S.

Expression and Purification of Cys-mutants

Expression of wild type and Cys-mutants of TR-γ was carried out by using a T7 RNA polymerase-based expression system (16). All forms of TR-γ were expressed as inclusion bodies. The inclusion bodies of recombinant proteins were solubilized, renatured, and purified as described previously (13).

Oxidation of Cys-mutants by copper ion

The oxidation of Cys-mutants is essentially as described by Yuan and Graves (11) with some modification. The Cys-mutants, after removing DTT, (0.2 mg/ml) were oxidized at pH 7.8 by incubating with 16 μM $CuCl_2$ at room temperature for approximately 20 min. After incubation the oxidized proteins were mixed with 1 mM EDTA to stop the reaction before subjecting them to titration with DTNB.

Determination of free sulfhydryl group

The number of titratable sulfhydryl groups of the Cys-mutants was determined by DTNB titration as described previously (11). Approximately 190 μl protein (0.2 mg/ml) with or without Cu^{2+} treatment were mixed with solid urea. An appropriate amount of 0.4 M Na_2HPO_4, pH 8.0 was added to give a total volume of 400 μl. The final concentration of urea and phosphate is 6 M and 0.1 M, respectively. The

reaction was initiated by the addition of DTNB to 1 mM. The mixture was incubated at room temperature for 15 min before measurement of absorbance at 414 nm. The concentration of sulfhydryl groups was calculated by using ε_{414} = 13,600 $M^{-1}cm^{-1}$ (17).

Activity assay

The serine kinase activity of wild type TR-γ and Cys- mutants is determined by the incorporation of ^{32}P into phosphorylase b. The assay is performed at pH 8.2 in the presence of 60 mM Tris-HCl, 60 mM HEPES, 10 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, and 100 μM phosphorylase b. The final concentrations of TR-γ and mutants are adjusted to around 0.6 nM. After incubating at 30 °C for 3 min a portion of aliquot is spotted on a square ET-31 paper (Whatman), washed with trichloroacetic acid, and analyzed by liquid scintillation counting (18). The tyrosine kinase activity is assayed as described previously (14). The reaction is performed in an assay mixture containing 10% glycerol, 50 mM Tris, 50 mM PIPES, pH 7.9, 3 mM $MnCl_2$, 4 mM angiotensin II, and 1 mM [γ-^{32}P]-ATP. The final concentrations of the truncated γ subunit and mutants were 20 μg/ml. The tyrosine kinase assay is allowed to proceed at 30°C for 15 min. After incubation, a portion of the reaction mixture is spotted on the phospho-cellulose paper and washed with cold 0.5% phosphoric acid.

Miscellaneous methods

The concentration of phosphorylase is determined spectrophotometrically (19). The concentrations of TR-γ and its mutants are determined by the Bradford assay with commercially prepared reagent from Bio-Rad (20). Peptide solutions are prepared based on their dry weight. SDS-polyacrylamide gel electrophoresis is carried out with Laemmli's single buffer system (21)

Kinetic data analysis - The kinetic data are analyzed with computer software Enzfitter (Elsevier Science Publishers). The initial velocity data are fitted to the equation for a two substrate random Bi Bi mechanism (eq 1)

$$1/v = 1/V\ (1 + K_a/A + K_b/B + K_{ia}K_b/AB) \quad \text{(eq 1)}$$

The inhibition data are fitted into the equations for competitive (eq 2) or non-competitive inhibition (eq 3).

$$1/v = 1/V\ [1 + K_a/A\ (1 + I/K_{is})] \quad \text{(eq 2)}$$

$$1/v = 1/V\ [(1 + I/K_{ii}) + K_a/A\ (1 + I/K_{is})] \quad \text{(eq 3)}$$

K_a and K_b are the Michaelis constants for substrates A (phosphorylase) and B (ATP), respectively. K_{ia} and K_{ib} are defined as the dissociation constants for A and B from their respective binary complexes with the kinase. Kis and Kii are defined as: Kis = (I)(E • ATP)/(E • ATP • I), and Kii = (I)(E • ATP • Phos)/(E • ATP • phos • I), respectively.

RESULTS

Primary characterization of Cys-mutants

To determine the function of each free sulfhydryl group, especially those in the active site region, Cys residues were replaced with serine by site-directed mutagenesis. All Cys-mutants can be renatured and purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis (Figure 2). The serine and tyrosine kinase activities of wild type and Cys-mutants are also compared (Figure 3). All Cys-mutants except C36S and C184S show similar kinase activities to those of wild type TR-γ (data for C36A not shown). Both serine and tyrosine kinase activities are low in C36S (2-3%). Interestingly, C184S has near normal serine kinase activity but shows only about 4-5% tyrosine kinase activity compared with that of wild type TR-γ.

Like wild type TR-γ, all mutants can be partly inactivated by the oxidation of Cu^{2+} (data not shown). The number of free sulfhydryl groups of each Cys-mutant after oxidization is also determined by DTNB titration (Table I). All but two Cys-mutants, C42S and C138S, show the number of titratable sulfhydryl groups decreasing by two (or one disulfide bond formed) after treating with 16 μM Cu^{2+}. This result suggests that the four free thiols, Cys-36, -172, -184, and -197, are

Figure 2. SDS-polyacrylamide gel electrophoresis of wild type truncated γ and Cys-mutants. Around 10 μg protein was used per lane. Samples were electrophorsed with 12.5% polyacrylamide gel.

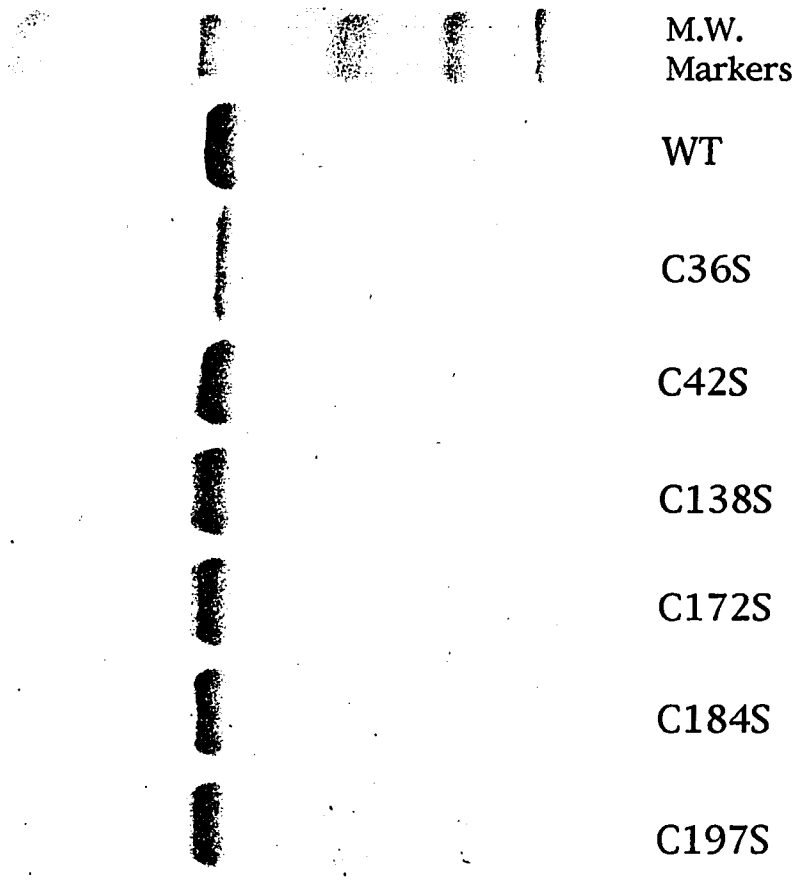
M.W.
Markers
WT
C36S
C42S
C138S
C172S
C184S
C197S

