Phosphorylase Kinase, a Metal Ion-dependent Dual Specificity Kinase

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Phosphorylase kinase is shown to be a dual specificity kinase. The specificity of phosphorylation is determined by divalent cation. Mg2+ causes seryl phosphorylation of phosphorylase b, but Mn2+ activates tyrosine phosphorylation of angiotensin II. In contrast to seryl phosphorylation, the tyrosine kinase activity of holoenzyme is not regulated by Ca2+. Preincubation of the holoenzyme with Ca2+, Mg2+, and ATP that causes autophosphorylation activates tyrosine kinase activity. The tyrosyl kinase activity is a property of the γ subunit. Addition of varying amounts of Mn2+ to a truncated form of the γ subunit of phosphorylase kinase containing MgATP inhibits serine kinase but activates tyrosine kinase activity. This result along with an oxidative reaction caused by Cu2+ 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 Mn2+ and ATP show that Km for nucleotide is not changed with a seryl or tyrosyl substrate. The Vmax 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 is caused by the binding of divalent cation at a second metal ion binding site.

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, 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 nucleotide triphosphate for effective phosphorylation. Serine/threonine kinases use Mg2+ more effectively than Mn2+, but tyrosyl kinases differ because Mn2+ stimulates phosphorylation equal to or better than that caused by Mg2+ (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 phospho-transfer activity, but binding at the second site inhibits the reaction. Phosphorylase kinase, a calcium-dependent protein kinase that 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. Mg2+, at the second site can activate the reaction with phosphorylase b, but the activity is inhibited by the binding of Mn2+ (10). Phosphorylase kinase acts on few other substrates, but it can phosphorylate a threonine residue in tropomisin I (16) and even inositol in phosphadiylinositol (17).

We report in this communication that the binding of a second divalent cation in the recombinant catalytic subunit of phosphorylase kinase determines enzyme specificity. With Mg2+, seryl phosphorylation occurs, but with Mn2+ tyrosine phosphorylation is favored. Similar results are seen with the holoenzyme, i.e. phosphorylase kinase has tyrosine kinase activity in the presence of Mn2+.

EXPERIMENTAL PROCEDURES

Materials—Highly purified rabbit skeletal muscle phosphorylase kinase was a kind gift from Dr. G. M. Carlson of the University of Tennessee. Recombinant truncated γ subunit (1-300) of phosphorylase kinase containing γ subunit is expressed, purified, and renatured as described by Huang et al. (18). Phosphorylase b is prepared as described (19). Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is from Sigma. Phosphocellulose paper and ET-31 filter paper are from Whatman. [γ-32P]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 32P into phosphorylase b as described with some modification (18). The standards assay contained 50 mM Tris, 50 mM Pipes, pH 8.2, 10 mM Mg2+, 10 mg/ml phosphorylase b, and 1 mM [γ-32P]ATP. The final concentration of truncated γ subunit is adjusted to around 0.3 μM. 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 MnCl2, 0.5 mM angiotensin II, and 1 mM [γ-32P]ATP. The final concentrations of the truncated

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The abbreviations used are: MAP, mitogen-activated protein; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid).
activated $\gamma$ subunit and phosphorylase kinase were 10 and 100 $\mu$g/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 $\gamma$ subunit is oxidized as essentially described by Landgraf et al. (23). To remove dithiothreitol, the truncated $\gamma$ 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 $\gamma$ and an appropriate amount of Cu$^{2+}$ (0.5–16 $\mu$m) for 20 min. After incubation, the oxidized truncated $\gamma$ subunits are subjected toeryl 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.

