Chemical Influences on the Specificity of Tyrosine Phosphorylation*

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Bruce L. Martini, Danlin Wu, Scott Jakes*, and Donald J. Graves|

From the Department of Biochemistry and Biophysics and *Department of Zoology, Iowa State University, Ames, Iowa 50011

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Biological tyrosine phosphorylation has become an extensively studied reaction. Little, however, is known of the chemistry involved. The acetylation of the tyrosyl phenolic hydroxyl group by N-acetylimidazole was studied as a model acylation reaction over the pH range 7.5–9.5. The reactivities of tyrosine and 3-fluorotyrosine were compared. The ratio of reactivities, k_下-k_上, decreases with increasing pH. Extrapolation to the state in which equal concentrations of the two derivatives exist indicates that, consistent with Brunsted theory, tyrosine is 17 times more reactive than fluorotyrosine. No reactivity was observed with tetrafluorotyrosine, 3-nitrotyrosine, or 3,5-dinitrotirosine.

A peptide containing fluorotyrosine was synthesized and compared with the tyrosine-containing peptide as a substrate for the insulin receptor/tyrosine kinase. In both the presence and absence of insulin, the tyrosine peptide was phosphorylated with higher V_m and K_m values than the fluorotyrosine peptide was. These results suggest that ionization of the tyrosyl hydroxyl group has an effect on both the chemical and enzymatic reactivities of the tyrosyl residue in acylation reactions. A model is suggested in which deprotonation of the acceptor occurs upon binding of the substrate to the kinase and implicates a role for the substrate site microenvironment in defining substrate specificity.

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† Present address: Section of Biochemistry, Division of Biology and Medicine, Brown University, Providence, RI 02912.
§ Present address: Dept. of Pharmacology, University of Connecticut Health Center, Farmington, CT 06032.

To whom correspondence should be addressed.

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Tyrosine phosphorylation is an integral aspect of cellular growth and transformation (1–4). A major emphasis in the field involves characterization of the substrate specificity displayed by tyrosine kinases. Many protein kinases, including the cAMP-dependent kinase, recognize phosphorylation sites within specific primary sequences. Tyrosine kinases also display a dependence on the primary sequence (5–10), but beyond an apparent requirement for adjacent acidic residues (8–10), it is not clear what determinants are responsible for the specificity of tyrosine phosphorylation. Primary sequence determinants flanking the phosphorylation site or in other parts of the primary structure may be important in the recognition of substrates or in the regulation of tyrosine residue reactivity. Tyrosine kinases also seem to require a high order structure (11) near the phosphorylation site. A preliminary investigation indicates requirements for some additional structural features, possibly including a β-turn (11) near the phosphorylation site.

Little, however, is known of the chemical features involved in the acylations of tyrosine residues. We have approached this problem by investigating the effect of substitution in the aryl ring of tyrosine on the reactivity of the tyrosyl hydroxyl group. Primarily fluorine derivatives were utilized because fluorine substitution enables a highly electronegative center to be introduced without steric factors. Specifically, we have examined the chemical and enzymatic modification of substituted tyrosine derivatives. Acetylation of the hydroxy group of various ring-substituted tyrosines by N-acetylimidazole (12) was examined as a model acylation reaction. The results demonstrate a dependence on the pH, K_m of the reacting phenolic group and are consistent with Brunsted theory. These results have been used to interpret the phosphorylation of peptides containing tyrosine or fluorotyrosine by the insulin receptor/kinase and provide insight into the mechanism of the tyrosine kinase reaction.

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**EXPERIMENTAL PROCEDURES**

**Materials**—N-Acetylimidazole, D,L-F-Tyr, 3-nitrotyrosine, 3,5-dinitrotirosine, phenylmethylsulfonyl fluoride, and all buffers except sodium borate were obtained from Sigma. Tetrafluorotyrosine was obtained from Columbia Organic Chemicals Co. Inc. All other materials were obtained from a suitable commercial source.

Preparation of L-3-F-Tyr-L-F-Tyr, used for incorporation into the peptide, was synthesized by a modification of Kirk's method (13). Kirk's protocol was followed through the silica gel step. Following this, L-F-Tyr was isolated by preparative thin layer chromatography on fluorescent-backed silica plates. The developing system consisted of 1-butanol, acetic acid, water, and pyridine in a ratio of 153:12:10. The hands containing F-Tyr were excised, and the F-Tyr was extracted with ethyl ether. The extracted material was dried to an orange-brown viscous liquid, decolorized, and loaded onto Sephadex G-10. The material was eluted with water and the fractions identified by UV-visible spectrophotometry. The purity of the F-Tyr material was evaluated by thin layer chromatography, high performance liquid chromatography, and amino acid analysis employing L-F-Tyr as a standard. The compound was also analyzed by 1H NMR on a Bruker WHM-300 spectrometer.