Figure 3. Serine and tyrosine kinases activity assay of wild type truncated γ and Cys-mutants. Serine (open bar) and tyrosine kinases (close bar) activity assays were carried out as described under Experimental Procedures.

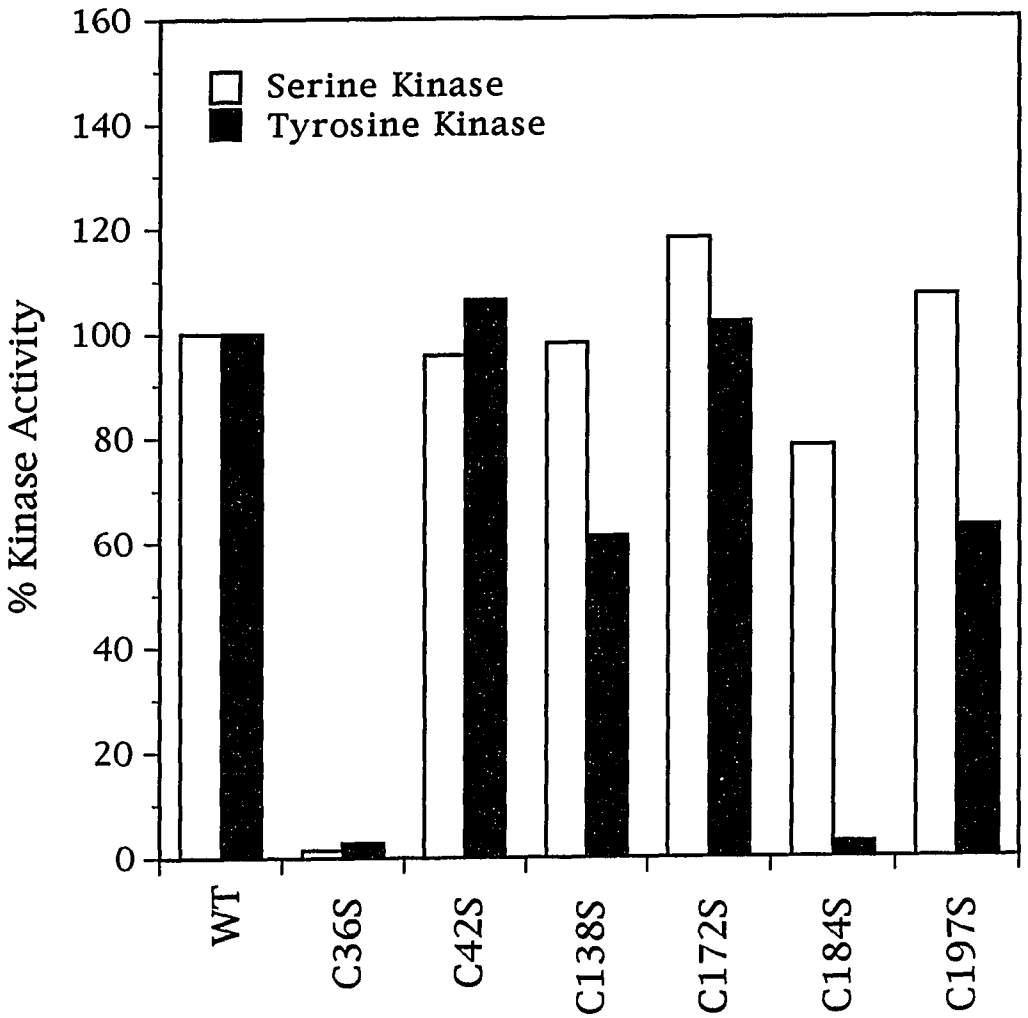
160
140
120
100
80
60
40
20
0
% Kinase Activity
Serine Kinase
Tyrosine Kinase
WT
C36S
C42S
C138S
C172S
C184S
C197S

Table I

Summary of DTNB-titratable Sulfhydryl Groups in Cys-Mutants after treating with 16 mM Cu^{2+}

Mutants	[SH]/molecule*	# of Disulfide bond
C36A@	3.1 ± 0.1	1
C42S	1.3 ± 0.2	2
C138S	1.6 ± 0.2	2
C172S	3.1 ± 0.1	1
C184S	2.6 ± 0.4	1
C197S	2.8 ± 0.3	1

* The oxidation and DTNB titration of Cys-mutants were done as described under Experimental Procedures.

@ C36A instead of C36S was used in this experiment because the yield of C36S after purification is low.

involved in disulfide bonds formation after oxidation with Cu^{2+}.

Kinetic study of Cys-36 mutants

The C36S mutant has been found to have about 2-3 % of both serine and tyrosine kinase activities of the wild type enzyme. The C36A mutant, however, was made and exhibited similar kinase activities compared to wild type TR-γ. Kinetic studies of both Cys-36 mutants, C36S and C36A, show similar K_a (20 ± 1 and 30.3 ± 0.8 μM, respectively) and K_{ia} (11 ± 2 and 32 ± 1 μM, respectively)(Table II). The K_b(ATP), however, are about 2-fold (133 ± 10 and 146 ± 5 μM, respectively) higher than that of the wild type kinase (79 ± 10 μM). The V_m of C36A (4.8 ± 0.1 μmol/min/nmol) is similar to that of Wild type TR-γ (4.10 ± 0.03 μmol/min/nmol), while that of C36S (0.50 ± 0.01 μmol/min/nmol) is about 8-fold lower (Table II). These results suggest that Cys-36 may not be directly involved in the binding of the ATP because the replacement of Cys with Ala, containing a smaller hydrophobic side chain, does not change much the affinity of the protein for ATP. The replacement of Cys-36 with a serine may twist the nucleotide binding site and change either the distance between the γ-phosphate and alcoholic group of Ser or the orientation of the γ-phosphate with respect to the phosphorylatable OH group.

