Supplementary Information

Characterization and application studies of ProxyPhos, a chemosensor for the detection of proximally phosphorylated peptides and proteins in aqueous solutions.

Supplementary Information

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Table of contents

List of Figures

List of Tables

List of Supplementary Notes

Supplementary Figures

The following figures (S4-6, S8-13, S24, S26) are presented in a logarithmic scale (x-axis).

Figure S1 Distribution of pS-(X)x-pS motifs in the human phosphoproteome

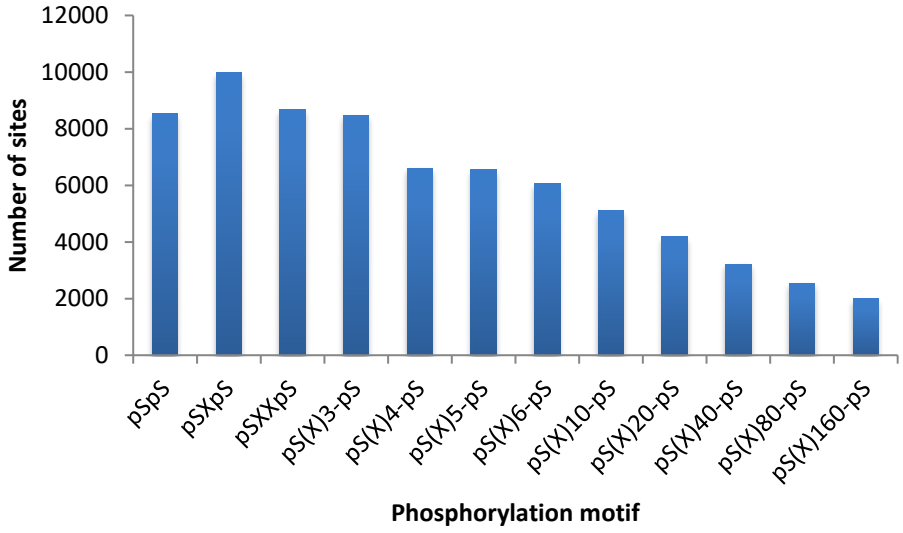


Figure S2 Distribution of pXpX motifs in the human phosphoproteome

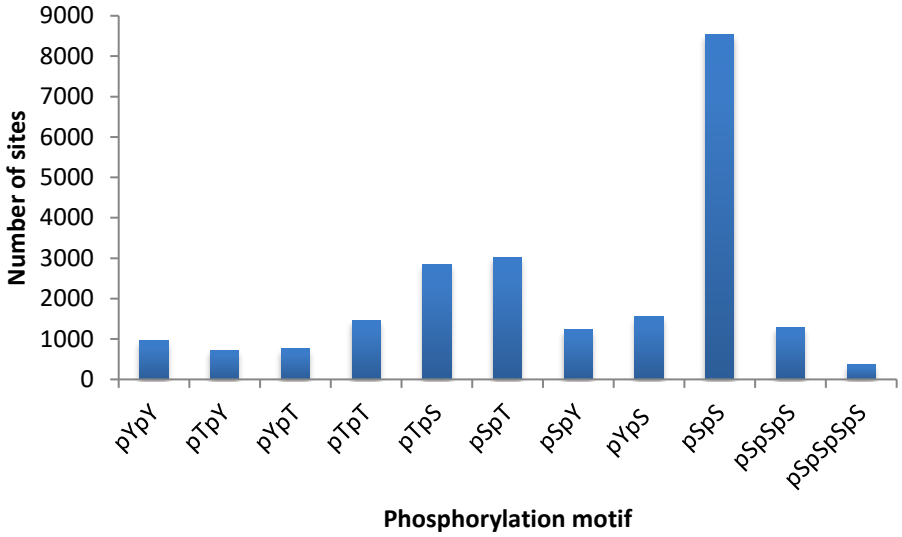
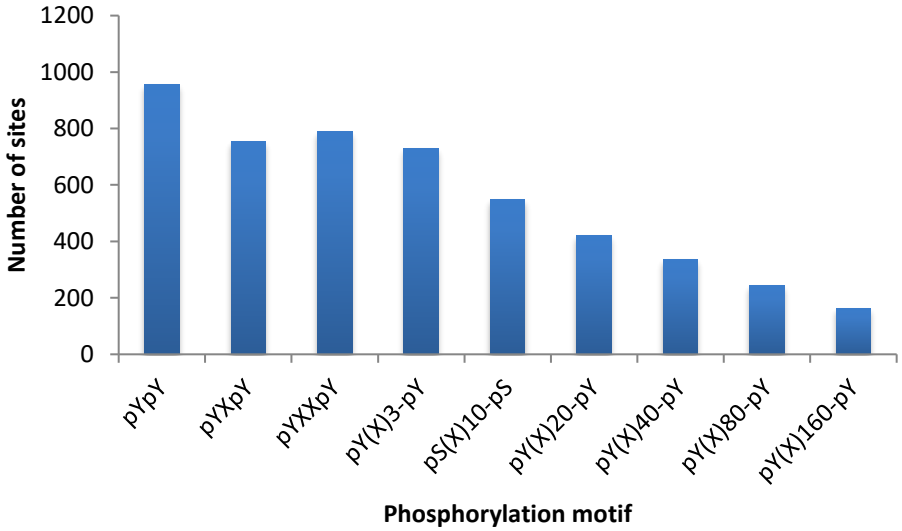


Figure S3 Distribution of pY-(X)x-pY motifs in the human phosphoproteome



Information for Figures S1-S3 was obtained from the PhosphoSite Plus® database.

