Optimizing thiophosphorylation by kinases in the presence of competing phosphorylation using MALDI-TOF-MS detection

Laurie L. Parker, Alexander B. Schilling, Stephen J. Kron, Stephen B. H. Kent

Supporting Information

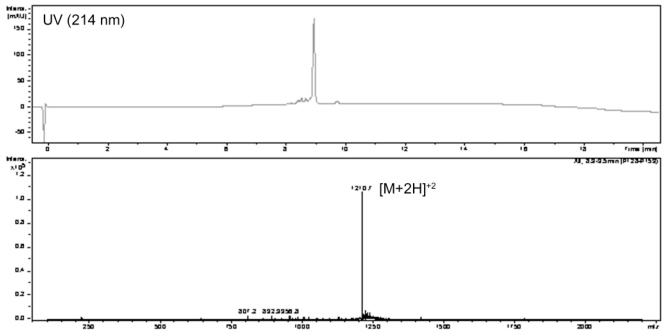
Contents

1. Peptide purity and identity data

Parent peptide 1

WSHPQFEKEAIYAAPFAKKK acetylated on the N-terminus (**1**) was synthesized on 0.1 mmol scale using manual Boc SPPS and cleaved from the resin with HF to give 273 mg crude peptide. The crude material was purified using preparative scale reverse phase HPLC (Waters Prep LC 4000 system): C18 column, gradient 5-35% B at 1%/min (A = 0.1% aqueous TFA; B = acetonitrile with 0.08% TFA). The gradient was held at 22% B until all **1** had eluted. Purification in eight lots gave 106 mg (44% yield) pure **1** (as shown by LC-MS, Fig. 1).

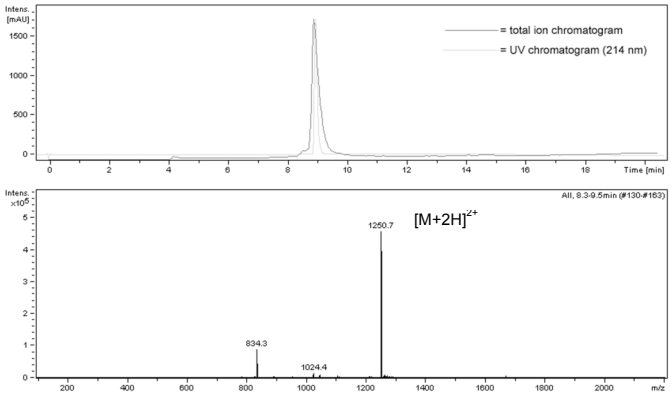
Fig. 1. LC-MS of parent peptide 1. C18 column, gradient 4% B/min.



Phosphopeptide 2

(N-acet)WSHPQFEKEAIYAAPFAKKK (1 μmol) was phosphorylated using Abl kinase domain (70 μg) in kinase buffer containing ATP (1 mM), 50 mM Tris-Cl pH 7.5, 30 μg/ul BSA, 1 mM DTT, 10 mM $MgCl_2$ and 1 mM $MnCl_2$ (reaction volume 2 ml). The reaction was carried out at 30 ºC for 3 h in order to ensure quantitative conversion of the substrate. A small amount of the resulting phosphopeptide was purified using preparative scale reverse phase HPLC (as before): C18 column, gradient 5-35% B at 1%/min (A = 0.1% aqueous TFA; B = acetonitrile with 0.08% TFA) to give (N-acet)WSHPQFEKEAIpYAAPFAKKK (**2**) (as shown by LC-MS, Fig. 2). Concentration of the resulting pure fraction was determined by UV (OD_{280} extinction coefficient [same as **1**] = 6970 $cm^{-1}M^{-1}$) and the sample was diluted in H_2O for preparing standard curve samples.

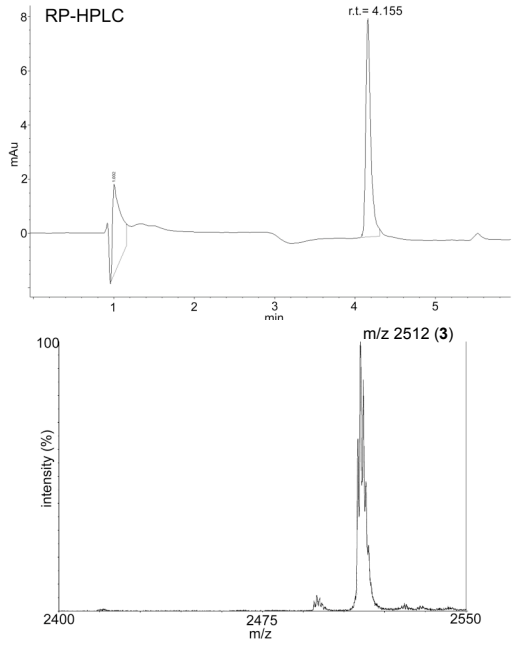
Fig. 2. LC-MS of phosphopeptide 2. C18 column, gradient 4% B/min.



