Enzymatic Synthesis of Phosphotyrosine-Containing Peptides via Adenylylated Intermediates

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Abstract: A novel two-part strategy has been developed for the specific O-phosphorylation of tyrosine residues in peptides. The first part involves the enzymatic transfer of an AMP moiety to the *O*-tyrosyl side chains of the peptide substrate to produce a stable adenylylated intermediate. This step is mediated by *Escherichia coli* glutamine synthetase adenylyltransferase, whose native function is the specific adenylylation of tyrosine-397 in glutamine synthetase. The second step consists of either the enzymatic or chemical degradation of the adenylylated intermediate to produce the corresponding phosphotyrosine-containing peptide. Both an enzymatic procedure using micrococcal nuclease and an oxidative degradation procedure using sodium m-periodate were used in this latter step. Several peptides, including [Tyr^5]bradykinin, angiotensin II, [Val^5]angiotensin II, neurotensin, Leu-enkephalin, and a 17-residue peptide encompassing the tyrosine adenylylation site in glutamine synthetase, were subjected to this synthetic procedure with overall yields ranging from 3 to 40%. High-performance liquid chromatography, liquid secondary ion mass spectrometry, tandem mass spectrometry, and amino acid analysis were used to isolate and characterize the adenylylated intermediates and phosphotyrosine-containing products. These analyses showed that both the micrococcal nuclease and $NaIO_4$ degradative methods produced a nearly quantitative yield of phosphorylated peptide from the adenylylated intermediate.

Introduction

Since the first isolation and characterization of a tyrosine kinase, the number of proteins identified as belonging to this important family has dramatically increased.[1] This family of proteins includes the insulin[2] and epidermal growth factor receptors,[3] the src family of kinases,[4] and various protooncogene and viral oncogene products.[5] The common feature among this rather diverse structural group of enzymes is their ability to phosphorylate specific tyrosine residues in various target proteins. The phosphorylation of these endogenous substrates by tyrosine kinases and their dephosphorylation by protein phosphatases[6] control a number of important cellular processes that can initiate a cascade of events including mitogenesis and, in the case of oncogene products, cellular transformation and carcinogenesis.[7] The role of oncogenic tyrosine kinases in cellular transformation has initiated several investigations toward the design of specific tyrosine kinase inhibitors.[8,9] While the exact mechanism of tyrosine kinases is not known, it is likely that a peptide could act as substrate- or product-type inhibitor for these kinases.

One of the major problems that has limited investigations into the development of peptide-based inhibitors of tyrosine kinases is the lack of synthetic approaches toward the preparation of peptides containing phosphorylated tyrosines. Only a few papers[10-14] have been published that present methods to synthesize phosphotyrosine-containing peptides, and these methods are complicated by side reactions, severe sequence limitations, and/or low yields. Recently, we investigated the use of *N*-(*tert*-butoxycarbonyl)-*O*-(dibenzylphosphono)-L-tyrosine[11] as a protected derivative of phosphotyrosine for peptide synthesis but encountered significant problems, particularly in dephosphorylation and other side reactions during HF deprotection. Valerio and co-workers[14] have reported the successful synthesis of the protected pentapeptide Z-Asn-Glu(OBzl)-Tyr(PO_3Me_2)-Thr(OBzl)-Ala-OBzl using Boc-Tyr(PO_3Me_2)-OH, although harsh deprotection conditions were needed to generate the final phosphorylated product in moderate yields. Alternatively, it would be desirable to have an enzymatic approach that could be used to synthesize phosphotyrosine-containing peptides using tyrosine kinases, since it would be particularly attractive to have a method of phosphorylating tyrosines of existing peptides. In such a scheme, peptides could be either isolated from natural sources or produced with high efficiency by using standard synthetic methodologies and then converted to their phosphotyrosine analogues. However, the extent of phosphorylation for peptide substrates with tyrosine kinases is generally quite low, due in part to high K_m values ($\geq$2 mM), contaminating phosphatases, and product and substrate inhibition.[15] The high specificity of tyrosine kinases for their native substrates could also place too great a restriction on sequence variations around a target tyrosine.[4] In addition, the purification and use of these kinases for synthetic purposes are complicated by the fact that these enzymes are membrane-associated and solubility problems are generally encountered.