Table II

Comparison of Kinetic Parameters of Wild Type TR-γ and Cys-36 Mutants of Phosphorylase Kinase[@]

TR-γ	Phos b (μM)		ATP (μM)		V_m (μmol/min/nmol)
	K_a	K_{ia}	K_b	K_{ib}	
Wild Type	18 ± 1	24 ± 3	79 ± 10	105 ± 21	4.10 ± 0.03
C36S	20 ± 1	11 ± 2	133 ± 10	73 ± 15	0.50 ± 0.01
C36A	30.3 ± 0.8	32 ± 1	146 ± 5	152 ± 5	4.8 ± 0.1

[@]Kinetic studies were done at pH 8.2 in the presence of 10 mM Mg^{2+} as described under Experimental Procedures. The reaction was carried out by using phosphorylase b as the variable substrate(10-80 μM) and ATP as the fixed variable substrate (50-400 μM).

Further characterization and kinetic studies of C184S

Initial study shows that C184S has near normal serine kinase activity but has only 4-5% tyrosine kinase activity of that of wild type TR-γ (Figure 3). The metal ion response of serine kinase activity of C184S (Figure 4A) is the same as that of wild type TR-γ (13), while that of tyrosine kinase activity is different (Figure 4B). To learn more about the function of Cys-184, kinetic studies of serine kinase activity are performed by using phosphorylase b as substrate. The results of kinetic studies of the serine kinase activity in the presence of Mg^{2+} (Table III) show that C184S has similar K_b (53 ± 1 μM), K_{ib} (51.6 ± 0.5 μM), and V_m (3.20 ± 0.05 μmol/min/nmol) to those of wild type TR-γ; however, K_{ia} (55.9 ± 0.2 μM) and K_a (58 ± 1 μM) are about 2-3 fold higher. This observation suggests that Cys-184 may be involved in the binding of the protein or peptide substrate for the serine kinase activity of TR-γ. Because the tyrosine kinase activity of C184S was very low using angiotensin II, it was not feasible to study its kinetics with this substrate. An alternate way to evaluate the binding characteristics of the mutant is to use angiotensin II as an inhibitor and study its effect on serine kinase activity. Angiotensin II inhibited the serine kinase activity of C184S in the presence of Mg^{2+} or Mn^{2+} (Figure 5) with the IC_{50} of around 6 and 4 mM, respectively. This is approximately the value of the K_m for

Figure 4. Metal ion responses of serine and tyrosine kinases of C184S. The serine (panel A) and tyrosine kinase (panel B) activity was assayed as described in Experimental Procedures except 0.2 mM ATP was used. Various amount (0 to 15 mM) of Mg^{2+} (-▲-) and Mn^{2+} (-Δ-) were used in the activity assay.

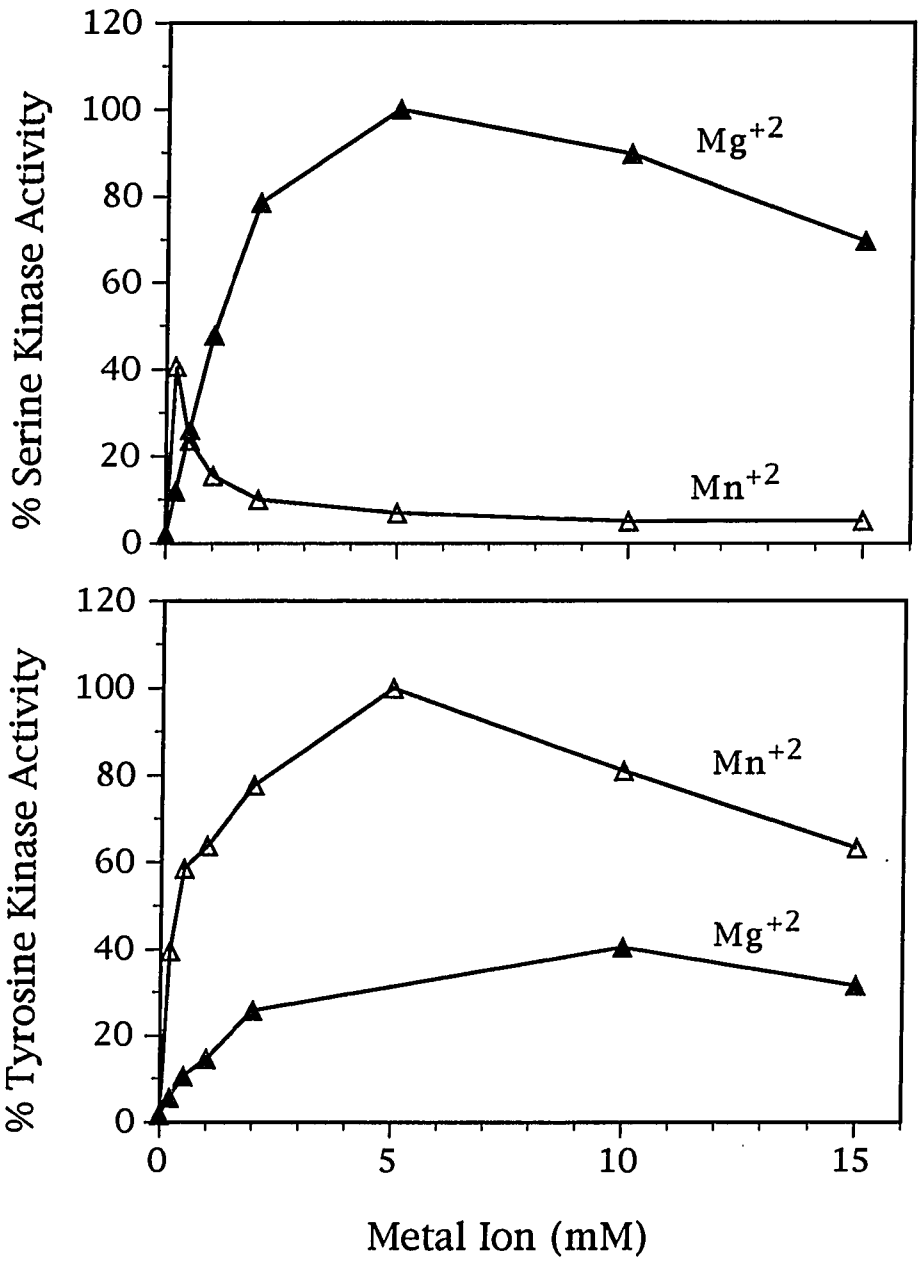
% Serine Kinase Activity
120
100
80
60
40
20
0
Mg+2
Mn+2
% Tyrosine Kinase Activity
120
100
80
60
40
20
0
Mn+2
Mg+2
0
5
10
15
Metal Ion (mM)

Table III

Comparison of Kinetic Parameters of Serine Kinase Activity of Wild Type TR-γ and C184S Mutant#

		Phos b (μM)		ATP (μM)		V_m (μmol/min/nmol)
	TR-γ	K_a	K_{ia}	K_b	K_{ib}	
Mg^{2+}	©WT	18 ± 1	24 ± 3	79 ± 10	105 ± 21	4.10 ± 0.03
	C184S	58 ± 1	55.9 ± 0.2	53 ± 1	51.6 ± 0.5	3.20 ± 0.05

Kinetic studies were done as described in Experimental procedures. The initial rates were determined by using phosphorylase b as the variable substrate (20-120 μM) and ATP as the fixed variable substrate (25-250 μM).