Thiophosphopeptide 3

(N-acet)WSHPQFEKEAIYAAPFAKKK (1 μmol) was thiophosphorylated using Abl kinase domain (70 μg) in kinase buffer containing ATPγS (1 mM), 50 mM Tris-Cl pH 7.5, 30 ug/ul BSA, 1 mM DTT, 10 mM $MgCl_2$ and 1 mM $MnCl_2$ (reaction volume 2 ml). The reaction was carried out at 30 ºC for 3 h in order to ensure quantitative conversion of the substrate. Due to the sensitivity of thiophosphopeptides to hydrolysis at low pH, **3** was purified on an analytical scale using A = triethylammonium phosphate (TEAP) aqueous buffer pH 7, B = acetonitrile (C4 column, 10-20% B at 0.5 %/min). The purest fraction recovered still contained trace phosphopeptide (Fig. 3) but was acceptable purity for the sensitivity/signal-to-noise capabilities of the MALDI-MS detection (i.e. <95% pure). A representative linear negative spectrum is shown in Fig. 3. The concentration of thiophosphopeptide in the purest fraction was determined by HPLC from the absorbance at 280 nm (using an HPLC calibration curve prepared from unphosphorylated peptide **1**, which has the same extinction coefficient as phosphopeptide **2** and thiophosphopeptide **3**) to be 4 μM. This stock was used immediately for preparation of known thiophosphopeptide/phosphopeptide ratios for the MALDI-TOF MS calibration curve.

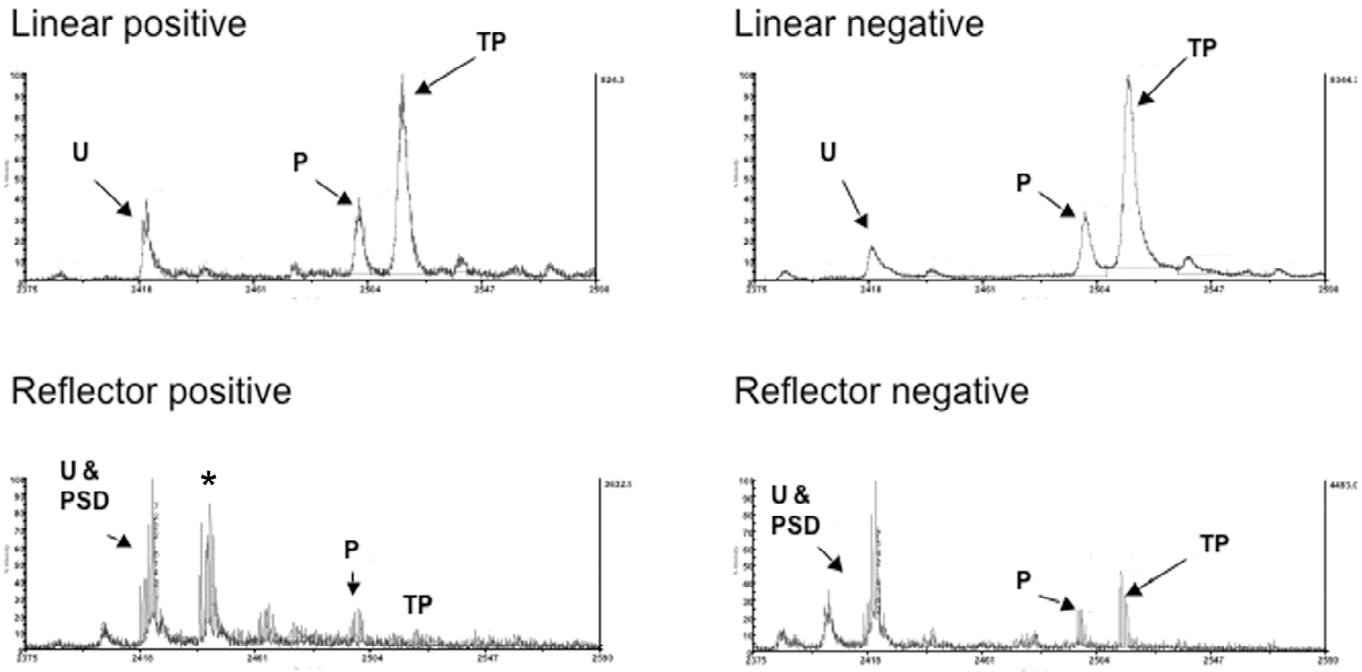
Fig. 3. Characterization of purified thiophosphopeptide by analytical RP-HPLC (214 nm) and MALDI-TOF-MS (linear negative mode)



