Lanthanide-mediated phosphoester hydrolysis and phosphate elimination from phosphopeptides†

Nathan W. Luedtke and Alanna Schepartz*

Received (in Cambridge, MA, USA) 19th July 2005, Accepted 9th September 2005
First published as an Advance Article on the web 30th September 2005
DOI: 10.1039/b510123a

Lanthanide ions can mediate both phosphomonoester hydrolysis and β-elimination of inorganic phosphate from polypeptide substrates under near-physiological conditions of pH, temperature, and salt.

Protein phosphorylation is involved in regulating virtually every cellular process, including cellular metabolism, organization and differentiation, signal transduction, transcription, cytoskeletal rearrangement, and cell–cell communication.[1] Molecules that identify or modify sites of protein phosphorylation are therefore important tools in biochemical and proteomic research,[2] and may provide new strategies for the treatment of human disease.[3]

Hydrolytically active metal ions constitute the active core of numerous metalloenzymes,[4] and have been incorporated into unnatural constructs that cleave nucleic acids,[5,6] as well as various small-molecule substrates.[7] In water, lanthanide ions and bis-tris propane (BTP) form dinuclear hydroxo complexes of the type $M_2(BTP)_2(OH)_n^{6-n}$ (where n = 2, 4, 5, or 6) that mediate phosphoester hydrolysis of electronically activated nitrophenyl-containing substrates.[8] To evaluate the possibility that the unactivated phosphomonoester bonds of phosphopeptide and protein substrates might also be cleaved by lanthanide–BTP complexes, we developed a high throughput screen suitable for evaluating large numbers of different metal ions and chelating agents (Fig. 1). This assay measures the time-dependent release of radioactive ^{32}P from an immobilized phosphopeptide–streptavidin complex. Seven biotinylated peptides (**P1–P7**, Fig. 2) were phosphorylated with γ-^{32}P ATP using an appropriate kinase, normalized for ^{32}P incorporation, and immobilized onto streptavidin-conjugated agarose beads. After extensive washing to remove non-biotinylated components, the beads were incubated with 1.0 mM of each metal ion in a BTP-containing buffer, and the amount of ^{32}P released into solution was quantified by scintillation counting. Using this method, the relative activities of 21 lanthanide, transition metal, and main-group ions were compared (Table 1). All mixtures containing lanthanide ions liberated ^{32}P from the immobilized peptides **P1**, **P2**, and **P7** (Table 1). No ^{32}P release was detected in the presence of any non-lanthanide ion tested (including Mg(II), Ca(II), Sc(III), Cu(II), and Zn(II)). The activities among group IIIB ions increased with increasing Lewis acidity and atomic number (Sc(III) < Y(III) < La(III)). The relative activities within the trivalent lanthanide series, on the other hand, decreased with increasing Lewis acidity and atomic number (La(III), Ce(III) > Pr(III), Nd(III), Sm(III), Eu(III),

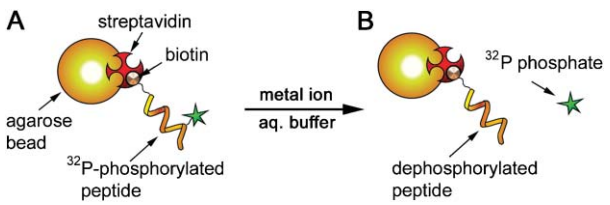

Fig. 1 (**A**) Schematic representation of a solid-phase immobilized ^{32}P-labeled phosphopeptide. (**B**) After incubating the assembly with a metal ion and chelating agent, the quantity of ^{32}P released into solution was determined by scintillation counting. Similar solid-phase-based approaches have proven useful for assessing both non-covalent binding and amide bond hydrolysis.[9,10]

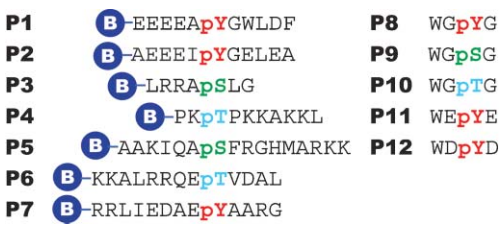

Fig. 2 Phosphopeptides used in this study. **P1–P7** contained an N-terminal biotin "B". **P8–P12** contained N-terminal free amines, and all peptides contained C-terminal amides.