In an effort to devise an alternative route to the synthesis of phosphotyrsine-containing peptides, we have investigated the synthetic utility of another enzymatically mediated modification of tyrosine residues in proteins, namely, the adenylylation of tyrosine. *Escherichia coli* glutamine synthetase is adenylylated at a specific tyrosine residue (Try-397) by adenylyltransferase (EC 2.7.7.42, ATase).[16] This enzyme has been isolated and purified from *E. coli*[17,18] and *Mycobacterium smegmatis*[19] and

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(16) Symbols used for amino acids and peptides are in accordance with the IUPAC-IUB Commission on Biochemical Nomenclature. Other abbreviations used: ATase, adenylyltransferase; LSIMS, liquid secondary ion mass spectrometry; MS/MS, tandem mass spectrometry; HPLC, high-performance liquid chromatography; MH^+, protonated molecular ion; TFA, trifluoroacetic acid.
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has been shown to mediate the transfer of an AMP moiety from ATP to tyrosine-397 only.[20,21] Given the quantitative extent of this reaction for its endogenous protein substrate, it seemed possible that this enzyme might also transfer AMP to tyrosine-containing peptide substrates in reasonable yields,and perhaps with better efficiency than in the analogous transfer of phosphate by protein tyrosine kinases. Since ATase's are cytoplasmic enzymes, they also lack the solubility problems inherent to the membrane-associated tyrosine kinases. This reaction is of interest since there exists at least two potential routes for the conversion of adenylylated tyrosine to the phosphorylated tyrosine analogues by either enzymatic cleavage with micrococcal nuclease[22,23] or chemical degradation of the adenosine group with sodium periodate.[24] In this paper we describe a general synthetic method for preparing phosphotyrosine-containing peptides from peptide substrates using the intermediate formation of adenylylated tyrosine prepared by treatment with *E. coli* ATase.

Experimental Section

Materials. Bradykinin, [Tyr^5]bradykinin, angiotensin II, [Val^5]angiotensin II, neurotensin, and Leu-enkephalin were all purchased from Peninsula Laboratories, Belmont, CA. All commercial peptides were assayed by HPLC and liquid secondary ion mass spectrometry (LSIMS), found to be homogeneous, and therefore used without further purification. A 17 amino acid peptide encompassing the site of tyrosine adenylylation in *E. coli* glutamine synthetase,[21,25] Gly(389)-Glu-Ala-Met-Asp-Lys-Asn-Leu-Tyr-Asp-Leu-Pro-Pro-Glu-Glu-Ala-Lys(405), was a generous gift from Dr. J. S. McMurray (M. D. Anderson Cancer Center, Houston, TX) and was synthesized by a 9-fluorenyl(methyloxycarbonyl)-*tert*-butyl strategy[26] using a Biosearch 9500 peptide synthesizer. Glutamine synthetase adenylyltransferase (ATase) from *E. coli* was kindly provided by Dr. S. G. Rhee at the National Heart, Lung and Blood Institute, NIH, and the procedures for the purification of this enzyme have been published elsewhere.[27] Sodium periodate and micrococcal nuclease were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of reagent grade except for those used for HPLC separations [acetonitrile, water, and trifluoroacetic acid (TFA)], which were obtained in their HPLC and/or sequencing grades.

Adenylylation of Peptides Substrates. In the first set of experiments, reaction conditions were followed based on those used for the quantitative adenylylation of glutamine synthetase by *E. coli* ATase.[27] An ATase solution containing 0.01–0.1 mg of ATase was prepared in a solution of 18 mM ATP, 0.12 M Tris-HCl (pH 7.8), 45 mM $MgCl_2$, and 0.12 M L-glutamine. This solution was preincubated for 15 min at room temperature and then added to 0.1–0.5 mg of various tyrosine-containing peptides dissolved in a solution consisting of 10 mM Tris-HCl (pH 7.8) and 20 mM $MgCl_2$. The final concentrations were 28 mM Tris-HCl (pH 7.8), 28 mM magnesium chloride, 3 mM ATP, and 20 mM L-glutamine in a total volume that ranged between 0.1 to 1 mL. These solutions were then incubated for 24 h at room temperature, and in some cases, an additional aliquot of ATase (~50 μg) was added and the reaction continued for an additional 24 h. To further investigate the conditions for increasing the adenylylation yields of the various peptide substrates, these conditions were varied as described under Results.

The adenylylated peptide mixtures were separated by reverse-phase HPLC using either a Kratos 783 variable-UV detector at 215 nm or an ABI 1000S UV diode array detector. All separations were carried out on a Vydac C_{18} reverse-phase analytical column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid in a H_2O mobile phase (solvent A). The peptides were eluted at 1 mL/min with a linear solvent gradient of 0.08% TFA in 30% H_2O/70% acetonitrile (solvent B) to a final composition of 50% B in 35 min. Peptide fractions were manually collected and directly analyzed by LSIMS to determine the identity of the reaction products. After identifying the adenylylated peptides by LSIMS, these peptide intermediates were dried with a Savant liquid concentrator and set aside for further enzymatic or chemical degradation. The yields of these adenylylation reactions in some cases were estimated by comparing amino acid analysis data for the unreacted peptides and their adenylylated products. For these analyses, the peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h, treated with phenylisothiocyanate to form their phenylthiocarbamyl derivatives,[28] and then analyzed by using a Pico-Tag analysis system on a Beckman 121 MB amino acid analyzer.