Preparation of Peptide Substrates—The peptides utilized in this study were derived from the sequence of gastrin, Arg-Arg-Leu-Glu-Glu-Glu-Glu-Ala-Lys-Tyr-Gly, as in Baldwin et al. (14). Gastrin was synthesized with a Beckman 990 peptide synthesizer. Following synthesis, the peptide was cleaved from the resin with HF, extracted with ethyl ether and acetic acid, and purified by HPLC. Chromatography was accomplished on a Beckman 332 chromatography system on a 10 × 250-mm Ultrasphere C18 column. Elution was performed with a gradient of 0-100% acetonitrile in 0.1% trifluoroacetic acid.
employing a gradient of acetonitrile in 0.1% trifluoroacetic acid over 25 min. Detection was performed at 275 nm with a Hitachi 100-40 UV-visible variable wavelength detector. Purity was confirmed by thin layer chromatography, analytical high performance liquid chromatography, and amino acid analysis on a Durrum D-400 analyzer.

**Preparation of the Insulin Receptor**—The insulin receptor was isolated from NIH 3T3 HIR5 cells transfected with the human insulin receptor cDNA (15). The cells were grown in Dulbecco's minimal essential medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 1000 units/ml penicillin, and 1 mg/ml streptomycin. Cells from confluent monolayers on 20 150-mm tissue culture dishes were harvested by solubilization in 10 mM Tris-HCl, pH 7.6, 1% Triton X-100, 250 mM sucrose, 2 mM EDTA, 5 mM benzamidine, 0.02% phenanthroline, 1 mM/ml pepstatin A, 10 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 50 μg/ml aprotinin inhibitio units/ml aprotinin. The solubilized mixture was incubated on ice for 1 h followed by centrifugation at 100,000 × g for 1 h. The supernatant was applied to 5 ml of wheat germ lectin-agarose equilibrated in 50 mM NaCl, 200 mM imidazole, and 10% heat-inactivated fetal calf serum, 1000 units/ml penicillin, and 200 μg/ml streptomycin.

**Assay of Insulin-Stimulated Receptor Kinase Activity**—Kinase activity was assayed in a reaction mix of 20 mM imidazole, 100 mM NaCl, 5% glycerol, 0.016% Triton X-100, 12 mM MgCl₂, 2 mM MnCl₂, 10 mM L-nitrophenyl phosphate, 10 μM vanadate, and 200 μM [γ-³²P]ATP. A single band was detected by sodium dodecyl sulfate-polyacrylamide electrophoresis and autoradiography. Consistent with identification as the β-subunit of the receptor, the band was localized between phosphohexose and β-galactosidase.

**Acetylation of Tyrosine Derivatives**—The reaction mix consisted of X-Tyr (1 mM) and NAI (10 mM) in 50 mM NaCl, 4.5% benzene in a total volume of 400 μl at 30°C. A specified time, 200 μl of the reaction mix was injected onto a Vydac C₁₈ column (10 X 150 mm). A separate reaction mix was prepared for each time point. Elution was accomplished with a gradient from 0.2 M triethylammonium acetate (TEAA), pH 6.0, to 0.2 M TEAA in 90% acetonitrile over 10 min. The gradient was preceded by a 5-min wash of 0.2 M TEAA. The chromatography system consisted of two Rainin Rabinatic HPLC pumps equipped with 10-ml heads and a Beckman 165 detector. The wavelengths for detection were selected from the UV absorption spectrum of each derivative. Data acquisition and analysis were accomplished with a Nelson analytical interface interfaced to an IBM PC. Rate constants were evaluated from linear regression fits of 1/C vs. ln[A] data. Standard curves of area vs. concentration were prepared for each compound. Reactions were done at pH 7.5 (sodium borate), pH 8.5 (sodium borate and Tricine), and pH 9.5 (sodium borate and glycine). The tyrosine derivatives used were tyrosine (X = H), 3-fluorotyrosine (X = F), 3-nitrotyrosine (X = NO₂), 3,5-dinitrotyrosine (X = (NO₂)₁₅), and 2,3,4,5-tetrafluorotyrosine (X = F₄). The kₚ values for the derivatives are: X = H, 10.07 (16); X = F, 9.21 (17); X = NO₂, 6.8 (18); and X = F₄, 5.40 (19).