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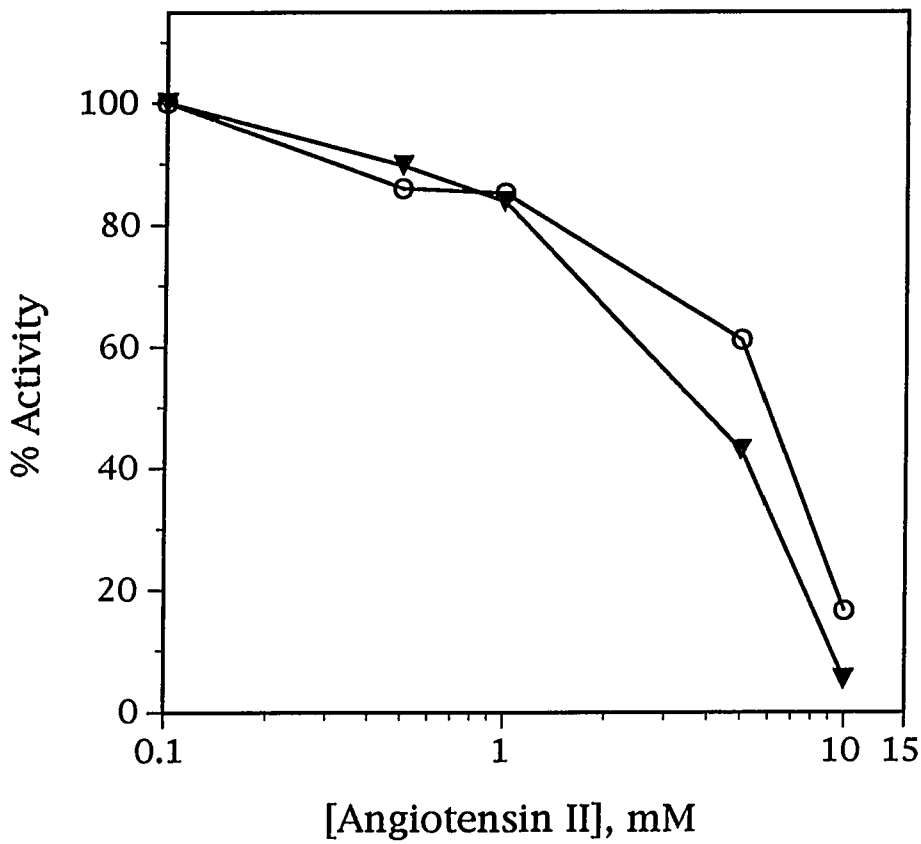
Figure 5. Inhibition of serine kinase activity of C184S mutant by angiotensin II in the presence of Mg^{2+} or Mn^{2+}. Serine kinase activity assay was performed as described under Experimental Procedures except phos 9-18 peptide (50 μM) was used as substrate. Inhibitory effect of angiotensin II (0.5 to 6 mM) was tested at pH 7.9 in the presence of 10 mM Mg^{2+} (-O-) or 3 mM Mn^{2+} (-▼-) and 1 mM ATP.

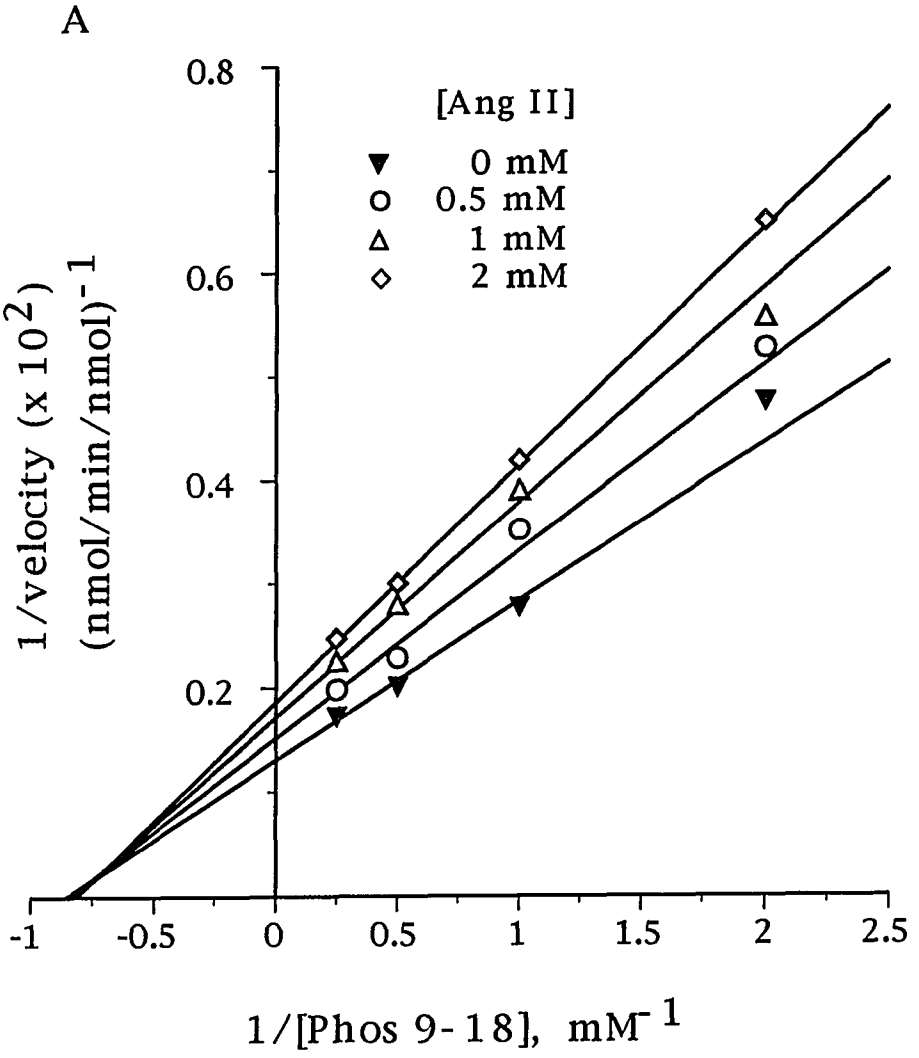

100
80
60
40
20
0
% Activity
0.1
1
10
15
[Angiotensin II], mM

angiotensin II of wild type truncated γ suggesting that the binding strength for a tyrosyl substrate is not changed much in the mutant.