Preparation of Phosphorylated Peptides. To prepare phosphorylated peptides from the adenylylated intermediates, both an enzymatic and chemical method were used. For the enzymatic cleavage, micrococcal nuclease was used to hydrolyze the ribose–phosphate linkage in adenylylated tyrosine to yield free adenosine and the phosphotyrosine peptides. For this reaction, an initial set of reaction conditions was used that consisted of an enzyme/substrate weight ratio of 1:1 in a Tris-HCl buffer (200 mM, pH 9) containing 10 mM $CaCl_2$. After incubation for 24 h at 37 °C, additional micrococcal nuclease was added and the reaction continued. At the end of 48 h (and sometimes longer), the reaction was stopped by freezing and the resulting peptides were separated by HPLC as previously described. The HPLC fractions were then analyzed by LSIMS and tandem mass spectrometry (MS/MS).

In addition to micrococcal nuclease digestion, an alternative two-step oxidative procedure was used to remove adenosine. For this reaction, the adenylylated peptides were first dissolved in 100 μL of sodium bicarbonate (50 mM, pH 10). Originally, a 5–10-fold excess of $NaIO_4$ was added to oxidize the ring C-2,3 diol to the corresponding dialdehyde,[24] although subsequent reactions were carried out successfully using only a 2-fold excess. After 25 min at room temperature, a 5-fold excess of ethylene glycol over $NaIO_4$ was added to destroy the excess periodate. The solution was incubated at 50 °C for 1 h to effect the β elimination of phoshate from the ring-opened nucleoside. The reaction was stopped by freezing, and the peptide mixture was separated by reverse-phase HPLC under the same conditions as previously described. Peptide fractions were then characterized by LSIMS and/or MS/MS analysis.

Mass Spectrometry. Peptide fractions were analyzed on a Kratos MS-50S mass spectrometer operating with a Cs^+ ion source.[29] Samples were dissolved in 0.1% TFA/H_2O and applied with 1 μL of glycerol/thioglycerol (1:1) to the LSIMS probe tip. A Cs^+ ion primary beam of 8 keV was used, and the secondary sample ions were accelerated at 8 kV. Scans were taken at 300 s/decade, and the spectra were recorded on a Gould electrostatic recorder and manually calibrated with Ultramark 1621 as external reference. To determine the site(s) of phosphorylation in these peptide substrates, the phosphorylated analogues of [Tyr^5]bradykinin and angiotensin II were analyzed by tandem mass spectrometry (MS/MS).[30,31] MS/MS spectra were taken primarily in the positive ion mode with a Cs^+ beam energy of 15–18 keV. A four-sector mass spectrometer (Kratos Concept II HH) equipped with an array detector on MS-II was used to acquire all MS/MS spectra, as previously described.[32] Samples were prepared as described above with approximately 1 μg of material. The isotopically pure ^{12}C component of the protonated (MH^+) or deprotonated molecular ions $(M - H)^-$ was selected in MS-I and collisionally activated in a helium collision cell located between MS-I and MS-II that was floated at 2 kV. The pressure of the helium in the cell was adjusted to attenuate the molecular ion abundance by $^2/_3$. The resulting fragment ions were then separated in MS-II and detected in successive adjacent 4% mass windows by use of a constant B/E field.

Results and Dicussion

Adenylylation of Peptide Substrates with ATase. The specific adenylylation of Tyr-397 in *E. coli* glutamine synthetase by ATase has been shown to occur rapidly and quantitatively in vitro.[27] However, the adenylylation of tyrosine with ATase on peptide substrates had not been previously attempted and would be expected to be slower than its native 52-kDa substrate.[25] Since this

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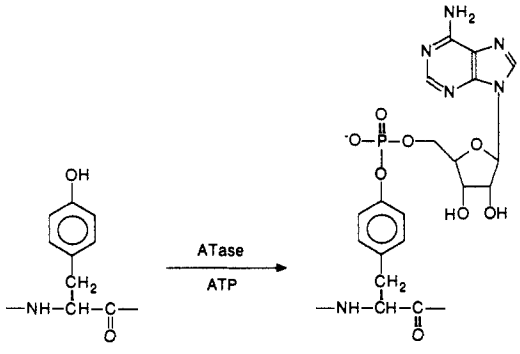
Table I. Sequence of Peptides Substrates Subjected to ATase-Mediated Adenylylation

Leu-Enkephalin:	Tyr-Gly-Gly-Phe-Leu
Neurotensin:	pyroGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
	pyroGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
[Tyr^5]-Bradykinin:	Arg-Pro-Pro-Gly-Tyr-Ser-Pro-Phe-Arg
Bradykinin:	Arg-Pro-Pro-Gly-Ser-Ser-Pro-Phe-Arg
Angiotensin II:	Asp-Arg-Val-Tyr-Ile-His Pro Phe
[Val^5]-Angiotensin II:	Asp-Arg-Val-Tyr-Val-His Pro Phe
Angiotensin I:	Asp-Arg-Val-Tyr-Ile-His Pro Phe-His Leu
GS-17:	Gly-Glu-Ala-Met-Asp-Lys-Asn-Leu-Tyr-Asp-Leu-Pro-Pro-Glu-Glu-Ala-Lys

