Nonenzymatic Phosphorylation of Tyrosine and Serine by ATP Is Catalyzed by Manganese but Not Magnesium*

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Many cellular and retroviral protein-tyrosine kinases display either a requirement or a preference for manganese over magnesium for maximal activity. We have observed that peptides and proteins are nonenzymatically phosphorylated at tyrosine and serine by ATP when heated in the presence of MnCl₂ at neutral pH. The extent of the reaction is negligible below 50 °C but increases rapidly at higher temperatures. The reaction proceeds in the presence of sodium dodecyl sulfate but is blocked by EDTA. No reaction is observed in the absence of Mn²⁺, even if Mg²⁺ is present. Manganese therefore acts as a catalyst for the nonenzymatic reaction, but magnesium does not. We propose that the preference for manganese shown by many protein tyrosine kinases is due at least in part to the intrinsic ability of Mn²⁺ to catalyze the transfer of phosphate from ATP to a phosphate acceptor such as tyrosine. The nonenzymatic phosphorylation reaction also offers a new synthetic pathway for the preparation of radiolabeled peptides containing phosphotyrosine and phosphoserine.

Protein phosphorylation at tyrosine is known to play an important role in cellular growth control and in the malignant transformation of vertebrate cells (1). A number of cellular proteins, including several growth factor receptors, possess protein-tyrosine kinase activity. Many of these proteins are protein-tyrosine kinases. All of the known protein-tyrosine kinases such as pp60⁺⁺ and ~210⁺⁺ have much lower activity with Mg²⁺ than with Mn²⁺. Although virtually all cellular ATP is present as Mg-ATP, many cellular and retroviral protein-tyrosine kinases display a divalent metal cation activity. Many cellular and oncogenic protein-tyrosine kinases display a preference for manganese over magnesium as the divalent cation for maximal activity (2). Some cellular protein-tyrosine kinases such as pp60⁺⁺ and p210⁺⁺ have much lower activity with Mg²⁺ than with Mn²⁺ (3, 4), even though virtually all cellular ATP is present as Mg-ATP (5).

We now report that tyrosine and serine in peptides and proteins are phosphorylated at neutral pH by ATP upon heating in the presence of MnCl₂. No reaction is observed with Mg²⁺ or in the absence of a divalent metal cation. Manganese, therefore, acts as a catalyst for the nonenzymatic reaction, but magnesium does not. The greater catalytic activity of manganese relative to magnesium offers a potential explanation for the preference for manganese shown by many protein-tyrosine kinases. The nonenzymatic phosphorylation reaction also offers a new synthetic route for the preparation of phosphotyrosine- and phosphoserine-containing peptides, particularly when the incorporation of a ³²P label is desired.

MATERIALS AND METHODS

Phosphorylation of poly(Glu₈₀Tyr₂₀)—Reaction mixtures contained 1.67 mg/ml poly(Glu₈₀Tyr₂₀) (Sigma), 100 mM buffer, 10 mM MnCl₂, and 270 μM [γ-³²P]ATP (4 Ci/mmol, prepared by a modification of the method of Walseth and Johnson (6) using the Gamma-Prep A system (Promega Biotec)) in a total volume of 30 μl. Reaction mixtures were heated at 80 °C for 10 min in sealed 1.5-ml tubes (Sarstedt). The pH of the buffer solutions employed (MES, pH 6.5, Tris-HCl, pH 8.0, or ethanolamine-HCl, pH 9.0) was adjusted at the temperature used for reactions. The reactions were initiated by the separate addition of manganese and ATP solutions to preheated reaction mixtures. The reactions were terminated by spotting the reaction mixtures onto 2.3-cm 3MM filter paper discs (Whatman) followed by immersion in ice-cold 10% trichloroacetic acid. The paper discs were then washed repeatedly with 10% trichloroacetic acid (7), dried, and counted in liquid scintillation acid.

Phosphorylation of Angiotensin Peptides—Reactions were carried out as described above, using 6 μg/ml angiotensin peptides (Sigma) in place of the poly(Glu₈₀Tyr₂₀) polypeptide. Reactions were terminated by spotting the reaction mix onto P81 phosphocellulose paper discs (Whatman), followed by washing in 0.5% phosphoric acid (8).

Phosphorylation of Enolase—Rabbit muscle enolase (Sigma) was acid denatured by incubation at pH 4 before use to enhance its ability to act as a substrate for phosphorylation (9). Reactions were carried out as described above, using 0.5 mg/ml enolase. For reactions which did not include SDS, reaction mixtures were cooled on ice before the addition of SDS sample buffer (10). These samples were incubated at 30 °C for 10 min before electrophoresis on SDS-polyacylamide gels.