Further studies show that angiotensin II is a non-competitive inhibitor (Figure 6A) for 9-18 peptide of phosphorylase b in the presence of Mg^{2+} with K_{is} and K_{ii} of 3.9 ± 0.4 and 4.2 ± 0.6 mM, respectively (Table IV). In the presence of Mn^{2+}, however, it is a competitive inhibitor (Figure 6B) with a K_{is} of 3.3 ± 0.1 mM. A similar non-competitive inhibition pattern is also obtained with wild type TR-γ in the presence of Mg^{2+} (Table IV) but with about 10-fold higher K_{is} (37 ± 2 mM) for angiotensin II. These results further suggest that the low tyrosine kinase activity of C184S is not due to a lack of binding of the substrate. A difference between two recombinant proteins in binding between angiotensin II, a tyrosyl substrate, and 9-18 peptide, a seryl substrate, is postulated according to the change in inhibition pattern with the binding of Mg^{2+} or Mn^{2+} (Table IV).

An active site containing two conformations in TR-γ with the binding of different metal ions, e.g., Mg^{2+} or Mn^{2+}, has been proposed by Yuan et al. (14) to explain the metal-ion dependent dual specificity of phosphorylase kinase. One active site conformation favors the seryl phosphorylation and the other tyrosyl phosphorylation. This conformational

Figure 6. Inhibition pattern of C184S by angiotensin II in the presence of Mg^{2+} or Mn^{2+}. Serine kinase activity was assayed as described in Experiment Procedures. A) inhibition of C184S by angiotensin II in the presence of Mg^{2+} with phos 9-18 peptide as the variable substrate (0.5-4 mM). The final concentrations of angiotensin II in the assay were from 0 to 2 mM as indicated. B) Inhibition of C184S by angiotensin II in the presence of Mn2+ with phos 9-18 peptide as variable substrate (0.6-6 mM). The final concentrations of angiotensin II were from 0 to 3 mM as indicated. The concentration of [γ-^{32}P]-ATP was 1 mM in both assays.


A
[Ang II]
0 mM
0.5 mM
1 mM
2 mM
1/velocity (x 10^2)
(nmol/min/nmol)^-1
0.8
0.6
0.4
0.2
-1
-0.5
0
0.5
1
1.5
2
2.5
1/[Phos 9-18], mM^-1

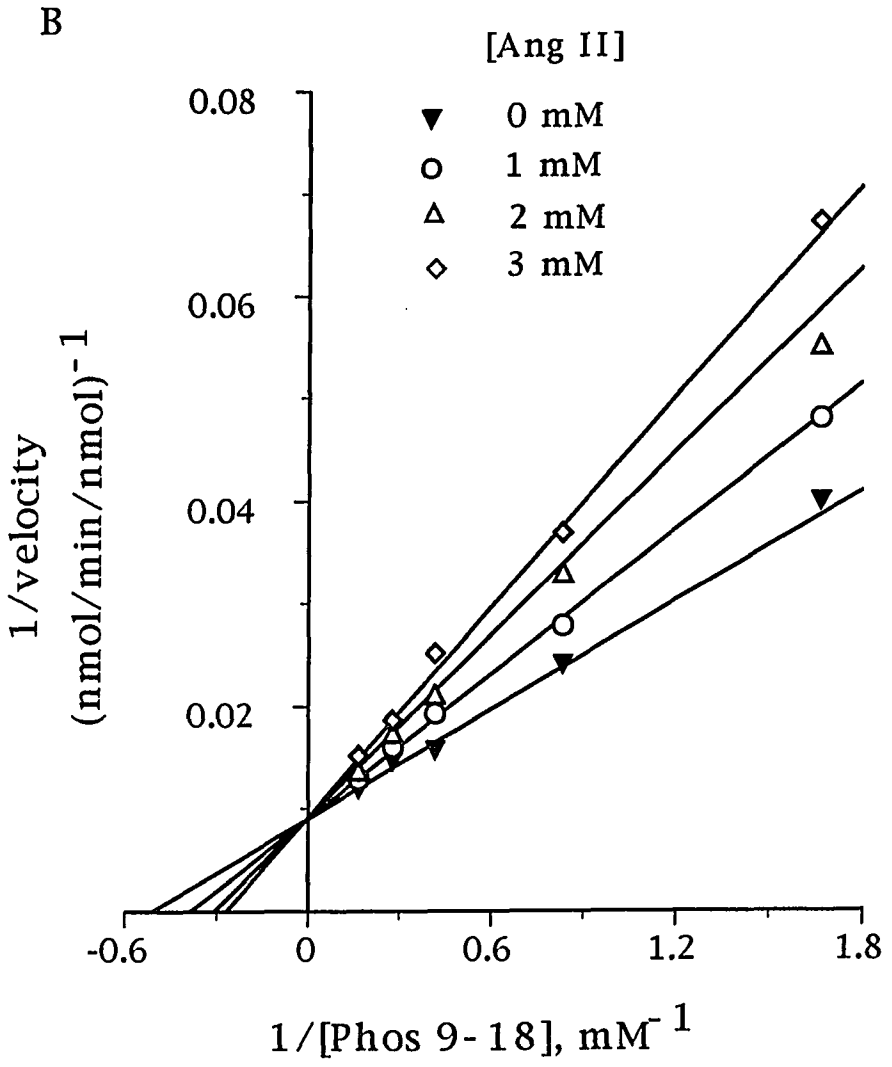


Figure 6 (continued)

Table IV

Summary of the kinetic studies of wild type truncated γ and C184S mutant using angiotensin II as substrate or inhibitor

TR-γ	Metal ion	K_{is}	K_{ii}	K_m	Inhibition Pattern
		(μM)	(μM)	(μM)	
WT	Mg	37 ± 2	8 ± 2	_	N C
	@Mn	3.4 ± 0.3	_	4.4 ± 0.2	_
C184S	Mg	3.9 ± 0.4	4.2 ± 0.6	_	N C
	Mn	3.3 ± 0.1	_	_	C

@ This is the K_{ia} and K_a values of wild type truncated γ for angiotensin II.

change caused by the binding of metal ions in the second metal ion binding site is further demonstrated by the observation that the sedimentation velocity of TR-γ with Mg^{2+} or Mn^{2+} was different in the XLA Beckman analytical centrifuge. The TR-γ subunit in the presence of Mn^{2+} exhibits a higher sedimentation velocity in ultracentrifuge than that in the presence of Mg^{2+} (Luo and Yuan unpublished data). This result suggests that TR-γ may have a more compact structure in the presence of Mn^{2+} than in the presence of Mg2+ because sedimentation velocity is inversely proportional to the size and the shape of the molecules with the same molecular mass.