Scheme I



reaction is the first step in our proposed route to the preparation of phosphotyrosine-containing peptides, an investigation of this reaction was carried out using several peptide substrates. In our initial experiments, several commercially available peptides containing one or more tyrosine residues(s) (i.e., neurotensin, angiotensin II, [Val^5]angiotensin II, [Tyr^5]bradykinin, and Leu-enkephalin) were used as substrates for *E. coli* ATase under conditions that gave quantitative adenylylation of the target tyrosine in glutamine synthetase (see Table I). Under the same initial reaction conditions for all these peptides, adenylylated peptide products were produced in each case with the exception of the non-tyrosine-containing peptide, bradykinin. Scheme I illustrates this reaction pathway.

Although all these peptides yielded some adenylylated product, the extent of this reaction varied considerably. For example, according to the HPLC separation of the adenylylated products (see Figure 1) from the unmodified commercial peptide substrates shown in Table I, [Tyr^5]bradykinin gave the highest yield on the basis of peak height (>25%, UV_{215} absorbance), followed by angiotensin II (15%, UV_{215} absorbance). Amino acid analysis indicated somewhat lower yields of 15% for bradykinin and 10% for angiotensin. This discrepancy is likely due to the increase in the UV absorbtion at 215 nm (40–50%) associated with the aromatic AMP group of the adenylylated peptide (see Figure 2). In comparison, neurotensin and Leu-enkephalin had somewhat poorer yields (~3–10%, corrected UV_{215} absorbance).

The complete UV spectrum of adenylylated and nonadenylylated [Tyr^5]bradykinin peaks was obtained from on-line UV diode array detection and clearly showed a shift in the chromophore for the adenylylated peptide product (see Figure 2). The identities of the adenylylated peptides were also confirmed by LSIMS analysis, which showed a shift of 329 Da (the mass difference for AMP addition) for the corresponding molecular ion species relative to the unmodified peptides (see Figure 3). In all cases, the adenylylated peptides eluted prior to the nonadenylylated peptides by C_{18} reverse-phase HPLC at approximately 1–2% less acetonitrile in the gradient program (see Figure 1). The disparity in yields among these peptides indicates that significant differences exist in the substrate specificity among these peptides under the conditions used in this study. Indeed, when a 17 amino

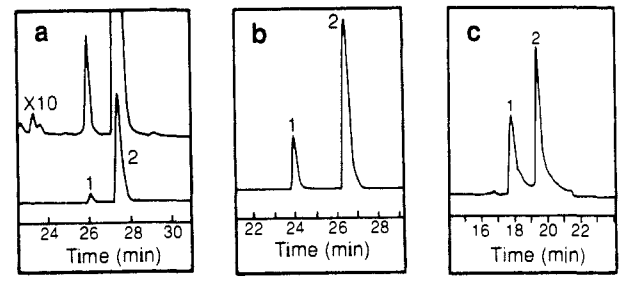


Figure 1. HPLC chromatograms of various peptides after treatment with *E. coli* ATase showing adenylylated (peak 1) and nonadenylylated (peak 2) forms of (a) angiotensin II, after 44-h incubation with E/S 1:2 (wt/wt); (b) [Tyr^5]bradykinin, after 44-h incubation with E/S 1:2 (wt/wt); and (c) GS-17, after 28-h incubation with E/S 1:8 (wt/wt).

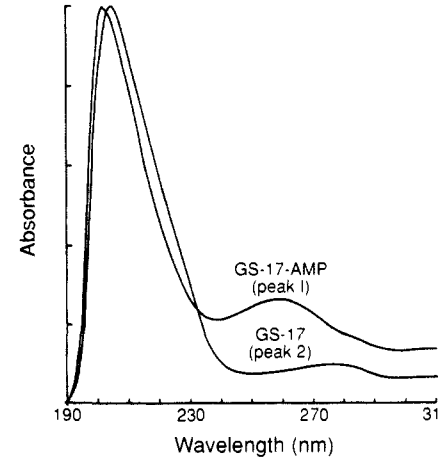


Figure 2. UV diode array spectrum of adenylylated and nonadenylylated GS-17 (peaks 1 and 2 in Figure 1c) normalized to their maximum absorbance.

acid peptide (GS-17) that encompasses the native Tyr-397 adenylylation site in glutamine synthetase (residues 389–405) was subjected to this same procedure, a yield of 35–40% (corrected UV_{215} absorbance) was obtained after incubation for 96 h at an E/S ratio of 1:8. However, it may be possible that changes in the reaction conditions could produce higher yields for some or all of these peptides.