Phosphoamino Acid Analysis—Following reaction with [γ-³²P]ATP, phosphorylated copolymer was precipitated in 20% trichloroacetic acid at 0 °C. The precipitated copolymer was washed once with 20% trichloroacetic acid and twice with ethanol. Partial acid hydrolysis of the phosphorylated copolymer was performed by dissolving the precipitate in 6 N HCl and heating at 110 °C for 80 min in a sealed tube. The hydrolysate was lyophilized and redissolved in water. Labeled phosphoamino acids in the hydrolysates were separated from one another and from partially hydrolyzed peptides and inorganic phosphate by two-dimensional electrophoresis on cellulose thin layer plates. Electrophoresis in the first dimension was performed at pH 1.9, and electrophoresis in the second dimension was performed at pH 3.5, as previously described (11). Unlabeled internal standards consisting of 2 μg each of phosphotyrosine, phosphoserine, and phosphothreonine were included in the sample subjected to two-dimensional electrophoresis. The location of the standards following electrophoresis was determined by staining with ninhydrin. The location of labeled compounds was determined by autoradiography. Films were exposed at −70 °C in the presence of an intensifying screen (Du Pont Lightning Plus). Amino acids phosphorylated in reactions with ATP were identified by comparison of the mobilities of labeled compounds detected by autoradiography with the mobilities of the phosphoamino acids.
acid internal standards detected by staining with ninhydrin. The amount of label incorporated into the phosphoamino acids was determined by scraping the ninhydrin-stained spots corresponding to each phosphoamino acid and measuring their radioactivity by liquid scintillation counting.

RESULTS AND DISCUSSION

The copolymer poly(Glu<sup>60</sup>Tyr<sup>20</sup>) can be isolated from other proteins and ATP by electrophoresis in Tris-glycine gels containing urea in the absence of any ionic detergent (12). When the copolymer was electrophoresed on such gels following heating in the presence of MnCl<sub>2</sub> and [γ-<sup>32</sup>P]ATP, the <sup>32</sup>P label comigrated with the copolymer bands on the gel (data not shown), indicating the incorporation of phosphate into the polypeptide. The γ-phosphate of ATP is known to be capable of reacting with carboxylic acid groups in the presence of divalent metal cations to form mixed anhydrides (13, 14). However, it was unlikely that the reaction of ATP with glutamic acid residues of the copolymer could account for the incorporation of label because the resultant mixed anhydrides are unstable and do not accumulate in solution (9). This was confirmed by performing reactions in which the poly(Glu<sup>60</sup>Tyr<sup>20</sup>) copolymer was replaced by an equal amount of polyglutamic acid homopolymer. In these reactions no incorporation of label into polyglutamic acid was observed (data not shown). Phosphoamino acid analysis of the phosphorylated copolymer revealed that the label was instead incorporated into phosphotyrosine (Fig. 1). In Fig. 1, the large spot at the upper right is inorganic phosphate liberated during the hydrolysis reaction and the spots at the lower left are partially hydrolyzed phosphopeptides (11). The extent of the reaction is negligible at temperatures below 50 °C but increases rapidly at higher temperatures (Fig. 2). Under the conditions used for the reactions described in Fig. 2, in which there is an 11-fold excess of tyrosine over ATP, the reaction at 100 °C is substoichiometric, resulting in the formation of 172 μmol of phosphotyrosine/mol of tyrosine, or 1975 μmol of phosphotyrosine/mol of ATP for 10-min reactions. With an increase in reaction time to 20 min, the total amount of phosphotyrosine generated increased by 25%, but no additional phosphotyrosine was generated upon longer reaction times of up to 2 h (data not shown). The yield of phosphotyrosine could be increased by increasing the concentration of ATP used in the reaction, but this approach was restricted by the limited solubility of Mn-ATP. Under conditions of 2.5 mM total tyrosine, 10 mM ATP, 18 mM MnCl<sub>2</sub>, pH 6.5, with heating at 100 °C for 10 min, approximately 0.5% of the tyrosine in the copolymer was converted to phosphotyrosine.

**Fig. 1. Phosphoamino acid analysis of phosphorylated poly (Glu<sup>60</sup>Tyr<sup>20</sup>).** The copolymer was phosphorylated by heating 1.67 mg/ml copolymer at 80 °C for 10 min in the presence of 100 mM MES, pH 6.5, 10 mM MnCl<sub>2</sub>, and 270 μM [γ-<sup>32</sup>P]ATP. Phosphoamino acid analysis was performed as described in the text. An autoradiogram of the thin layer plate following two-dimensional electrophoresis is shown. Y = phosphotyrosine.