2. Fragmentation of thiophosphopeptide

Post-source decay of a peptide is indicated by the presence of a signal in reflector mode (sometimes poorly resolved) that was not displayed in linear mode.[1] Phosphopeptides are known to exhibit neutral loss of phosphate due to post-source decay in MALDI-TOF-MS. With phosphoserine and phosphothreonine this loss is typically –98 Da, and is thought to represent elimination of the entire phosphate upon deprotonation at the α-carbon of the residue. Phosphotyrosine has been reported typically to undergo loss of 80 Da (fragmentation at the O-P bond between tyrosine and the phospho group). However, other data from peptides containing multiple Arg or Lys residues shows that phosphotyrosine can sometimes lose

Fig. 4. Fragmentation of thiophosphopeptides in reflectron mode. Spectra shown for the same sample spot in all four detection modes of the instrument (linear positive and negative, reflector positive and negative). Peptide **1** was thiophos- and phosphorylated with 40:1 ATPγS:ATP using Abl kinase domain. **U** = unphosphorylated; **PSD** = thiophosphopeptide post-source decay fragment signal; **P** = phosphopeptide; **TP** = thiophosphopeptide; * = Na^+ adduct of **U** and **PSD**. Linear mode is also shown to emphasize absence of **PSD** peak in linear, as well as to confirm presence of thiophosphopeptide in the sample.

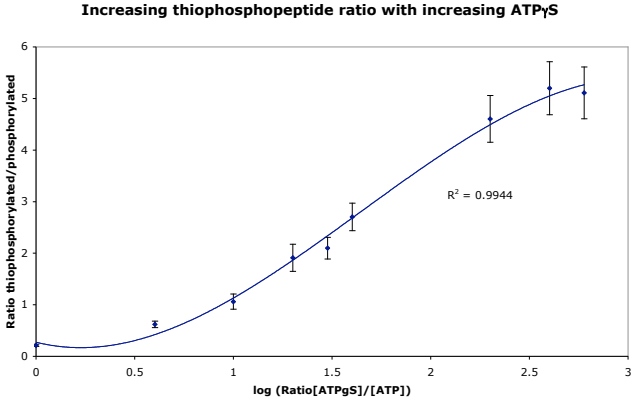


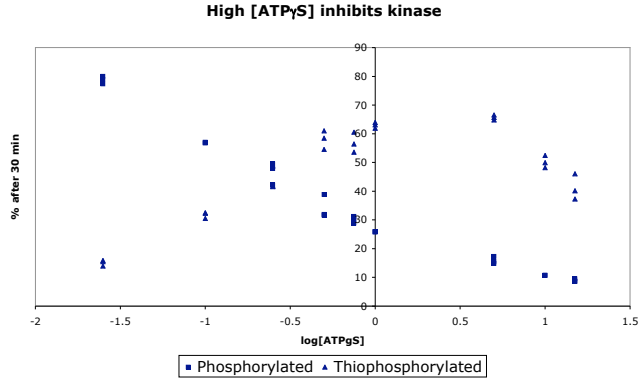
predominantly H_3PO_4 (-98 Da), which seems to be related to protonation of the phosphate by basic residues in the peptide.[2-4] The tyrosine peptide used in this study contained multiple Lys residues, and the phosphorylated peptide did display loss of 98 Da in both reflector mode and MS/MS analysis (data not shown). However, the thiophosphorylated analogue almost exclusively gave apparent loss of HPO_2S. The resulting signal at m/z 2421 (reflector positive mode) was relatively well resolved (Fig. 4), indicating possible ion extraction-related processes contributing to the fragmentation.[5] MALDI-TOF/TOF tandem MS analysis reveals the major fragment from thiophosphate loss at m/z 2418, confirming loss of HPO_2S. We are currently performing *ab initio* calculations on the differences in bond character between phospho- and thiophosphopeptides and further characterizing this 'signature' for thiophosphotyrosine in MALDI-TOF-MS.