Table 1 Moles of phosphate released (× 10^{12}) from immobilized peptides **P1–P7** after incubation for 18 h (25 °C) in 50 mM bis-tris propane (pH 8.0) containing 150 mM NaCl, 0.1% Tween-20 and 1 mM metal ion[a]

Ion	P1	P2	P3	P4	P5	P6	P7
None	0.00	0.00	0.00	0.01	0.02	0.02	0.00
Mg(II)	0.01[b]	0.01[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]
Sc(III)	0.00	0.01	0.02	0.00	0.02	0.00	0.00
Y(III)	0.11	0.07	0.09	0.02	0.06	0.00	0.12
La(III)	0.57	0.56	0.19	0.00	0.01	0.02	0.14
Ce(III)	0.76	0.91	0.29	0.00	0.12	0.12	1.18
Ce(IV)	2.78	1.99	0.06	0.02	0.10	0.18	3.06
Sm(III)	0.14[c]	0.12[c]	0.08[c]	0.00[c]	0.00[c]	0.02[c]	0.19[c]
Lu(III)	0.09[d]	0.06[d]	0.03[d]	0.02[d]	0.01[d]	0.00[d]	0.07[d]

[a] Estimated standard deviations were less than or equal to ± 30% of each value. [b] Similar values measured for Ca(II), Sc(III), Cu(II), and Zn(II). [c] Similar values measured for Pr(III), Nd(III), Eu(III), and Gd(III). [d] Similar values measured for Tb(III), Dy(III), Ho(III), Er(III), Tm(III), and Yb(III).

Departments of Chemistry and Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06520-8107, USA. E-mail: Alanna.Schepartz@yale.edu; Fax: (203) 432-3486

† Electronic supplementary information (ESI) available: Details describing the ^{32}P release assay, a complete set of values for Table 1, product characterization, kinetic analysis, and changes in Ce(IV) activity with time. See DOI: 10.1039/b510123a

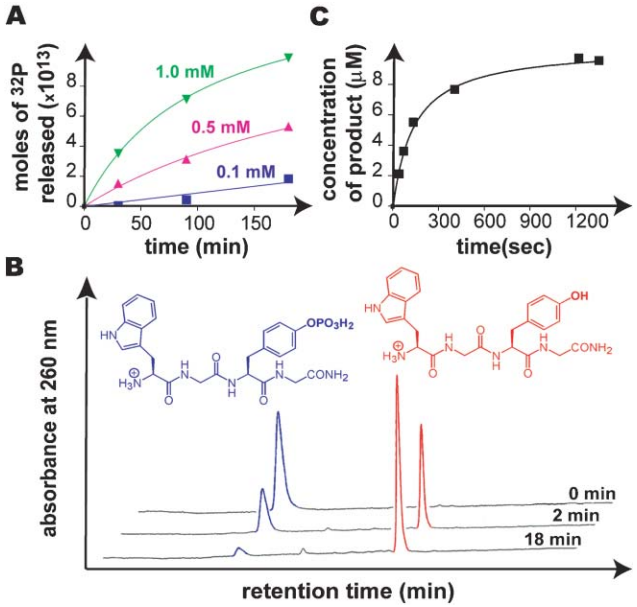

Fig. 3 (**A**) Time-dependent release of ^{32}P from the solid-phase immobilized phosphopeptide **P1** in the presence of 0.1, 0.5, and 1.0 mM Ce(IV). (**B**) HPLC analysis of a reaction containing **P8** (10 μM) and Ce(IV) (1 mM) after 0, 2, and 18 min in the presence of 50 mM bis-tris propane (pH 8.0), 150 mM NaCl, and 0.1% Tween-20. Similar results were obtained with **P11** and **P12**. For all reactions, product identities were confirmed by mass spectrometry and co-injection with an authentic sample. Mock reactions that lacked lanthanide ions showed no product formation after 2 weeks. (**C**) Concentration of product *vs.* time for a reaction containing 1 mM Ce(IV) and 10 μM of **P8**. Points were collected from three independent trials and fit to a hyperbolic curve to determine an initial reaction rate (8×10^{-8} M s^{-1}). For reactions conducted in parallel, error bars (standard deviations) were similar in size to the points shown on the graph.