**RESULTS AND DISCUSSION**

The acetylation of the phenolic hydroxyl group by N-acetyl-l-methacrole in is a convenient model reaction for biological phosphorylation. Both reactions are nucleophilic attacks on an electron-rich site in an acyl functional group. At pH values of 7.5, 8.5, and 9.5 only tyrosine and 3-fluorotyrosine reacted with NAI. The reaction followed second-order kinetics under the conditions utilized. No reaction was observed with tetrafluorotyrosine, 3-nitrotyrosine, or 3,5-dinitrotyrosine. The ratios of rate constants evaluated for these reactions are collected in Table I. The ratio of reactivity was used because of the uncertain stability of NAI at these conditions (20).

The activities of tyrosine and fluorotyrosine were analyzed in more detail. The introduction of an electron withdrawing substituent has a strong influence on the acetylation reaction. Initially, it seems that 3-F-Tyr is more reactive than tyrosine. Additional analysis reveals the opposite. As demonstrated in Table I, the relative activities of tyrosine and fluorotyrosine are dependent upon the pH of the reaction mixture. At lower pH values, fluorotyrosine is much more reactive, presumably because of its lower kₚ value. Upon an increase in pH, the reactivity of tyrosine more closely matches that of fluorotyrosine and finally, the reactivity of tyrosine becomes greater than that of fluorotyrosine. The change in relative reactivities is correlated to the relative concentration of the reactive species, the phosphonides, as illustrated in Fig. 1.

The relationships shown in Fig. 1 can be fit to the equation: log (kₚ-X TYR/kₚ-tyr) = 0.32 (A-X TYR/A-tyr) - 1.55. At the point

**FIG. 1. Relationship between relative reactivity and concentration of the reactive anion.** The values for relative reactivity are from Table I. The anion concentrations are calculated using kₚ values of 10.07 and 9.21 for tyrosine and fluorotyrosine, respectively.
Enzyme assays were performed as described under "Experimental Procedures." The enzyme was present at a final concentration of 10 
μg/ml; insulin, when included, was present at a final concentration of 10 
μM. The fluorotyrosyl peptide was phosphorylated with the reaction was decreased more than 8-fold in the presence of 10 
μM fluorotyrosine. Replacement of the target residue by a fluorotyrosine were used as substrates for the insulin receptor/kinase in comparison with serine kinases. Cyclic GMP- and AMP-dependent protein kinases, for example, have values from 1000 to 4000 nmol/min/mg (25, 26), but the insulin receptor kinase has values from 6 to 72 nmol/min/mg (27-29). Generation of a reactive anionic form of the substrate on the enzyme surface is not unknown, and it has been demonstrated that a thiolate anion is generated at the active site of glutathione transferase (30). It was suggested that the enzyme's binding energy is used to position the thiol in such a way to induce a decrease in the pKₐ of the thiol. Binding of the fluorotyrosyl peptide may have a lower pKₐ because of the presence of a more easily deprotonated hydroxyl requires a lower expenditure of binding energy.

Although sharing common features in catalysis, tyrosine and serine kinases do not have overlapping specificities. For the insulin receptor/kinase, serine and threonine substrate analogues serve as inhibitors of the tyrosine kinase activity (31). The Kₐ values of the peptide substrates are approximately 2 mM; each of the inhibitory peptides has a Kₐ of the same magnitude (2-4 mM). Because the rest of the peptide is identical, it is clear that the kinase must be able to distinguish the tyrosine from serine and threonine. Similar discrimination can be demonstrated with other kinases. Autophosphorylation of the tyrosine kinase, P130 -ERK1, was completely blocked by mutation of tyrosine to serine or threonine (32). Enzymic functional groups other than the conserved aspartic acid residue must be involved to provide additional determinants of specificity.

This study suggests another aspect to the regulation of the substrate specificity of tyrosine kinases, particularly the insulin receptor/kinase. Previous studies (5-10) have provided evidence that acidic residues on the N-terminal side of the modified tyrosine residue are important determinants of kinase specificity. The results of the present study demonstrate that the chemical environment about the phenolic hydroxyl group is an important feature in the specificity of tyrosine kinases. We propose that, in good substrates, the acceptor tyrosine residue is situated within an environment enabling efficient deprotonation of the tyrosine residue. The importance of the β-turn structure (11) may be related to the generation of the proper environment.