Substrate specificity of C184S

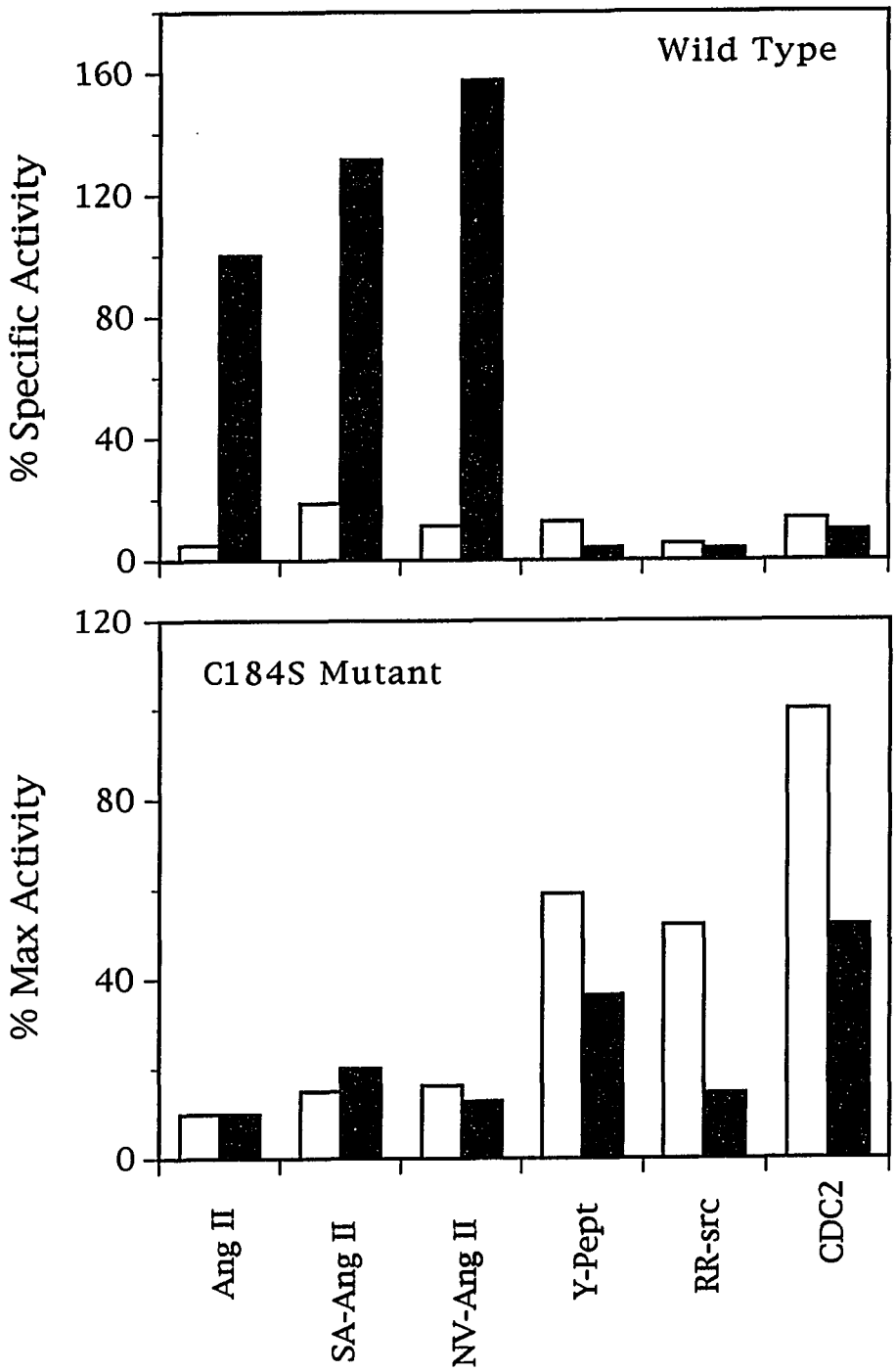
Several common tyrosine kinase substrates, angiotensin II and derivatives, CDC2 peptide, and RR-Src peptide, and other tyrosine bearing peptides were used (Table V) to study the specificity of tyrosine phosphorylation with the wild type and C184S mutant TR-γ. As shown in the top panel of Figure 7 wild type TR-γ strongly prefers angiotensin II and its derivatives, Sar^1,Ala^8- and Asn^1,Val^5-angiotensin II, as tyrosine kinase substrates. Mn^{2+} is required for these reactions. In contrast, the Cys-184 mutant shows a different substrate specificity and a metal ion requirement (bottom panel of Figure 7). C184S prefers CDC2 peptide, RR-Src

Table V

Peptide Substrates for the Tyrosine Kinase Activity of truncated γ of Phosphorylase Kinase

Peptides	Sequences
Phos 9-18	K R K Q I S V R G L
Ang II	D R V Y I H P F
SA-Ang II	S R V Y I H P A
NV-Ang II	N R V Y V H P F
Y-Peptide	K R K Q I Y V R G L
CDC2	R R K I E K I G E G A Y G V V F K
RR-Src	R R L I E D A E Y A A R G

Figure 7. Substrate specificity of the tyrosine kinase activity of wild type truncated γ and C184S mutant. Tyrosine kinase activity of wild type truncated γ (top penal) and C184S mutant (bottom penal) was assayed as described in Experimental Procedures in the presence of 1 mM [γ-^{32}P]-ATP and 10 mM Mg^{2+} (open bar) or 3 mM Mn^{2+} (close bar). The final concentrations of peptide substrates were 1 mM.


Wild Type
% Specific Activity
160
120
80
40
0
C184S Mutant
% Max Activity
120
80
40
0
Ang II
SA-Ang II
NV-Ang II
Y-Pept
RR-src
CDC2

peptide, and Y-peptide as substrates in comparison to angiotensin II. In addition, Mg^{2+} is more effective than Mn2+ for the tyrosine kinase activity of C184S. This result suggests that Cys-184 is important for the determination of tyrosine kinase substrate specificity.

DISCUSSION

Four out of seven free sulfhydryl groups, C36S, C172, C184S, and C197S, have been predicted to be located in or near the active site region of the catalytic subunit of phosphorylase kinase based upon the crystal structure of cAMP-dependent protein kinase (8-10) and primary sequence alignment of the kinases (4). Previous studies (11) suggest that two intramolecular disulfide bridges can be formed by the oxidation caused by Cu^{2+}. One of these two disulfide bonds has been located between Cys-36 and Cys-172 by peptide mapping and sequencing (11). As shown in Table I, only C42S and C138S can form two disulfide bond (around four free sulfhydryl groups/molecule disappear) after treating with 16 μM Cu^{2+}. With this concentration of Cu^{2+}, two disulfide bonds could be formed in wild type TR-γ. If a certain cysteine in the wild type kinase is involved in the formation of the disulfide bond, two disulfide bonds should not be expected to be found in the mutant which replaced that Cys with Ser or Ala. Hence, we conclude that Cys-36, -172, -184, and -197, are involved in the formation of disulfide bonds. Accordingly, the second disulfide bridge should be located between Cys-184 and Cys-197.