In the LSIMS spectra of the adenylylated [Tyr^5]bradykinin products, MH^+ ions were found for the adenylylated peptide (m/z 1405.7) as well as several smaller species that were also adenylylated, but that were missing amino acids from the N terminus. For example, a MH^+ ion at m/z 1249.6 was found that corresponds to the loss of N-terminal Arg. A MH^+ ion was also found for unreacted [Tyr^5]bradykinin (m/z 1076.5) along with at least one truncated version lacking the N-terminal Arg at m/z 920.5. Further analysis of several smaller peaks indicated that additional N-terminally clipped peptides were present in the adenylylated peptide mixture and suggested the presence of a contaminating

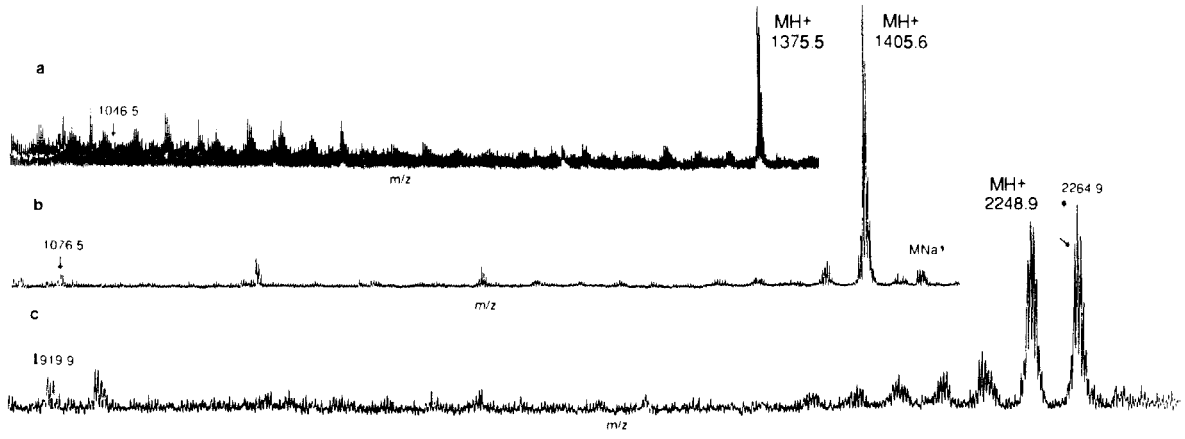


Figure 3. Partial LSIMS spectra of the molecular ion region of adenylylated (a) angiotensin II (MH^+ m/z 1375.6), (b) [Tyr^5]bradykinin (MH^+ m/z 1405.6), and (c) GS-17 (MH^+ m/z 2248.9). Oxidized adenylylated GS-17 (MH^+ m/z 2264.9) was also found in this latter preparation corresponding to formation of methionine sulfoxide, which can be reversed by treatment with dithiothreitol.

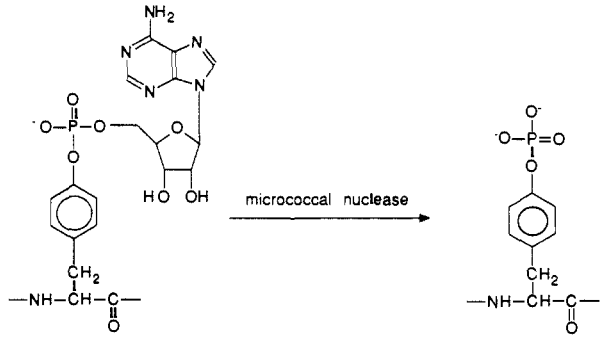
amino peptidase in the ATase preparation. Subsequent to our initial studies, however, additional preparations of the ATase no longer contained significant amounts of this activity, and only intact adenylylated and unreacted peptide peaks were found after HPLC separation.

Within the limits of detection, the LSIMS analyses indicate that the adenylylation is completely specific for tyrosine residues in these peptides, i.e., no adenylylation of either serine or threonine residues occurs. For example, when native bradykinin containing Phe instead of Tyr at the fifth position (see Table I) was incubated with ATase as a control, no reaction was observed at the alternative Ser-6 site. This compares to a 10–20% adenylylation yield for [Tyr^5]bradykinin at Tyr-5 under the same reaction conditions. The specificity observed in the peptide substrates for tyrosine is consistent with what is known about the selectivity of ATase for the adenylylation of glutamine synthetase at tyrosine-397.[33] It is also interesting to note that ATase appears to have some selectivity for different tyrosine residues within a single peptide substrate. When neurotensin is incubated with ATase, an analysis of the adenylylated peptide products by LSIMS showed that only the first tyrosine (position 3), and not the second tyrosine (position 11), was significantly adenylylated. This may reflect the similarity of the two amino acids adjacent to Tyr-3 in neurotensin (Leu-2 and Glu-4) to those found on either side of Tyr-397 in glutamine synthetase (Leu-396 and Asp-398).