**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$^{2+}$ (Fig. 1). Poly(Glu,Tyr) and Raytide, two other common tyrosine peptide substrates with a poly-negative charge, can be phosphorylated at a very low level. The tyrosine kinase activity of holoenzyme is affected little by ATP, a condition that causes autophosphorylation, can also activate the enzyme C24–26. To determine whether preincubation of the enzyme with Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ more than Mg$^{2+}$ for their activity (1). At the 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 residue, Asn, was used to replace Asp$^{150}$ in $\gamma$. 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

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**FIG. 2. Effect of preincubation on tyrosine kinase activity.** The tyrosine kinase activity of holoenzyme is determined with (○) or without (□) 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 66 mM Tris, 56 mM PIPES, pH 7.9, 10% glycerol, 3.3 mM MnCl$_2$, 2.8 mM angiotensin II, and 1.1 mM $[\gamma-^{32}P]$ATP and incubated at 30 °C for 5, 20, and 50 min. Phosphorylase kinase preincubated at 30 °C without MgATP is used as the control. The substrate mixture for the control experiment is about the same as above except 0.3 mM Mg$^{2+}$, 0.1 mM ATP, and 10 µm Ca$^{2+}$ were added along with 3.3 mM Mn$^{2+}$ and 1.1 mM $[\gamma-^{32}P]$ATP. 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 kinase protein in truncated $\gamma$ preparation is excluded by the fact that recombinant protein is renatured and purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis (18). The recombinant truncated $\gamma$ 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 $\gamma$. 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-Phc) shows some similarity in primary sequence to that of phosphorylase (Arg$^{10}$-Lys-Gln-Ile-Ser-Val-Amy-Gly-Leu$^{16}$).

It has been demonstrated that the serine/threonine kinase activity of the truncated $\gamma$ subunit can be inhibited by the oxidation of Cu$^{2+}$ (27). The oxidation caused 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 $\gamma$ subunit, oxidation should interfere with the binding of both substrates. As shown in Fig. 3, both serine/threonine and tyrosine kinase activities of the truncated $\gamma$ subunit are inhibited identically with increasing Cu$^{2+}$. The idea of two kinase activities sharing one active site is further supported by the result of site-directed mutagenesis at an invariant residue of the catalytic loop at Asp$^{150}$ corresponding to Asp$^{166}$, the putative catalytic base of cAMP-dependent protein kinase (28). A similar but uncharged residue, Asn, was used to replace Asp$^{150}$ in $\gamma$. The mutant is constructed, expressed, and purified to homogeneity. Both serine/threonine and tyrosine kinase activities are abolished by this mutagenesis.

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truncated γ subunit of phosphofructokinase is shown in Fig. 4. Inhibition of serine/threonine kinase activity and activation of tyrosine kinase activity of truncated γ show parallel changes by increasing Mn²⁺ in the presence of an equivalent amount of Mg²⁺ and ATP. The two activity curves intersect at a concentration of approximately 250 μM Mn²⁺, which is also the EC₅₀ for both curves, suggesting this effect is due to the binding of Mn²⁺ 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, Mg²⁺ or Mn²⁺, may hold the nucleotide in the active site and cause inhibition (9, 29). For phosphorylase kinase, the second metal ion, especially Mn²⁺, may have a different role, modulating substrate specificity. Bolten et al. (30) suggested that the catalytic subunit of phosphorylase kinase might have two active conformations to recognize different serine/threonine residues. Ohla et al. (31) demonstrated that the large lobe of the catalytic subunit of CAMP-dependent protein kinase can rotate about 35 degrees around a hinge residue, Gly¹⁹⁵, a residue that 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, Mn²⁺ and Mg²⁺, 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).

Kinetic results (Table I) show that in the presence of Mn²⁺ the Kₘ for ATP (54 ± 3 μM) is about the same as that measured in the presence of Mg²⁺ (79 μM) (18). The apparent Kₘ for phosphorylase b (4.0 ± 0.2 μM), however, is about 5-fold lower. In the presence of Mn²⁺ 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 μM, respectively, using angiotensin II as substrate in the presence of Mn²⁺. This result suggests that a conformational change at the active site region caused by the binding of Mn²⁺ may further expose the binding site to its substrate and give more room for a phenolic group. It is known that the binding of a second metal ion can increase the affinity of enzyme for its substrate with the insulin receptor tyrosine kinase (34). A conformational change in γ induced by Mn²⁺ may turn the alcoholic group of Ser¹⁰ of phosphorylase b away from the putative catalytic base, Asp¹⁸², and reduce the rate of phosphoryl transfer (apparent Vₘ = 163 ± 8 nmol/min/nmol) (Table I). 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 1/30 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 aad 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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