All seven Cys-mutants, C36A, C36S, C42S, C138S, C172S, C184S, and C197S, were tested for their serine and tyrosine

kinase activities. Among the seven mutants only C36S and C184S show interesting results. Cys-36 is interesting because it is on β-sheet 2 of the small lobe, which is close to the nucleotide binding site of γ, according to the crystal structure of cAPK (4,8). This observation suggests that the side chain of Cys-36 may be in the region of the ATP binding domain and play a role in nucleotide binding and/or recognition. In the case of CaM-dependent protein kinase II, Cys-30, corresponding to Cys-36 of γ, was also suggested to be involved in nucleotide binding (12). However, kinetic studies of C36S and C36A (Table II) reveal that Cys-36 may not be directly involved in nucleotide binding and/or recognition in the γ subunit. This is because the replacement of Cys by a smaller hydrophobic residue, Ala, did not change much the affinity of Cys-mutant for ATP. An alteration in the structure of β-sheet 2 by the replacement of Cys with Ser could occur. This structure alteration may change either the orientation of the γ-phosphate of ATP or the distance between γ-phosphate and the acceptor hydroxyl group and affect the phosphotransferase activity.

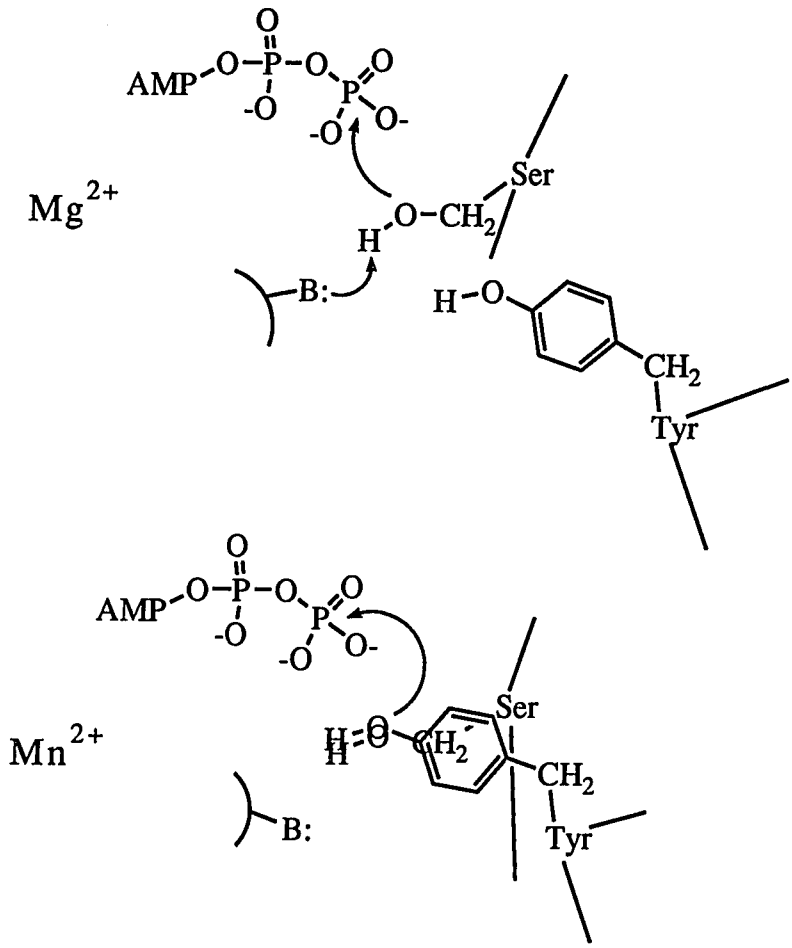
Although the catalytic domain among kinases is highly conserved according to primary sequence alignment (11), several variable sites are proposed to play a role in determining the substrate specificity in the kinase family (9). Among these variable sites the protein substrate

binding loop (PSB loop), between β-sheet 9 and F-helix, is important (8,9). For example, it has an important role in anchoring inhibitory peptide, PKI(5-24), in cAPK (9). The modification of Cys-199 in the PSB loop by a thiol specific reagent or a peptide analog specific for thiol group could totally block the enzyme activity (23,24). The crystal structure of cAPK suggests that the sulfhydryl group of Cys-199 is in close proximity with the P+1 site of PKI peptide (9) and contributes to the general hydrophobic environment around the P+1 site. In addition, the mutagenesis of Cys-199 to Val increases the K_m for peptide substrate without changing kinase activity (25). In case of CDK2, the T loop, which corresponds to the PSB loop of cAPK, was suggested to be highly dynamic and flexible and act as an auto-inhibitor to block the entrance of the active site cleft before autophosphorylation and cyclin binding (22). In the γ subunit of phosphorylase kinase this region is even more important because it is involved not only in the binding of serine kinase substrate but also in the determination of substrate specificity of tyrosine kinase activity. When Cys-184 of TR-γ, corresponding to Cys-199 of cAPK, is replaced with serine, the K_m of C184S mutant for phosphorylase b increases about 3-fold (Table III). This result suggests that Cys-184 may not be crucial in the interaction of serine kinase substrate. More dramatic results were obtained from the effect of

mutagenesis of Cys-184 on the tyrosine kinase activity. The primary characterization shows that C184S contains only 4-5% tyrosine kinase activity with angiotensin II as substrate. Further characterizations of C184S show that mutagenesis does not abolish the binding of angiotensin II (Table IV) but switches the substrate preference from angiotensin II to CDC2 peptide, RR-Src peptide, and Y-peptide (Figure 7).

An active site model is proposed (Figure 8) to explain the metal ion-dependent dual specificity of phosphorylase kinase. In the presence of Mg^{2+} the active site region may be shallower than in the presence of Mn^{2+} and just fit the alcoholic group of serine kinase substrate. With this conformation the binding of tyrosine kinase substrate will be restricted and make phenolic group of tyrosyl residue out of the active site region. With Mn^{2+} at the second metal ion binding site, however, the active site region of γ becomes deeper and can accommodate the bulky phenolic group of tyrosyl residue. A conformational change may also occur in the flexible PSB loop in the presence of Mn^{2+} and help to anchor the tyrosine kinase substrate, angiotensin II, in the active site region. The conformational change resulting from the binding of Mn^{2+} could also affect the catalytic base of the kinase by twisting it away from the phosphorylation site. This may partly explain the inhibitory effect of Mn^{2+} to serine kinase activity. The replacement of Cys-184 to Ser

Figure 8. Active site model of the γ subunit of phosphorylase kinase for substrates of serine and tyrosine kinases in the presence of Mg^{2+} or Mn^{2+}. The catalytic base is shown as -B:.