The adenylylation of glutamine synthetase by *E. coli* ATase has been reported to occur rapidly in vitro, requiring only a few minutes under the reaction conditions employed in our peptide experiments.[27] However, when peptides are used as substrates for adenylylation, the reaction rate was found to be considerably slower in all cases. In fact, the reaction was incomplete after 5 days of incubation with ATase, even though the enzyme still retained considerable activity. We do not know at this point what factors limit the reaction, although it is possible that product inhibition of ATase by the adenylylated peptide products may be partially responsible. Alternatively, some preparations of ATase have been reported to possess deadenylylation activity[19,34] (P_i + peptide-AMP → peptide + ADP) which may, in the presence of contaminating P_i (formed from hydrolysis of ATP), convert adenylylated product back to the starting material.

Conversion of Adenylylated Peptides to Phosphotyrosine Peptides by Micrococcal Nuclease. To complete the conversion of the adenylylated peptides to their phosphorylated forms, a second step was employed in this synthetic strategy consisting of cleavage of the adenosine group from the adenylylated intermediate to produce phosphotyrosine-containing peptides. In our first experiments,

Scheme II



an enzymatic procedure using micrococcal nuclease[22,23] was used according to Scheme II.

The cleavage of adenosine from the adenylylated peptides with micrococcal nuclease was considerably more difficult than that reported for a similar reaction with adenylylated *E. coli* glutamine synthetase.[22,23] For the adenylylated peptide substrates, larger concentrations of micrococcal nuclease and longer incubation times were needed for complete conversion. For example, after digestion of adenylylated [Tyr^5]bradykinin by micrococcal nuclease for 24 h with an enzyme/substrate ratio of 1:1 (wt/wt), significant adenylylated material still remained. However, by increasing both the reaction time to 48 h and micrococcal nuclease concentration 2-fold (2:1, enzyme/substrate weight ratio), the adenylylated material disappeared completely and a large peak corresponding to phosphorylated [Tyr^5]bradykinin appeared in the HPLC profile (see Figure 4a). In some cases, we also observed a small amount of the original unphosphorylated peptide that was apparently being formed by hydrolysis of the deadenylylated peptide. In all cases, LSIMS analysis of the separated products identified the phosphorylated analogues of these peptides by their characteristic mass shifts of +80 Da for the MH^+ ions compared to their nonphosphorylated counterparts e.g., m/z 1156.5 (phosphorylated bradykinin) and 1126.5 (phosphorylated angiotensin II). Several large peaks also appeared in the HPLC traces of both incubation mixtures at identical retention times and are derived from impurities in the micrococcal nuclease preparation.

Conversion via Oxidative Degradation with Sodium Periodate. In an alternative approach to converting the adenylylated peptide intermediates to their phosphotyrosine peptides, a chemical reaction scheme pioneered by Frey and co-workers[24] was used that involves the oxidative degradation of the ribose followed by β elimination of the phosphate moiety. In this reaction, sodium periodate first oxidizes the ribose ring at the C-2,3 diol to form the ring-opened intermediate. However, this intermediate is unstable under basic conditions and undergoes complete elimination (pH 10.5, 50 °C) to yield the phosphorylated peptide by specific C–O bond cleavage at the ribosyl C-5 position. In order

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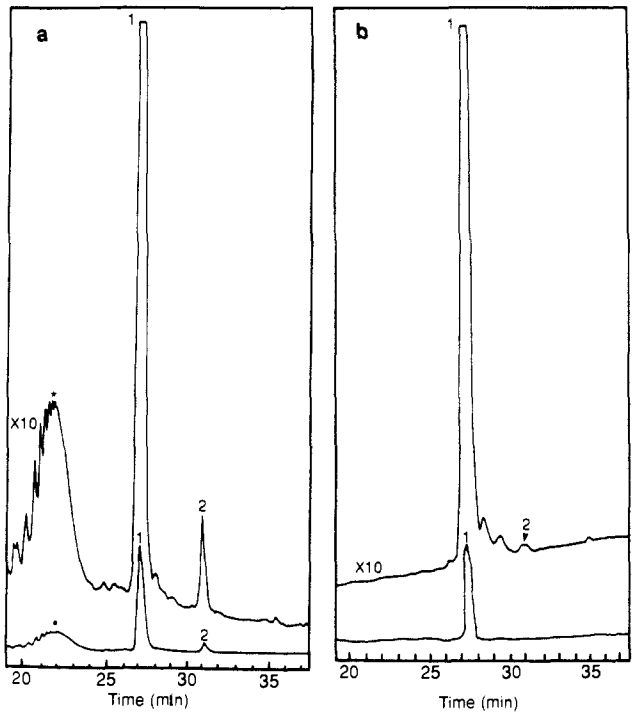
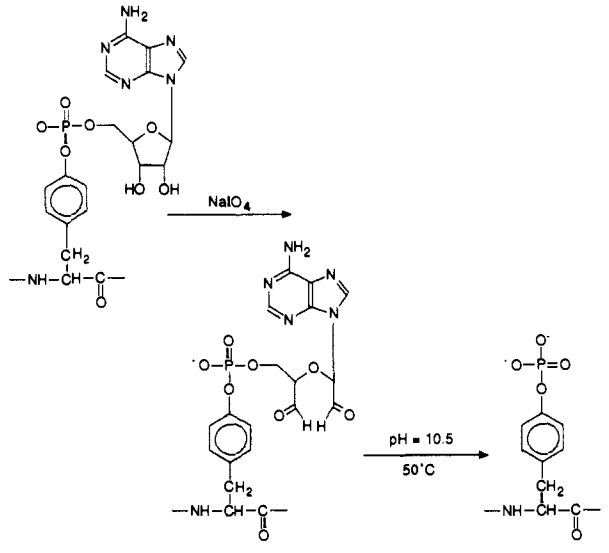