The relatively low yields that have been observed indicate that the nonenzymatic reaction would be of limited use in the preparation of large quantities of phosphorylated proteins. However, the reaction does provide a ready means to introduce a <sup>32</sup>P label into a peptide or protein.

**Fig. 2. Effect of reaction temperature on the manganese catalyzed phosphorylation of poly(Glu<sup>60</sup>Tyr<sup>20</sup>) by ATP.** Reactions were performed by heating 1.67 mg/ml copolymer for 10 min in the presence of 100 mM MES, pH 6.5, 10 mM MnCl<sub>2</sub> and 270 μM [γ-<sup>32</sup>P]ATP.

The ability of divalent metal cations to catalyze the hydrolysis of ATP has been studied extensively (15). In these studies it was found that although Mn<sup>2+</sup> was an efficient catalyst of the hydrolysis of ATP, Mg<sup>2+</sup> had no catalytic activity at pH 5 and only very low reactivity at pH 9. Mn<sup>2+</sup> was found to have 45% lower activity at pH 9 than at pH 5. Zinc and copper were found to catalyze the hydrolysis of ATP at pH 5 at 4- and 17-fold greater rates, respectively, than manganese. At pH 9, calcium was reported to be a better catalyst than manganese. In light of these observations, it was of interest to investigate the abilities of various divalent metal cations to catalyze the phosphorylation of tyrosine.

In the absence of any divalent metal cation, no phosphorylation of tyrosine was detected. As shown in Fig. 3, Mn<sup>2+</sup> was by far the best catalyst for the phosphorylation reaction. Under alkaline conditions reactions with Mn<sup>2+</sup> gave greater yields than those conducted at pH 6.5. This is in contrast to the lower yields reported for the hydrolysis of ATP at alkaline pH (13). The overall increase in yield observed under alkaline conditions may be due to the increased formation of tyrosylate ions, which as stronger nucleophiles would be expected to be more reactive than tyrosine. A brown precipitate of manganese hydroxide formed within 10 min at pH 8 and almost

**Fig. 3. Catalysis of tyrosine phosphorylation by metal cations.** Reactions were performed by heating poly(Glu<sup>60</sup>Tyr<sup>20</sup>) at 80 °C in 100 mM buffer and 270 μM [γ-<sup>32</sup>P]ATP with each metal cation present as a chloride salt at a concentration of 10 mM. The yield of phosphotyrosine for each reaction is shown in μmol of phosphotyrosine/mol of tyrosine.
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immediately at pH 9. The rapid precipitation of manganese hydroxide probably accounts for the lower yields observed at pH 9 than at pH 8.

$\text{Mg}^{2+}$ showed no catalytic activity at pH 6.5 and only slight activity under more alkaline conditions. The lack of activity toward tyrosine parallels the inability of $\text{Mg}^{2+}$ to catalyze the hydrolysis of ATP. Both copper and zinc showed little ability to catalyze the phosphorylation of tyrosine, in marked contrast to their strong catalytic activity in ATP hydrolysis. This disparity may reflect differences in specific interactions between the metal cations and tyrosine. Only calcium showed catalytic activity comparable to manganese.

The observation that tyrosine residues in the poly(Glu$^{\alpha}$-Tyr$^{\alpha}$) copolymer could be phosphorylated in a nonenzymatic reaction raised the question of whether other tyrosine-containing peptides, particularly those lacking such a high proportion of acidic amino acid residues, would be equally reactive. Reactions were carried out utilizing angiotensin II, [Val$^{\beta}$]-angiotensin II, [Asn$^{\gamma}$,Val$^{\alpha}$]-angiotensin II, and angiotensin III. The first two peptides contain one aspartic acid residue at the NH$_2$ terminus, the second two peptides lack any acidic residues. At both pH 6.5 and 8.0, the yields from these peptides were equal or greater than the yields observed for the poly(Glu$^{\alpha}$-Tyr$^{\alpha}$) copolymer at identical total tyrosine concentrations (not shown). No product was observed when Mg$^{2+}$ was substituted for Mn$^{2+}$.