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| Peptide | Kₐ* | Vₐ* |
|---------|-----|-----|
| -Insulin | +Insulin | -Insulin | +Insulin |
| Gastrin | 0.40 ± 0.18 | 0.41 ± 0.05 | 1.2 ± 0.2 | 4.9 ± 0.2 |
| F'-Tyr-gastrin | 0.080 ± 0.035 | 0.14 ± 0.02 | 0.18 ± 0.03 | 0.59 ± 0.03 |

* Kinetic constants were evaluated using a weighted linear regression program. The substrate concentration was varied from 0.5 Kₐ to 5 Kₐ.

where [A']₇₃₀/[A']₇₅₀ = 1.0 (both fully deprotonated), the ratio R₇₃₀/R₇₅₀ becomes equal to 17. This reveals that, consistent with Brønsted theory, tyrosine is 17-fold more reactive than fluorotyrosine under conditions at which the hydroxyl group of each compound is fully deprotonated. The seemingly higher reactivity of F-Tyr at pH 7.5 and 8.5 is because of the higher concentrations of reactive (anionic) species at these pH values. Assuming a similar relationship between tyrosine and tetrafluorotyrosine (Kₐ 5.40), analysis suggests that tyrosine would be 83-fold more reactive than tetrafluorotyrosine at pH 7.5. This may explain the lack of reactivity of tetrafluorotyrosine with NAI at pH 7.5.

The phosphorylation of tyrosyl-containing peptides by the insulin receptor/kinase is also modulated by chemical features of the acceptor substrate. Peptides containing either tyrosine or fluorotyrosine were used as substrates for the insulin receptor/tyrosine kinase. Both the tyrosyl- and fluorotyrosyl-containing peptides were phosphorylated in both the absence and presence of insulin; insulin-stimulated peptide phosphorylation 3-4-fold. Replacement of the target residue by a derivative containing a hydroxyl group with a lower pKₐ, 2.91 versus 10.07, dramatically affects the phosphorylation of the peptide. The fluorotyrosyl peptide was phosphorylated with an approximately 3-fold lower Kₐ value, but the Vₐ value for the reaction was decreased more than 8-fold in the presence of insulin. The kinetic parameters for both reactions are presented in Table II.

These results indicate that the ionization state of the hydroxyl group may be an important regulatory feature in both the binding of substrate and the phosphorylation reaction. The substrate with the lower pKₐ seems to bind better but is phosphorylated less well. As a first approximation, these results suggest that the enzyme has higher affinity for the anionic form of the substrate. By the same criterion, it seems unclear why, if the enzyme has higher affinity for the anionic species, the tyrosine phosphate is phosphorylated with a higher Vₐ. This result is consistent, however, with the results of the model acylation reaction provided the anionic species is formed upon binding to the enzyme. When bound to the kinase, the hydroxyl group of each peptide is deprotonated equally well. It is predicated that once deprotonated, tyrosine will be more reactive, as in the model reaction.

Formation of a deprotonated seryl residue has been postulated for the phosphorylation of Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) by the cAMP-dependent protein kinase (21, 22) via general base catalysis. The deprotonation is proposed to occur simultaneously with binding of the substrate to the enzyme and to utilize aspartic acid at position 184 of the catalytic subunit in the deprotonation of the serine residue (22). Because this aspartic acid is conserved in all protein kinases (23), deprotonation of the acceptor residue may be a feature of catalysis common to all protein kinases, with the deprotonated species being the reactive nucleophile. Although tyrosine, with pKₐ = 10.07 (16) versus 13.6 (24) for serine, should be more easily deprotonated than serine is, the alkoxide of serine should be a more potent nucleophile than the phenoxide of tyrosine is. The chemical difference of the substrates may contribute to the lower Vₐ value seen for tyrosine kinases in comparison with serine kinases. Cyclic GMP- and AMP-dependent protein kinases, for example, have values from 1000 to 4000 nmol/min/mg (25, 26), but the insulin receptor kinase has values from 6 to 72 nmol/min/mg (27-29). Generation of a reactive anionic form of the substrate on the enzyme surface is not unknown, and it has been demonstrated that a thiolate anion is generated at the active site of glutathione transferase (30). It was suggested that the enzyme's binding energy is used to position the thiol in such a way to induce a decrease in the pKₐ of the thiol. Binding of the fluorotyrosyl peptide may have a lower pKₐ because of the presence of a more easily deprotonated hydroxyl requires a lower expenditure of binding energy.
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