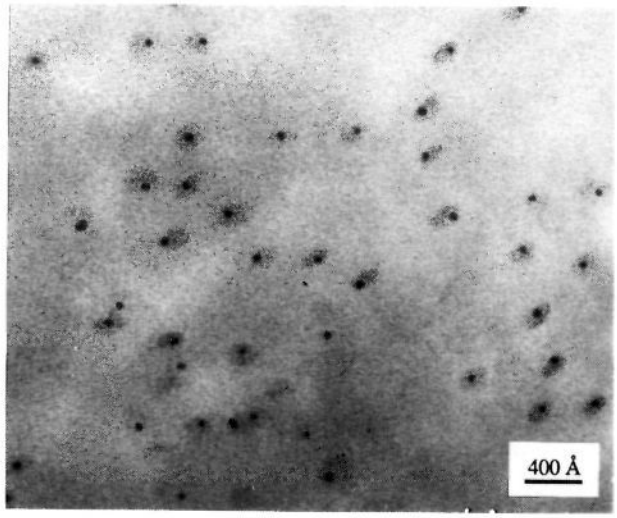

Figure 2. Electron micrograph of Ag-1 after thermal treatment. The gray areas surrounding each particle are the phosphorus-rich original microdomains.

containing microdomains ca. 180 Å in diameter. Analysis by X-ray fluorescence on a scanning transmission electron microscope (STEM) confirmed the presence of silver and phosphine within the spherical microdomains only.

The color of the Ag-1 film changed to yellow and then to red-orange upon heating to 90 °C in water for 5 days. (The relationship of the color to the plasmon frequency of the silver particles is uncertain at this point because a sample of poly(MTD) also changes slowly to yellow-orange when heated under similar conditions.) A TEM micrograph of a ca. 200 Å thick (microtomed) section (Figure 2) revealed that approximately spherical silver clusters formed within the original microdomains and that usually only one cluster is found in each microdomain. The residual phosphorus atoms provide enough contrast to image the dimensions of the original microdomain. The clusters have mean diameters of 55 Å with a standard deviation of about 20% and did not grow larger upon further heating of the sample. X-ray fluorescence microprobe analysis performed on the STEM confirmed that the clusters were located within the phosphine-containing microdomains and that decomposition of the silver complexes was complete. Although the narrow size distribution of the clusters is consistent with one cluster growing within each microdomain, a few clusters (ca. 1% or less) were found to be >100 Å in diameter, a result that would require either that the microdomain in which they originate be >310 Å in diameter[13] or that silver atoms or small clusters migrate through the MTD from one microdomain to the other. When the sample was heated at 120 °C, a much broader size distribution of clusters was observed. The phosphine centers within the microdomains may assist cluster growth, but cluster mobility through the poly(MTD) matrix between microdomains is likely to be severely restricted once the cluster reaches a certain size.

Observation of lattice fringes [(111) planes] by high-resolution TEM suggests that the particles have the same structure as the bulk metal and many of them actually are single crystals. The crystalline nature of the clusters was also confirmed by the observation of weak (111) and (200) peaks by X-ray powder diffraction of the bulk sample.

A film of $[Ag_2(Hfacac)_2(NORPHOS)]_{150}[MTD]_{300}/[MTD]_{200}$ (Ag-2), prepared in a manner analogous to Ag-1 with the same metal content, was found to contain spherical microdomains 280 Å in diameter. After decomposition of the silver complexes in the film under similar conditions, ca. 85 Å clusters were formed within the microdomains, but two clusters were observed within some microdomains.

We conclude that the method described here is useful for synthesizing clusters <100 Å in diameter and are continuing to investigate the scope and limitations of this polymer-based approach to the synthesis of size-selected clusters.

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(13) A calculation based on the density of the $[Ag_2(Hfacac)_2(NORPHOS)]_{150}$ homopolymer (1.56 g/cm^3) gave the following relation: cluster size = 0.31 microdomain size, assuming that the microdomain comprises silver-containing segments only, with very little or no poly(MTD) being present. The density was measured by the flotation method in H_2O/KI solution to which a small amount of soap had been added.