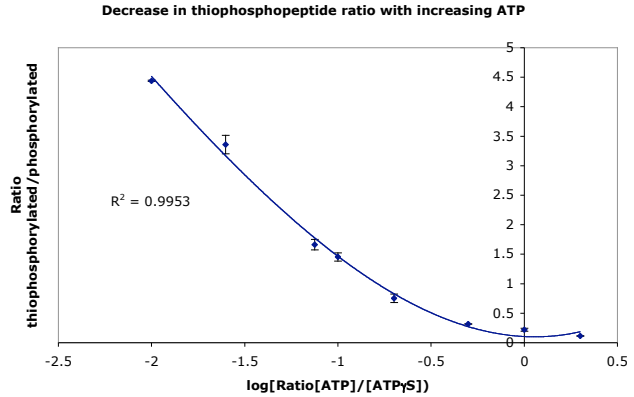
3. Additional data from optimization of Abl thiophosphorylation (Fig. 5)

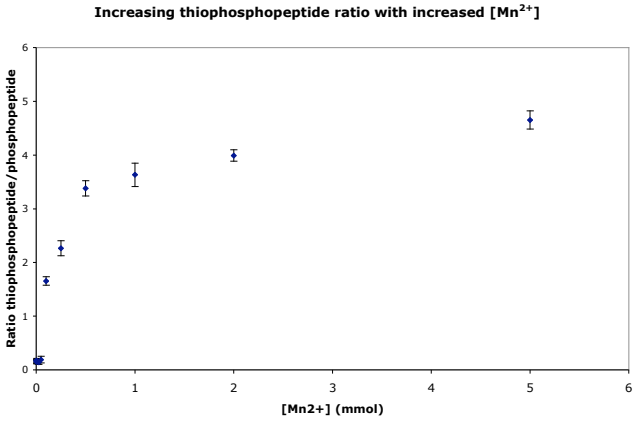
The solid-phase assay described in the Experimental section for this manuscript was used to characterize different reaction components for thiophosphorylation by Abl kinase. The optimal Mn^{2+} concentration was found to be 0.5-1 mM, as higher concentrations of Mn^{2+} caused inhibition of the enzyme. The optimal ATPγS concentration was found to be 1-2 mM, with higher concentrations inhibiting enzyme activity. Competition experiments with

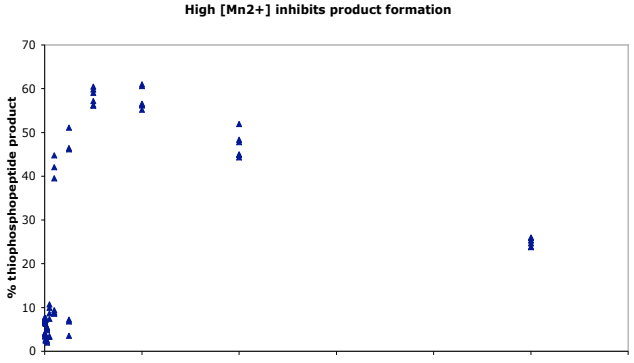
Fig. 5. Optimization of reaction components. "% after 30 min" refers to the percentage total peak intensity (sum of unphosphorylated, phosphorylated and thiophosphorylated) in linear negative mode. While these values are not calibrated to ionization efficiency and thus not 'absolute' determinations of reaction completion, they provide an estimation of the degree of conversion of the peptide to either phospho- or thiophospho-product under various conditions.











ATPγS and ATP revealed that at 10:1 ATPγS:ATP, thio- to phosphorylation efficiency was 1:1.

1. Spengler, B., Post-source decay analysis in matrix-assisted laser desorption/ionization mass spectrometry of biomolecules. *Journal of Mass Spectrometry* **1997**, *32*, (10), 1019-1036.
2. Moyer, S. C.; VonSeggern, C. E.; Cotter, R. J., Fragmentation of cationized phosphotyrosine containing peptides by atmospheric pressure MALDI/ion trap mass spectrometry. *Journal Of The American Society For Mass Spectrometry* **2003**, *14*, (6), 581-592.
3. Moyer, S. C.; Cotter, R. J.; Woods, A. S., Fragmentation of phosphopeptides by atmospheric pressure MALDI and ESI/ion trap mass spectrometry. *Journal Of The American Society For Mass Spectrometry* **2002**, *13*, (3), 274-283.
4. Metzger, S.; Hoffmann, R., Studies on the dephosphorylation of phosphotyrosine-containing peptides during post-source decay in matrix-assisted laser desorption/ionization. *Journal Of Mass Spectrometry* **2000**, *35*, (10), 1165-1177.
5. Gabelica, V.; Schulz, E.; Karas, M., Internal energy build-up in matrix-assisted laser desorption/ionization. *Journal Of Mass Spectrometry* **2004**, *39*, (6), 579-593.