Figure 4. HPLC chromatograms showing phosphorylated [Tyr^5]bradykinin produced from conversion of the adenylated intermediate by treatment with (a) micrococcal nuclease (E/S 2:1 (wt/wt), 48 h) or (b) oxidative degradation with $NaIO_4$ (5× molar excess, 30 min) followed by β elimination.

to prevent overoxidation with possible side reactions at other sensitive side chains, the $NaIO_4$ was destroyed by the addition of ethylene glycol at the end of the reaction period.

This two-step oxidative degradation scheme gave a virtually quantitative yield of the desired phosphotyrosine products for [Tyr^5]bradykinin, angiotensin II, and GS-17. After this simple reaction, the majority of the adenylylated peptides disappeared and were converted to their phosphorylated products as determined by HPLC separation (Figure 4b) and LSIMS analysis of the

Scheme III



resulting product(s), which showed a mass shift of −249 Da from the adenylylated intermediates e.g., m/z 2248.9 → 1999.9 (GS-17), m/z 1375.6 → 1126.5 (angiotensin II), and m/z 1405.6 → 1156.5 ([Tyr^5]bradykinin). The conversion of the adenylylated peptides to their phosphorylated analogues via this oxidation procedure is fast and easily carried out to completion. It is interesting to note that while the single methionine in GS-17 was found partially oxidized to methionine sulfoxide after this procedure, its percentage relative to the unoxidized form had not increased over that formed during the long incubation with ATase (see Figure 1c). One should be cautious, however, as a reaction using greater than 2-fold excess of $NaIO_4$ can produce other side reactions.

Mass Spectrometry of Phosphorylated Peptides. To identify tyrosine as the site of phosphorylation in the final products, both LSIMS and tandem mass spectrometry were carried out on several of the phosphorylated peptides. The LSIMS spectrum of [Tyr^5]bradykinin contained many of the features that we have previously identified as being unique to phosphotyrosine peptides,[11]

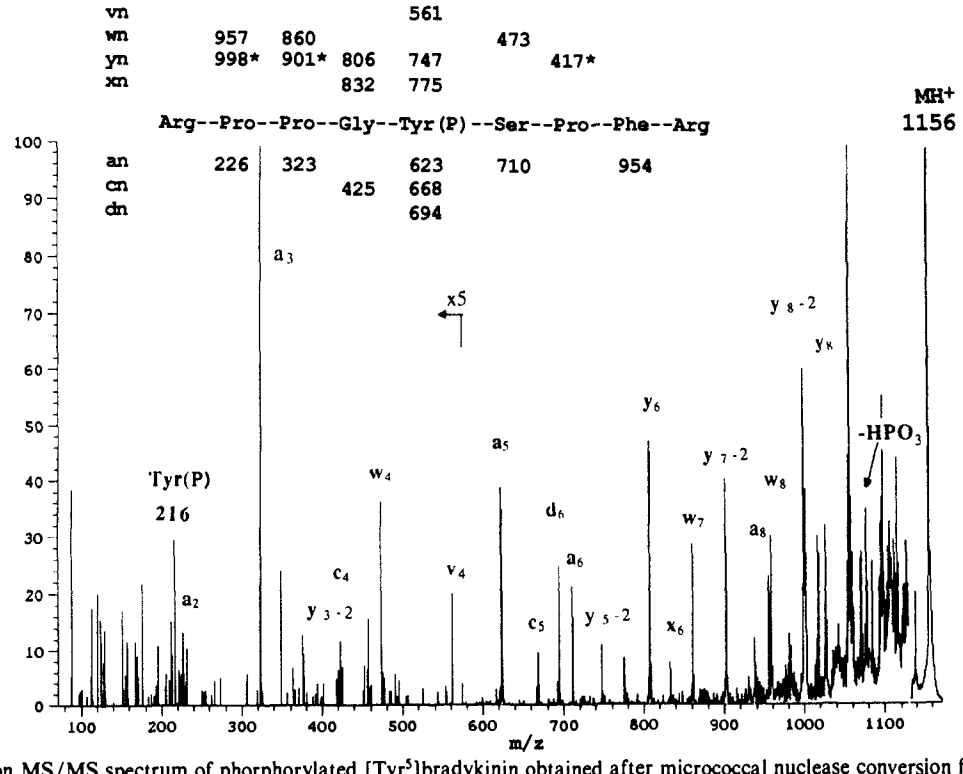


Figure 5. Positive ion MS/MS spectrum of phorphorylated [Tyr^5]bradykinin obtained after micrococcal nuclease conversion from the adenylylated intermediate. Fragmentation nomenclature is used according to Johnson et al.[35]