Isolation and Structural Elucidation of a Novel Phosphocysteine Intermediate in the LAR Protein Tyrosine Phosphatase Enzymatic Pathway

Hyeongjin Cho,† Ravichandran Krishnaraj,† Eric Kitas,‡ Willi Bannwarth,‡ Christopher T. Walsh,† and Karen S. Anderson*,§

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School 240 Longwood Avenue, Boston, Massachusetts 02115 Pharma Research New Technologies Hoffmann-La Roche Ltd. Grenzacherstrasse 124 Basel, Switzerland Department of Pharmacology Yale University Medical School 333 Cedar Street New Haven, Connecticut 06510

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Protein tyrosine phosphatases (PTPases) are of growing importance in understanding the regulation of signal transduction pathways which control hormones and growth factors. Phosphorylation of tyrosyl protein residues is one of the key modes involved in intracellular signaling for selective gene activation. The PTPases are thought to be the counterparts to the protein tyrosine kinases in switching signaling pathways off[1] and on[2-5] by controlling the lifetime of phosphorylated tyrosyl groups. The PTPases are found both in cytoplasmic locations and as a family of transmembrane proteins with intracellular phosphatase domains and extracellular domains presumed to be involved in specific ligand and/or cell–cell interactions. PTPases contain signature motifs including an absolutely conserved and required cysteine residue.

We have overexpressed and purified the catalytic domains of the 200-kDa transmembrane LAR (leukocyte antigen related) PTPase.[6] In addition, the specificity toward several synthetic phosphotyrosyl peptides corresponding to known phosphorylation sites in protein components of cellular signal transduction pathways was determined, and a 40-kDa single-domain fragment, LAR-D1, was shown to retain high catalytic efficiency. We have therefore utilized this representative membrane PTPase fragment for mechanistic studies.[6,7]

* Author to whom correspondence should be addressed.
† Harvard Medical School.
‡ Hoffmann-La Roche Ltd.
§ Yale University Medical School.

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A covalent phosphoryl enzyme intermediate has been implicated in several PTPase reaction pathways.[6,8,9] Most recently a low molecular weight 18-kDa bovine heart PTPase has been studied with the alternate substrate *p*-nitrophenyl phosphate, and accumulation of a covalent phosphoryl enzyme was detected and described as an unusual *S*-phosphocysteinyl enzyme by comparison with phosphocystamine.[10] On the basis of a series of mutagenesis studies on the conserved cysteine in other PTPases, it has been suggested that the conserved active site cysteine acts as the catalytic nucleophile.[10-15] In this report, we describe the isolation and kinetic and structural characterization of a phosphorylcysteine intermediate for LAR-D1 PTPase which forms *transiently* at the enzyme active site using a peptide substrate analog of the insulin receptor.

Rapid chemical quench flow experiments provided the first direct evidence of a covalent phosphoryl intermediate, Enz-X-PO_3^{2-}. In these experiments a ^{32}P-labeled monophosphotyrosyl dodecapeptide, TRDIpYETDFFRK, was used as a substrate. This pY peptide, synthesized as previously described,[16] is an analog of residues 1142–1153 of the autophosphorylation site of the insulin receptor in which the two phenylalanines are replaced by tyrosyl groups to reduce regiospecificity issues in pY peptide synthesis and enzymatic dephosphorylation. A single turnover time course (enzyme in excess over substrate) demonstrated the transient formation and decay of a covalent phosphoryl intermediate as shown in Figure 1A. The intermediate was formed on the enzyme within 3 ms and decayed to form products (dephosphorylated tyrosyl substrate and inorganic phosphate) over the next 50 ms as determined by SDS–PAGE analysis. This analysis allows the phosphoryl intermediate (MW 40000) to be easily distinguished from phosphorylated tyrosyl substrate (MW 1400) and inorganic phosphate (MW 164). The rates of formation (1200 s^{-1}) and decay (80 s^{-1}) of the phosphoryl enzyme intermediate paralleled the disappearance of substrate and the formation of product and are substantially faster than the steady-state turnover rate (24 s^{-1}), indicating that product release is most likely rate limiting. This experiment establishes that the intermediate is kinetically competent and a *true* intermediate along the catalytic reaction pathway.