Having observed that peptides could be phosphorylated on tyrosine, it was of interest to determine whether the reaction would occur with proteins as well. When enolase was heated at $80^\circ$C in the presence of Mn$^{2+}$ and ATP, the protein was phosphorylated (Fig. 4A). No reaction occurred at $30^\circ$C, demonstrating that the phosphorylation was not due to the presence of kinase activity in the enolase sample. The results of protein kinase assays are frequently determined by heating the reaction mixtures in SDS sample buffer and analyzing the phosphorylated proteins by SDS-polyacrylamide gel electrophoresis. The non-enzymatic phosphorylation reaction also occurred in the presence of 2% SDS (Fig. 4B). When excess EDTA was present as well, no reaction occurred. Thus, when protein kinase assays are carried out, the addition of EDTA should be sufficient to prevent the artifactual phosphorylation of proteins heated in SDS sample buffer in the presence of manganese and ATP. Phosphoamino acid analysis (Fig. 4C) revealed that 80% of the conformational phosphorylation was in phosphoserine (12.3 mmol of phosphoserine/mol of enolase) and 20% in phosphotyrosine (3 mmol of phosphotyrosine/mol enolase). A trace amount of phosphothreonine was also detected. Rabbit muscle enolase is known to contain serine, threonine, and tyrosine (9), and human and rat enolase contain 17 and 18 residues of threonine, respectively (16). The lack of phosphothreonine is therefore not due to a lack of threonine in enolase. In the reaction of alcohols with anhydrides to form esters, primary alcohols and phenols are much more reactive than secondary alcohols (17). In the analogous reaction of amino acids with ATP to form phosphoesters, the secondary alcohol moiety on threonine would be expected to be much less reactive than the primary alcohol moiety on serine or the phenol moiety on tyrosine.

All known phosphotransferases, including the kinases, are believed to require divalent metal ions complexed to their nucleotide substrates (18), with the metal ions acting as superacid catalysts within the active site of the enzyme (19). We have observed that manganese catalyzes the phosphorylation of tyrosine by ATP in free solution, whereas magnesium does not. It seems likely that manganese has a similarly greater catalytic activity than magnesium in the active site of protein-tyrosine kinase enzymes. We propose that this greater catalytic activity may, at least in part, explain why many tyrosine kinases display higher activities with Mn$^{2+}$ than with Mg$^{2+}$.

The effect of greater catalysis by manganese relative to magnesium on overall enzymatic activity might be expected to be most apparent for enzymes which have only low levels of activity. A number of cellular protein-tyrosine kinases are under regulatory control. For example, pp60$^{\text{cbl}}$ displays a substantially lowered activity when tyrosine 527 is phosphorylated (3). In this less active form, the enzyme displays much higher activity with Mn-ATP than with Mg-ATP, whereas there is little difference between magnesium and manganese when tyrosine 527 is not phosphorylated (3). We propose that the additional catalytic activity of manganese provides a substantial rate acceleration for the enzyme in its less active form, but contributes proportionally less to the activated enzyme, where the reaction proceeds readily without additional catalysis. The activated enzyme may duplicate some aspects of the additional catalytic activity of manganese, rendering it no more effective than magnesium. Characterization of the divalent cation requirements of protein-tyrosine kinases may therefore prove useful in examining the regulation of these enzymes.

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![Fig. 4. Phosphorylation of enolase. A, enolase was incubated at 80 °C (lane 1) or at 30 °C (lane 2) in the presence of Mn$^{2+}$ and $[\gamma^32P]ATP$ as described in the text, in the absence of SDS. SDS sample buffer was added prior to electrophoresis in 10% polyacrylamide gel. The position of the labeled enolase band observed following autoradiography is indicated by the arrow. B, enolase, 10 mM Mn$^{2+}$, and $[\gamma^32P]ATP$ was heated in SDS sample buffer in the absence (lane 1) or presence (lane 2) of 30 mM EDTA. Arrow indicates the position of the labeled enolase band, whose mobility upon SDS-polyacrylamide gel electrophoresis varies depending on whether it was heated in SDS sample buffer. C, the labeled enolase band shown in part A, lane 2 was excised from the dried gel and rehydrated in 800 µl of 6 M HCl. Following partial acid hydrolysis at 110 °C for 80 min, the solution containing the hydrolysate was removed from the remaining polyacrylamide gel slice. The hydrolysate was then lyophilized, redissolved in water, and subjected to anion exchange chromatography on Dowex AG1-X8 (Cl$^-$ form, Bio-Rad) to separate the phosphoamino acids from contaminating material, as described previously (11). The eluted phosphoamino acids were lyophilized and redissolved in water prior to separation by two-dimensional electrophoresis as described under "Materials and Methods." An autoradiogram of the thin layer plate following electrophoresis is shown. Spots are identified by their comigration with unlabeled phosphoamino acid standards included in the sample and detected by staining with ninhydrin. S = phosphoserine, T = phosphothreonine, Y = phosphotyrosine.](image)
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