such as losses of HPO_3 (-80 Da) and HPO_4 (-96 Da). The MS/MS spectrum of phosphorylated [Tyr^5]bradykinin (Figure 5) contained definitive fragment ions that clearly identified phosphotyrosine and its sequence position. The peak at m/z 216 is the immonium ion fragment of a phosphoryltyrosine residue, $CH(CH_2C_5H_4OPO_3H_2)=NH_2^+$, and has been observed in other MS/MS spectra of phosphotyrosine-containing peptides.[30,31] One also observes significant fragment ions for $(M + H - HPO_3)^+$ and $(M + H - H_2PO_3)^+$, which is observed primarily as $(M + H - H_3PO_4)^+$ in peptides containing phosphoserine.[36]

Conclusions

The importance of tyrosine kinase activity associated with growth factor receptors and oncogenic and protooncogenic protein products is well established. To study this important class of enzymes in more detail, more versatile and efficient synthetic methods for the preparation of peptides and peptide analogues containing phosphotyrosine need to be developed. Some of these peptides may find use as product inhibitors of these tyrosyl protein kinase enzymes, leading to their development as pharmaceuticals. In addition, a synthetic route for phostyrosine-containing peptides will undoubtedly be essential to determine the substrate specificity of the associated tyrosine phosphatase enzymes.

The strategy described here provides an efficient route in the synthesis of phosphotyrosine-containing peptides directly from their parent nonphosphorylated peptides. The conversion of the adenylylated peptide intermediates by either treatment with micrococcal nuclease (a commercially available enzyme) or oxidative degradation with sodium periodate has been found to be virtually quantitative, with very little side product formation. The transfer of AMP to peptide substrates catalyzed by ATase is versatile and highly specific for tyrosine moieties. Although ATase is not commerically available, it has reportedly been overproduced to levels approaching 500-fold in *E. coli* using the plasmid vector pKC30.[37] It is also the only available method that can work on intact peptides with selectivity for tyrosine residues. It is encouraging that all peptides that have been subjected to the ATase treatment have been adenylylated to some extent (≥3%), although this may not have been the case for the second tyrosine (Tyr-11) in neurotensin. In this latter case, it is not clear whether Tyr-11 is a poor substrate or if the relatively faster adenylylation of Tyr-3, whose neighboring amino acids resemble those in glutamine synthetase, occurs to such an extent as to preclude adenylylation of the second tyrosine.

It may be possible to increase significantly this reaction yield by coupling the conversion of the adenylylated intermediate to the formation of its phosphotyrosine product by finding conditions where both ATase and micrococcal nuclease can function simultaneously. We are currently exploring this possibility along with immobilization of both enzymes with the aim of finding conditions that will yield a quantitative overall conversion to the phosphotyrosine peptide product. Beside the obvious importance of a synthetic strategy for phosphotyrosine peptides, the production of the corresponding adenylylated tyrosine analogues also has some interesting implications. For example, peptides containing adenylylated tyrosine may find use as bisubstrate inhibitors of tyrosine kinases, as they incorporate potential binding features of both ATP and tyrosine-containing substrates.

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Laser Photochemistry of DNA: Two-Photon Absorption and Optical Breakdown Using High-Intensity, 532-nm Radiation

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Abstract: Formation of cyclobutylpyrimidine dimers and strand breaks in double-stranded DNA was investigated by using 532-nm, 28-ps pulses from a frequency-doubled, mode-locked Nd:YAG laser at intensities below and above the threshold for optical breakdown. Two-photon absorption by DNA was detected in the absence of optical breakdown by measuring the yields of cyclobutylpyrimidine dimers formed in supercoiled pBR322 DNA. The yield of cyclobutylpyrimidine dimers per laser pulse was measured at seven peak intensities between 1.03 and 8.04 GW/cm^2. A plot of the ln (dimer yield/pulse) versus ln (photon flux) was linear with a slope of 1.88 ± 0.26. The two-photon cross section for absorption at 532 nm was calculated to be 0.5 (±0.2) × 10^{-52} cm^4 s photon^{-1} per nucleotide. Experiments performed by using intensities above the threshold for optical breakdown caused breaks in the DNA strands but no cyclobutylpyrimidine dimers. The free-radical quencher, mannitol, partially inhibited formation of the strand breaks, indicating that mechanical processes initiated by the plasma also contribute to the creation of DNA strand breaks.

Intensity-dependent laser photochemistry of organic molecules has received increasing attention recently.[1-3] Most of these studies have involved sequential two-photon absorption; the first photon is absorbed by the ground state and the second by an electronically excited state, usually the triplet, or a transient intermediate. In some cases, a molecule in an upper excited state reacts prior to relaxation to the lowest excited state of that manifold.[2] Absorption

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