The next step was to identify the active site residue, Enz-X-PO_3^{2-}, which becomes phosphorylated during catalysis. Quantities of LAR-D1 phosphoryl intermediate were prepared for ^{31}P NMR analysis to establish the identity of the active site residue. This was accomplished by reacting the TRDIpYETDFFRK peptide substrate (0.6 mM) with the LAR catalytic fragment D1 (0.66 mM) for 10 ms followed by quenching with 0.2 N NaOH in D_2O (final concentrations). A small amount of radiolabeled peptide substrate was included to verify the reaction by SDS–PAGE analysis.

The ^{31}P NMR spectrum obtained after quenching is shown in Figure 1B. Three major phosphate signals were observed. The two upfield signals correspond to substrate (0.6 ppm) and the product, inorganic phosphate (5.5 ppm). A third signal was observed downfield at 16.1 ppm, suggesting a phosphorylcysteine intermediate. This chemical shift is consistent with that previously reported for the phosphocysteine intermediate in the phosphotransferase system and bovine heart PTPase.[10,15] An authentic phosphocysteine peptide, HpCSAGVGRTG, corresponding to residues 1521–1530 of LAR and containing the active site Cys 1522, was prepared by phosphorylation of a precursor blocked on Ser and Thr[17] and served as a ^{31}P NMR standard, yielding a ^{31}P resonance at 16.1 ppm, validating the Enz-X-PO_3^{2-} as an Enz-CysSPO_3^{2-}. The relative distribution of species at the active site as determined by radioactivity/SDS–PAGE analysis was