Gd(III) > Tb(III), Dy(III), Ho(III), Er(III), Tm(III), Yb(III), Lu(III)). For nearly all peptides, Ce(IV) was the most active metal evaluated (Table 1). ^{32}P release from the immobilized peptide **P1** was time-dependent and exhibited first-order behavior in Ce(IV) concentration (Fig. 3A).

To characterize the products of lanthanide-mediated phosphopeptide dephosphorylation we treated **P3**, **P7**, and **P8**–**P12** with 1 mM La(III), Ce(III), Ce(IV), or Lu(III) in a BTP-containing buffer and monitored each reaction using HPLC and mass spectrometry. Ce(IV)-mediated dephosphorylation of **P8**, **P11**, and **P12** was nearly quantitative after only 18 min at RT (Fig. 3B).‡ For all substrates tested, Ce(IV)-treatment produced a single product consistent with phosphate hydrolysis (see ESI for details). Likewise, treatment of the phosphotyrosine-containing peptides **P7**, **P8**, **P11**, and **P12** with all lanthanide ions evaluated produced a single product with a retention time and molecular mass consistent with phosphate hydrolysis (see Fig. 3B for a representative reaction). Surprisingly, the addition of trivalent lanthanide ions (Ce(III), La(III), and Lu(III)) to **P3**, **P9**, and **P10** generated products consistent with β-elimination, furnishing dehydroalanine- and methyldehydroalanine-containing peptides (see ESI for details). For Ce(III), products consistent with a 3 : 1 ratio of phosphate elimination to hydrolysis were observed, while La(III) and Lu(III) provided, almost exclusively, the elimination products of **P9** and **P10** (Table 2). These reactions represent, to the best of our knowledge, the first examples of β-elimination of phosphate from phosphoserine- and phosphothreonine-containing substrates under near-physiological conditions. Since the resulting products are reactive Michael acceptors,[11] the site-specific covalent modification of phosphoproteins under native conditions might be accomplished by sequential addition of a trivalent lanthanide ion and thiol-containing nucleophile. Indeed, the addition of 2-aminoethanethiol to a crude mixture of La(III) and **P9** results in quantitative conversion of the dehydroalanine-containing product into a diastereoisomeric mixture of thioether-containing peptides. Similar methods are currently used to identify phosphorylated residues in proteins,[11] but employ harsh conditions for phosphate elimination (40 mM barium hydroxide, 50 °C, pH > 12).[12]

To compare the relative rates of lanthanide-mediated phosphopeptide dephosphorylation, apparent second-order rate constants were calculated from initial reaction rates (see Fig. 3C for representative kinetic data) by assuming each reaction is first-order in both metal ion (1 mM) and substrate (10 μM). Ce(IV)-mediated hydrolysis does, in fact, exhibit first-order behavior from 0.3 μM–2.0 μM of metal ion, and from 2.5 μM–10 μM of substrate **P8** (see ESI), but multiple species may contribute to the observed reaction rates,[7a,8] and product inhibition by inorganic phosphate is observed. The apparent second-order rate constants (summarized in Table 2) are therefore used for comparison purposes. Consistent with the ^{32}P-release data, the apparent second-order rate constants for dephosphorylation of **P8**–**P10** indicate the following trend in reactivities: Ce(IV) >> Ce(III) > La(III) > Lu(III) (Table 1 and Table 2). Also consistent with the ^{32}P-release data, the relative rates for **P8**–**P10** dephosphorylation depended on the identity of the phosphorylated residue, where pTyr >> pSer > pThr (Table 1 and Table 2). The Ce(IV)-mediated hydrolysis of **P8** was 200- and 8,000-times faster than **P9** and **P10**, respectively (Table 2). This