AMP
O
P
O
-O
O-
Mg2+
H
O-CH2
Ser
B:
H-O
CH2
Tyr
AMP
O
P
O
-O
O-
Mn2+
H
O
CH2
Ser
B:
CH2
Tyr

may either change the local conformation or flexibility of PSB loop and make it unable to bring a tyrosyl residue of angiotensin II to an optimal position in the active site. Instead other tyrosyl residue bearing peptides, CDC2 peptide, RR-Src-peptide, and Y-peptide, might reach the γ-phosphoryl group of ATP in the active site region with this conformational change.

Angiotensin II was reported by Garcia et al. (26) that it could form two tight turns when bound to its receptor. The possibility that protein tyrosine kinases recognize tyrosyl residue in a β-turn was also proposed by Tinker et al. (27). Phosphorylase kinase recognizes its tyrosine kinase substrate by a certain secondary structure in the phosphorylation site region is also speculated. Although the secondary structure of CDC2, RR-Src peptide, and Y-peptide are unknown so far, we believe they will be proved to be distinct from that of angiotensin II.

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GENERAL SUMMARY AND DISCUSSION

Phosphorylase kinase is a complicated enzyme not only because of its subunit composition but also because of its regulation and substrate specificity. Evidence from our lab and others suggest that it can phosphorylate not only serine/threonine (67-70,73) but also on tyrosine residues (83). This unique property makes phosphorylase kinase a different class of protein kinase, termed dual-specificity protein kinase (84). Different from other dual-specificity kinases, e.g., MAP kinase kinase (85-87), phosphorylase kinase requires metal ions, i.e., Mg^{2+} and Mn^{2+}, as a switch for different kinase activities (83). One metal ion activates one kinase activity and inhibits the activity of another and *vice versa*. One active site containing two different kinase activities of phosphorylase kinase was proposed (83) according to the observation that both serine and tyrosine kinase activities are inactivated by the oxidation by Cu^{2+}. A conformational change in the active site region upon the binding of metal ion, i.e., Mn^{2+}, is proposed. This speculation is supported by the observation that TR-γ exhibits different sedimentation velocities in the presence of Mg^{2+} or Mn^{2+}. In the presence of Mn^{2+}, TR-γ was found to move faster than in the presence of Mg^{2+} in the ultracentrifuge. Because sedimentation velocity is inversely

proportional to the size of the molecules with the same molecular mass, a more compact structure of the TR-γ subunit caused by the binding of Mn^{2+} was speculated. The results from Yuan and Graves (39) and Bollen et al. (82) further support the idea of one active site containing two conformations in γ. The Dansyl-calmodulin•Ca^{2+} complex was found to bind differently with the HPLC-purified renatured γ subunit in the presence of Mg^{2+} or Mn^{2+}. A conformational change in γ by the binding of different metal ions was suggested to make Dansyl-calmodulin bind differently (39). Phosphorylase kinase was also found to recognize two different peptide substrates, syntide 2 and syntide 3, in the presence of Ca^{2+} or heparin (82). One active site containing two different conformations caused by the binding of different ligands was also proposed.

The γ subunit of phosphorylase kinase may share some structural features with those of cAMP-dependent protein kinase (cAPK) (88-91) based on the primary sequence alignment among kinases (26). However, some unique structural features in γ are also expected based on its metal ion response, substrate specificity, and regulation. By using the 3D structure of cAPK as a model, we compared the structure of γ and cAPK based on the studies of the function of free sulfhydryl groups by biochemical and molecular biological approaches and the tyrosine kinase activity of γ. We conclude

that first, like cAPK, γ of phosphorylase kinase also has a flexible catalytic domain. It has been found that cAPK contains the open and closed structures upon the binding of substrate (92,93). The small lobe of cAPK can move 15-39° related to large lobe. However, the movement between small and large lobes in γ may be larger. This is suggested by the location of first disulfide linkage between Cys-36 and Cys-172. Since Cys-36 and Cys-172 could be readily oxidized by low concentration of Cu^{2+}, the thiol groups from two Cys residues may be in close proximity. In the other words, the active site cleft between small and large lobes may be much narrower than it is in the crystal structure of cAPK (88-91). The binding of substrates may fix the active site at a certain conformation. This idea was supported by the observation that MgATP could protect truncated γ from inactivation at low concentration of Cu^{2+}.

Second, E-helix and Cys-138 in γ may play a role in the interaction with other subunits, α and/or β, or its own C-terminal calmodulin binding domain. The Glu-155 in E-helix of cAPK, corresponding to Cys-138 of γ, was found to face the acyl-binding pocket and interact with a myristyl group. The binding of myristyl group stabilizes the structure of C subunit of cAPK (92,94). In TR-γ the cross-linking of Cys-peptide to Cys-138 results in slightly increasing Km values for phosphorylase b and ATP (about 2-fold) and decreasing Vm

(about 3-fold). A restriction in the conformational change could be provided by Cys-peptide. Meanwhile a different pH-activity curve of Cys-peptide coupled TR-γ was found from that of wild type kinase. A higher pKa value for the catalytic base in the Cys-peptide coupled TR-γ subunit is suggested by this result. Accordingly, the environment of the catalytic base in Cys-peptide coupled TR-γ becomes more hydrophobic because of the coupling of Cys-peptide. Without the restriction provided by the coupled Cys-peptide, TR-γ may adjust its active site region more effectively to an optimal conformation. The conformational change in active site region of γ results in the exposure of its catalytic base and accommodating the incoming substrates. A similar pH-activity curve as in Cys-peptide coupled TR-γ was also seen in αγδ complex and non-activated phosphorylase kinase holoenzyme (22). These results suggest that Cys-138 in γ may have a interaction with either regulatory subunit, α or β, or with its C-terminal calmodulin binding domain. The interaction of the regulatory subunits or C-terminal calmodulin binding domain to this region may regulate the phosphotransferase activity of the γ subunit. This speculation is partly suggested by the results that TR-γ has higher V_m and lower K_m values for both substrates (34) than those of various forms of phosphorylase kinase (1,22,25,61).

Third, Cys-184 may have a role in determining the substrate specificity of tyrosine kinase activity of phosphorylase kinase. The Cys-199 of cAPK, which corresponds to Cys-184 of γ, was found to be in close proximity with the P+1 site of PKI peptide (90) and contribute to the general hydrophobic environment around the P+1 site. The replacement of Cys-199 to Val change the Km for peptide substrate but not the Vm of cAPK (95). A steric hindrance provided by Val side chain was suggested. In TR-γ, when replacing Cys-184 with Ser, the tyrosine kinase activity of TR-γ was reduced by approximately 20-fold by using angiotensin II as substrate. Meanwhile the serine kinase activity of TR-γ is roughly the same. Kinetic studies show that the mutation changed the Km for phosphorylase b by 3-fold but the binding strength of mutant for angiotensin II is about the same. Further study suggests that C184S has a different preference for substrates, i.e., CDC2 peptide, RR-Src peptide, and Y-peptide, from that of wild type truncated γ. This result along with others suggests that Cys-184 may play a role in the binding of serine kinase substrate and most importantly in the determining the substrate specificity of tyrosine kinase activity.

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