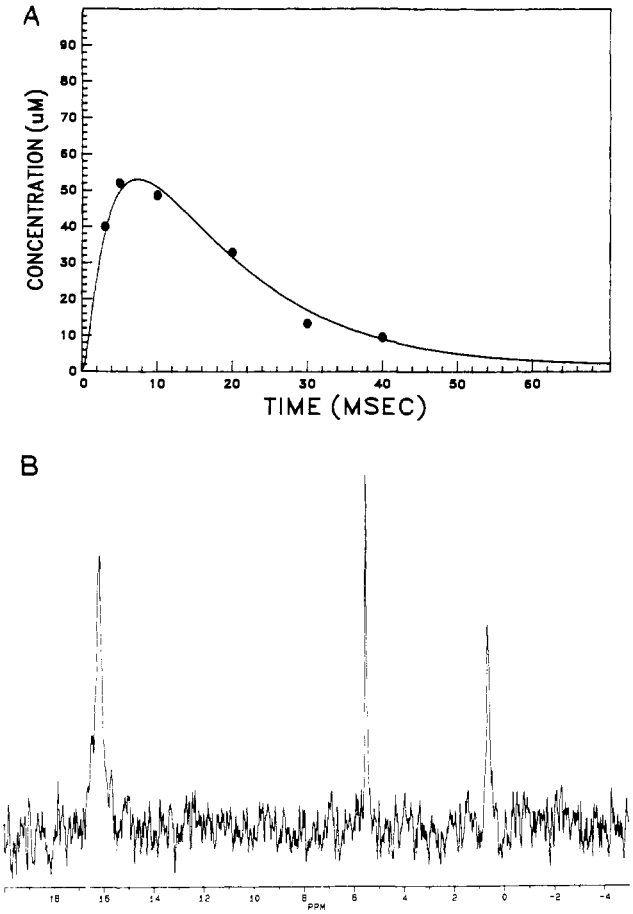

Figure 1. (A). Single turnover time course for the PTPase reaction. A solution of ^{32}P-labeled monophosphorylated dodecapeptide substrate, TRDIpYETDFFRK, was mixed with enzyme. The final concentrations were 100 μM substrate and 160 μM enzyme. The formation and disappearance of phosphoryl intermediate (●) was monitored by radioactivity/SDS–PAGE analysis. The curve was simulated by numerical integration with Kinsim using the mechanism depicted in eq 1.[20] The values for the rate constants are as follows: $k_1 = 2\ \mu m^{-1}\ s^{-1}$, $k_{-1} = 50\ s^{-1}$, $k_2 = 1200\ s^{-1}$, $k_{-2} = 3\ s^{-1}$, $k_3 = 80\ s^{-1}$, $k_{-3} = 2\ s^{-1}$, $k_4 = 24\ s^{-1}$, and $k_{-4} = 0.01\ \mu M^{-1}\ s^{-1}$. (B) ^{31}P NMR spectra of the phosphoryl intermediate. The phosphorus-31 spectrum of a mixture of substrate (20%), intermediate (40%), and inorganic phosphate product (40%) was obtained at 4 °C on a Bruker 11.7 T at 202.49 MHz in D_2O. Chemical shifts are reported in ppm relative to 80% H_3PO_4. The spectra were obtained with a 0.4-s delay between pulses and represent 2000 scans. Three major resonances are observed. The resonance at 0.6 ppm corresponds to phosphorylated tyrosyl substrate and that at 5.5 ppm to the product, inorganic phosphate. The resonance at 16.1 ppm corresponds to the phosphocysteine intermediate. The chemical shift assignments for the substrate and product were verified by obtaining the spectra for each separately under identical conditions. Methods: To synthesize the phosphoryl intermediate, enzyme (1 mL at 2 mM) was mixed with an equal volume of substrate (1.8 mM) and allowed to react for 10 ms, and then the reaction was terminated by mixing with 0.6 N NaOH in a rapid quench apparatus.[21] All enzymatic reactions were carried out at 20 °C in 100 mM MES buffer, 10 mM EDTA at pH 6.0.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P \qquad (1)$$

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(17) The peptide HCSAGUGRTG blocked on His(Trt)Arg(Pmc)Ser(Bu^t) and Thr(Bu^t) was phosphorylated and deprotected as previously described,[16] purified on C_{18}RPHPLC, and yielded upon ion spray MS, MH^- 1022.4 (calcd MW = 1023.2).

substrate (40%), intermediate (40%), and product (20%). The phosphoryl intermediate appeared to be moderately base stable at pH 13, with no significant decomposition after 4 h at 4 °C. However, after overnight scanning there were detectable changes in the ^{31}P NMR spectrum. The integral area for the peak at 16.1 ppm (phosphocysteine intermediate) was reduced while the peak at 5.5 ppm (inorganic phosphate) was increased, indicating decomposition. In addition, a small resonance appeared at −1.0 ppm. This resonance could be an Enz-N-PO_3^{2-} resulting from intramolecular transfer of the phosphate from the active site cysteine to the adjacent histidine (transfer from Cys 1522 to His 1521). The spectral characterization coupled with our rapid quench kinetics along with previous data provides definitive identification of the covalent phosphorylcysteine intermediate in the LAR PTPase reaction pathway. We can now conclude with confidence that the reaction proceeds by forming a covalent phosphocysteine intermediate which is subsequently hydrolyzed to product.

Although there are numerous examples of covalent phosphoryl intermediates utilizing oxygen as the nucleophilic species,[18,19] there are relatively few examples which employ cysteine.[10,15] It is likely that the utilization of the cysteine active site nucleophile is a common mechanistic feature of both low molecular weight and high molecular weight PTPases. The implications of this reaction mechanism are not fully understood; however, they may have a major impact on designing inhibitors and modulating the activity of the PTPase enzymes.