Table 2 Apparent second-order rate constants ($M^{-1}s^{-1}$) for phosphopeptide dephosphorylation and the cleavage of dinucleotides in 50 mM bis-tris propane (pH 8.0), 150 mM NaCl, and 0.1% Tween-20 at RT[a]

Substrate	Ce(IV)	Ce(III)	La(III)	Lu(III)
P8[b]	0.2–8[c,d]	7×10^{-3}[c]	2×10^{-3}[c]	5×10^{-4}[c]
P9	4×10^{-2}[c,e]	2×10^{-3}[f,g]	2×10^{-3}[f,h]	2×10^{-4}[f,h]
P10	1×10^{-3}[c,e]	6×10^{-4}[f,g]	8×10^{-4}[f,h]	5×10^{-5}[f,h]
CpG	1×10^{-2}–3×10^{-4}[d]	1×10^{-2}	2×10^{-3}	4×10^{-3}
dCpdG	$< 1 \times 10^{-6}$[i]	$< 1 \times 10^{-6}$[i]	$< 1 \times 10^{-6}$[i]	$< 1 \times 10^{-6}$[i]

[a] Estimated standard deviations for all rates were less than or equal to ± 30% of each reported value. [b] Similar rates observed for **P11** and **P12**. [c] Phosphate hydrolysis observed. [d] The cited range is for a fresh (10 min-old) or 100 day-old stock solution of Ce(IV). [e] Reaction rate is for a 100 day-old Ce(IV) stock solution. [f] Phosphate elimination observed. [g] A 3 : 1 ratio of phosphate elimination to phosphoester hydrolysis is observed. [h] A 10 : 1 ratio of phosphate elimination to phosphoester hydrolysis is observed. [i] No reaction observed after one week.

selectivity is consistent with the well-established relationship between reaction rate and leaving group pK_a.[13]

The rates of Ce(IV)-mediated dephosphorylation of **P8**–**P10** were up to 10,000-fold faster than those reported for metal-mediated RNA and DNA cleavage.[5] To provide a direct comparison of these differences under our conditions, the cleavage rates of an RNA (CpG) and DNA (dCpdG) dinucleotide were measured in the presence of 1 mM Ce(IV), Ce(III), La(III), or Lu(III) (Table 2). The rates for Ce(IV)-mediated phosphomonoester hydrolysis of **P9** and **P10** were more than 1,000-times faster than that of dCpdG phosphodiester hydrolysis (Table 2). These differences are consistent with a report that 5′-deoxyadenosine monophosphate (pdA) was hydrolyzed by Ce(IV) up to 500-times faster than the adenosine dinucleotide dApdA.[14] Interestingly, Ce(IV)-mediated dephosphorylation of **P8** is over one million times faster than dCdpG hydrolysis and up to 27,000 times faster than CpG hydrolysis (Table 2). Along with the noteable progress made towards the site-selective cleavage of nucleic acids using mixtures of hydrolytic metal ions and various chelating/targeting agents,[15] these results suggest it may be possible to effect site-specific dephosphorylation of phosphoproteins using lanthanide ions in conjunction with high affinity ligands for protein surfaces.

In summary, we have developed a solid-phase-based assay for phosphoester cleavage suitable for screening large numbers of different lanthanide ions, peptide sequences, and chelating agents. Using this assay, we found that certain lanthanide ion–BTP complexes dephosphorylate phosphopeptides under near-physiological conditions of pH, temperature, and salt. Control experiments conducted in solution confirmed the trends from the solid-phase assay and, unexpectedly, revealed that trivalent lanthanide ions can mediate β-elimination of phosphate from phosphomonoesters. In agreement with previous studies using other types of substrates,[5b,16] Ce(IV) was the most reactive ion tested, in all cases generating a single product consistent with phosphomonoester hydrolysis. For Ce(III), La(III), and Lu(III), β-elimination of the phosphate group from **P3**, **P9**, and **P10** was faster than hydrolysis, generating dehydroalanine- and methyldehydroalanine-containing peptides. Dephosphorylation of phosphotyrosine-containing peptides by Ce(IV) was particularly rapid with apparent second-order rate constants approaching 10 $M^{-1}s^{-1}$. This value is 10^6-fold lower than typical k_{cat}/K_m values for phosphotyrosine protein phosphatases, but is 10^{13}-fold higher than the estimated hydrolysis rate of phenylphosphate at high pH.[17] ‡

This work was supported by the NIH (GM65453 and GM59843), the National Foundation for Cancer Research, and in part by a grant to Yale University, in support of A. S., from the Howard Hughes Medical Institute. N. W. L. was supported by NIH-NRSA GM074401.

Notes and references

‡ A 40-fold increase in the apparent rate of **P8** dephosphorylation (see Table 2 and ESI) was observed for Ce(IV) stock solutions of increasing age (prepared at 100 mM in deionized water and stored in borosilicate glass at RT). This observation may indicate that in the absence of BTP, a slow-forming multimeric complex is the most reactive Ce(IV) species. Indeed, multinuclear lanthanide–hydroxide complexes form spontaneously in water,[7a] and dimeric lanthanide complexes cleave phosphodiester bonds up to 1,000-times faster than analogous monomers.[5a,18] In contrast to the trend observed for the hydrolysis of **P8**, aqueous solutions of Ce(IV) became less active for RNA hydrolysis over time (Table 2). In contrast to Ce(IV), the activities of Ce(III), La(III), and Lu(III) did not appreciably change over 100 days.

1 G. Manning, D. B. Whyte, R. Martinez, T. Hunter and S. Sudarsnam, *Science*, 2002, **298**, 1912.
2 M. Bollen, *Trends Biochem. Sci.*, 2001, **26**, 426.
3 P. Cohen, *Nat. Rev. Drug Discovery*, 2002, **1**, 309.
4 S. J. Lippard and J. M. Berg, *Principles of Bioinorganic Chemistry*, University Science Books, Mill Valley, CA, 1994.
5 For reviews see: (*a*) J. Chin, *Curr. Opin. Chem. Biol.*, 1997, **1**, 514; (*b*) M. Komiyama, *Chem. Commun.*, 1999, 1443; (*c*) J. A. Cowan, *Curr. Opin. Chem. Biol.*, 2001, **5**, 634; (*d*) S. J. Franklin, *Curr. Opin. Chem. Biol.*, 2001, **5**, 201.
6 For recent papers see: (*a*) M. E. Branum, A. K. Tipton, S. Zhu and L. Que, Jr., *J. Am. Chem. Soc.*, 2001, **23**, 1898; (*b*) C. Sissi, P. Rossi, F. Felluga, F. Formaggio, M. Palumbo, P. Tecilla, C. Toniolo and P. Scrimin, *J. Am. Chem. Soc.*, 2001, **123**, 3169; (*c*) C. Wang, S. Choudhary, C. B. Vink, E. A. Secord and J. R. Morrow, *Chem. Commun.*, 2000, 2509; (*d*) J. B. Delehanty, T. C. Stuart, D. A. Knight, E. R. Goldman, D. C. Thach, J. E. Bongard and E. L. Chang, *RNA*, 2005, **11**, 831.