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Regioselectivity in Intramolecular Cycloaddition of Double Bonds to Triplet Benzenes

Peter J. Wagner,* Masami Sakamoto, and Ahmad E. Madkour

Chemistry Department, Michigan State University East Lansing, Michigan 48824

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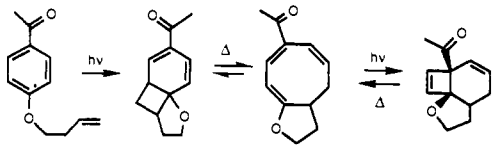
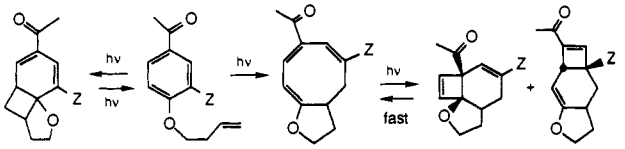
A few years ago we reported that double bonds undergo intramolecular 2 + 2 ortho cycloaddition to the π,π^* triplet states of acylbenzenes, when tethered ortho or para to the acyl group.[1-3] In the cases first studied, the initial bicyclo[4.2.0]octa-2,4-dienes undergo rapid thermal opening to cyclooctatrienes, which undergo further photochemistry as shown in Scheme I.[2] We now report that ring substituents promote high regioselectivity in the formation of stable cycloadducts. The selectivity appears to reflect inductive effects both on the initial triplet-state cycloaddition and on the competing thermal and photochemical electrocyclization reactions of the photoproducts.

We have prepared[4] and studied several meta-substituted *p*-butenoxyacetophenones **1a–e**. Scheme II summarizes the results in terms of stable, isolable products. Irradiation[5] of the amide

Scheme I



Scheme II



2a **1a-d** **3b,c,d** **4b,c,d** **5c**

a : Z = OMe **b** : Z = $CONH_2$ **c** : Z = CN **d** : Z = CH_3 **e** : SCH_3

1b produces a >90% yield of a single photoproduct, **4b**.[6] Whereas the cyclobutenes reported earlier take days to open to cyclooctatrienes,[7] **4b** opens to **3b**[8] in a few hours. Near-UV irradiation of isolated **3b** converts it quantitatively to **4b**, which begins to revert to **3b** as its NMR spectrum is being recorded.

Similar irradiation of the nitrile **1c** produces, in >90% total yield, the two isomeric cyclobutenes **4c** and **5c**[9] produced by disrotatory electrocyclization of each diene unit in cyclooctatriene **3c**. Cyclobutene **4c** is formed by closure of the same diene unit that closes in **3b**, while **5c** represents the first example that we have seen of the other diene unit closing so that the acetyl group ends up on the cyclobutene double bond. (Gilbert reported an analogous structure as the only product from *p*-butenoxybenzonitrile.[10]) Upon standing, the mixture of **4c** and **5c** converts to **3c** and **4c** within 1 day, while **5c** requires 2 weeks. The linear cyano group at the bridgehead position apparently produces less steric driving force for opening than do the acetyl and carboamido groups, although the photoinduced closure does not show the converse effect. Irradiation of isolated **3c**[11] again produces a mixture of **4c** and **5c**.

Irradiation of methyl-substituted **1d** at 313 nm produces mainly **4d** plus a minor amount of a di-π-methane rearrangement product

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(4) Fries rearrangement of the acetates of ortho-substituted phenols provide the phenol precursors to **1** in good yields. All materials were fully characterized as to structure and purity before use.