7 (*a*) R. A. Moss, H. Morales-Rojas, S. Vijayaraghavan and J. Tian, *J. Am. Chem. Soc.*, 2004, **126**, 10923; (*b*) F. Medrano, A. Calderon and A. K. Yatsimirsky, *Chem. Commun.*, 2003, 1968; (*c*) B. Zhu, D. Xue and K. Wang, *BioMetals*, 2004, **17**, 423; (*d*) R. Kluger and L. L. Cameron, *J. Am. Chem. Soc.*, 2002, **124**, 3303; (*e*) J. Sumaoka, M. Yashiro and M. Komiyama, *Nucleic Acids Symp. Ser.*, 1992, **27**, 37; (*f*) P. Rossi, F. Felluga, P. Tecilla, F. Formaggio, M. Crisma, C. Toniolo and P. Scrimin, *Biopolymers*, 2000, **55**, 496; (*g*) E. Bamann, H. Trapmann and F. Fischler, *Biochem. Z.*, 1954, **326**, 89; (*h*) M. Shimomura and F. Egami, *Bull. Chem. Soc. Jpn.*, 1953, **26**, 263; (*i*) R. A. Moss and K. G. Ragunathan, *Chem. Commun.*, 1998, 1871; (*j*) J. Chin, M. Banaszczyk, V. Jubian and X. Zou, *J. Am. Chem. Soc.*, 1989, **111**, 186; (*k*) J. Rawlings, W. W. Cleland and A. C. Hengge, *J. Inorg. Biochem.*, 2003, **93**, 61.
8 P. Gomez-Tagle and A. K. Yatsimirsky, *Inorg. Chem.*, 2001, **40**, 3786.
9 N. W. Luedtke and Y. Tor, *Angew. Chem., Int. Ed.*, 2000, **39**, 1788.
10 D. Kahne and W. C. Still, *J. Am. Chem. Soc.*, 1988, **110**, 7529.
11 (*a*) H. Jaffe, Veeranna and H. C. Pant, *Biochemistry*, 1998, **37**, 16211; (*b*) F. Rusnak, J. Zhou and G. M. Hathaway, *J. Biomol. Tech.*, 2002, **13**, 228; (*c*) Z. A. Knight, B. Schilling, R. H. Row, D. M. Kenski, B. W. Gibson and K. M. Shokat, *Nat. Biotechnol.*, 2003, **21**, 1047.
12 (*a*) H. E. Meyer, B. Eisermann, A. Donella-Deanna, J. W. Perich, E. Hoffmann-Posorske and H. Korte, *Protein Sequences Data Anal.*, 1993, **5**, 197; (*b*) M. P. Molly and P. C. Andrews, *Anal. Chem.*, 2000, **73**, 5387; (*c*) M. F. Byford, *Biochem. J.*, 1991, **280 (Pt 1)**, 261.
13 A. J. Kirby and A. G. Varvoglis, *J. Am. Chem. Soc.*, 1977, **89**, 415.
14 S. Miyama, H. Asanuma and M. Komiyama, *J. Chem. Soc., Perkin Trans. 2*, 1997, 1685.
15 (*a*) K. D. Copeland, M. P. Fitzsimons, R. P. Houser and J. K. Barton, *Biochemistry*, 2002, **41**, 343; (*b*) W. Chen, Y. Kitamura, J. M. Zhou, J. Sumaoka and M. Komiyama, *J. Am. Chem. Soc.*, 2004, **126**, 10285; (*c*) R. T. Kovacic, J. T. Welch and S. J. Franklin, *J. Am. Chem. Soc.*, 2003, **125**, 6656; (*d*) A. Sreedhara and J. A. Cowan, *JBIC, J. Biol. Inorg. Chem.*, 2001, **6**, 166; (*e*) T. Nakatsukasa, Y. Shiraishi, S. Negi, M. Imanishi, S. Futaki and Y. Sugiura, *Biochem. Biophys. Res. Commun.*, 2005, **330**, 247; (*f*) L. Huang, L. L. Chappell, O. Iranzo, B. F. Baker and J. R. Morrow, *JBIC, J. Biol. Inorg. Chem.*, 2000, **5**, 85.
16 T. Takarada, M. Yashiro and M. Komiyama, *Chem.–Eur. J.*, 2000, **6**, 3906.
17 R. Kluger and L. L. Cameron, *J. Am. Chem. Soc.*, 2002, **124**, 3303.
18 (*a*) P. Hurst, B. K. Takasaki and J. Chin, *J. Am. Chem. Soc.*, 1996, **118**, 9982; (*b*) M. Yashiro, A. Ishikubo and M. Komiyama, *J. Biochem. (Tokyo)*, 1996, **120**, 1067.