(5) Benzene solutions 0.02 M in ketone were prepared in argon-flushed, sealed NMR tubes that were attached to the outside of a quartz immersion well containing a medium-pressure mercury arc filtered only by Pyrex (λ > 290 nm). They were irradiated until no starting material remained (3 h for **1a,e**, 1 h for **1b–d**). (These times represent quantum yields in the 0.03–0.10 range.) Reaction progress was monitored by NMR spectroscopy. In preparative runs, 100 mg in 200 mL of argon-flushed solvent was irradiated in a Pyrex-filtered immersion well. After solvent was removed, the crude product was examined by NMR and then chromatographed on silica gel. Decoupling experiments on the products were consistent with the proposed structures.
(6) **4b**: ^1H NMR (CD_3OD) δ 1.77 (m, 1 H), 1.89 (m, 1 H), 2.21 (s, 3 H), 2.24 (ddd, J = 17.0, 5.7, 3.0 Hz, 1 H), 2.45 (m, 1 H), 2.71 (dd, J = 17.0, 3.0 Hz, 1 H), 3.71 (ddd, J = 8.6, 8.2, 3.7 Hz, 1 H), 3.78 (ddd, J = 9.9, 8.2, 6.9 Hz, 1 H), 6.31 (d, J = 2.8 Hz, 1 H), 6.48 (dd, J = 2.8, 0.6 Hz, 1 H), 6.58 (d, J = 3.0 Hz, 1 H); ^{13}C NMR δ 24.14, 28.02, 30.12, 31.40, 40.76, 68.03, 132.51, 132.89, 139.35, 140.99, 173.31, 212.84.
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(8) **3b**: ^1H NMR (CD_3OD) δ 1.89 (m, 1 H), 2.25 (m, 1 H), 2.37 (s, 3 H), 2.44 (dd, J = 13.8, 7.9 Hz, 1 H), 2.89 (dd, J = 13.8, 2.8 Hz, 1 H), 3.05 (m, 1 H), 4.16 (ddd, J = 10.1, 8.3, 5.7 Hz, 1 H), 4.25 (ddd, J = 8.3, 8.3, 2.5 Hz, 1 H), 5.49 (dd, J = 8.5, 2.0 Hz, 1 H), 7.16 (s, 1 H), 7.30 (d, J = 8.5 Hz, 1 H).
(9) **5c**: ^1H NMR (C_6D_6) δ 0.87 (tdd, J = 11.7, 11.1, 8.6 Hz, 1 H), 0.99 (dd, J = 13.0, 11.9 Hz, 1 H), 1.23 (m, 1 H), 1.55 (m, 1 H), 1.61 (s, 3 H), 1.7 (dd, J = 13.0, 5.2 Hz, 1 H), 3.29 (ddd, J = 11.7, 8.6, 5.7 Hz, 1 H), 3.59 (d, J = 6.5 Hz, 1 H), 3.62 (ddd, J = 8.6, 8.6, 0.8 Hz, 1 H), 4.99 (dd, J = 6.5, 2.5 Hz, 1 H), 5.54 (s, 1 H). **4c**: δ 6.34 (br d, J = 2.8 Hz, 1 H), 5.80 (dd, J = 2.8, 0.5 Hz, 1 H), 5.37 (d, J = 2.8 Hz, 1 H), 3.44 (m, 1 H), 1.85 (s, 3 H). **5c** was isolated; the partial spectrum of **4c** is from the initial ~3:1 mixture of the two formed by irradiation. The peaks attributed to **4c** disappear upon standing and are replaced by those for **3c**.
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(11) **3c**: ^1H NMR (C_6D_6) δ 1.00 (m, 1 H), 1.13 (m, 1 H), 1.82 (ddd, J = 15.0, 8.7, 1.1 Hz, 1 H), 1.84 (s, 3 H), 1.95 (dd, J = 15.0, 3.0 Hz, 1 H), 2.28 (m, 1 H), 3.39 (ddd, J = 8.7, 8.6, 6.5 Hz, 1 H), 3.46 (ddd, J = 8.7, 7.6, 4.3 Hz, 1 H), 5.28 (dd, J = 8.2, 1.75 Hz, 1 H), 6.69 (d, J = 8.3 Hz, 1 H), 7.20 (br s, 1 H).

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