Figure S4 Titration of 40 µM and 20 µM ProxyPhos with pY or pYpY peptides (top) and control proteins (bottom) in 50 mM HEPES, pH 7.5, 5% DMSO ($\lambda_{ex/em}$ = 350/476 nm)

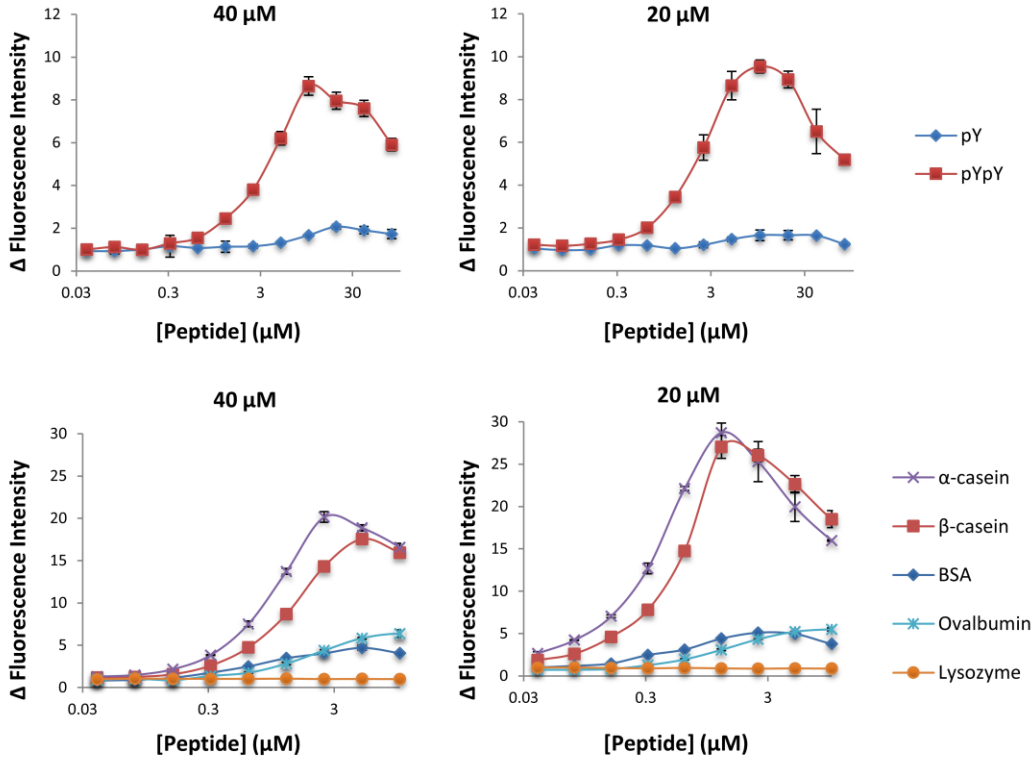


Figure S5 Titration of 40µM ProxyPhos with increasing concentrations of pY (A) or pYpY (B) in buffers of different pH. 50 mM HEPES, 5% DMSO was used for pH 6.5 and 7.5; 50 mM Acetate, 5% DMSO was used for pH 5.5, 4.5 and 3.5 ($\lambda_{ex/em}$ = 350/476 nm)

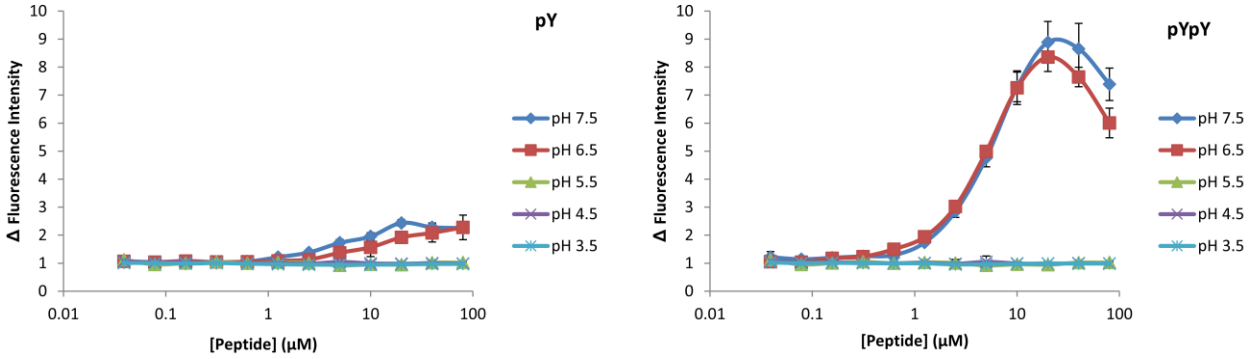


Figure S6 Titration of 40 µM ProxyPhos with pY or pYpY peptides with varied NaCl concentration and pH. 50 mM HEPES, 5% DMSO was used for pH 6.5 and 7.5; 50 mM Acetate, 5% DMSO was used for pH 5.5 ($\lambda_{ex/em}$ = 350/476 nm)

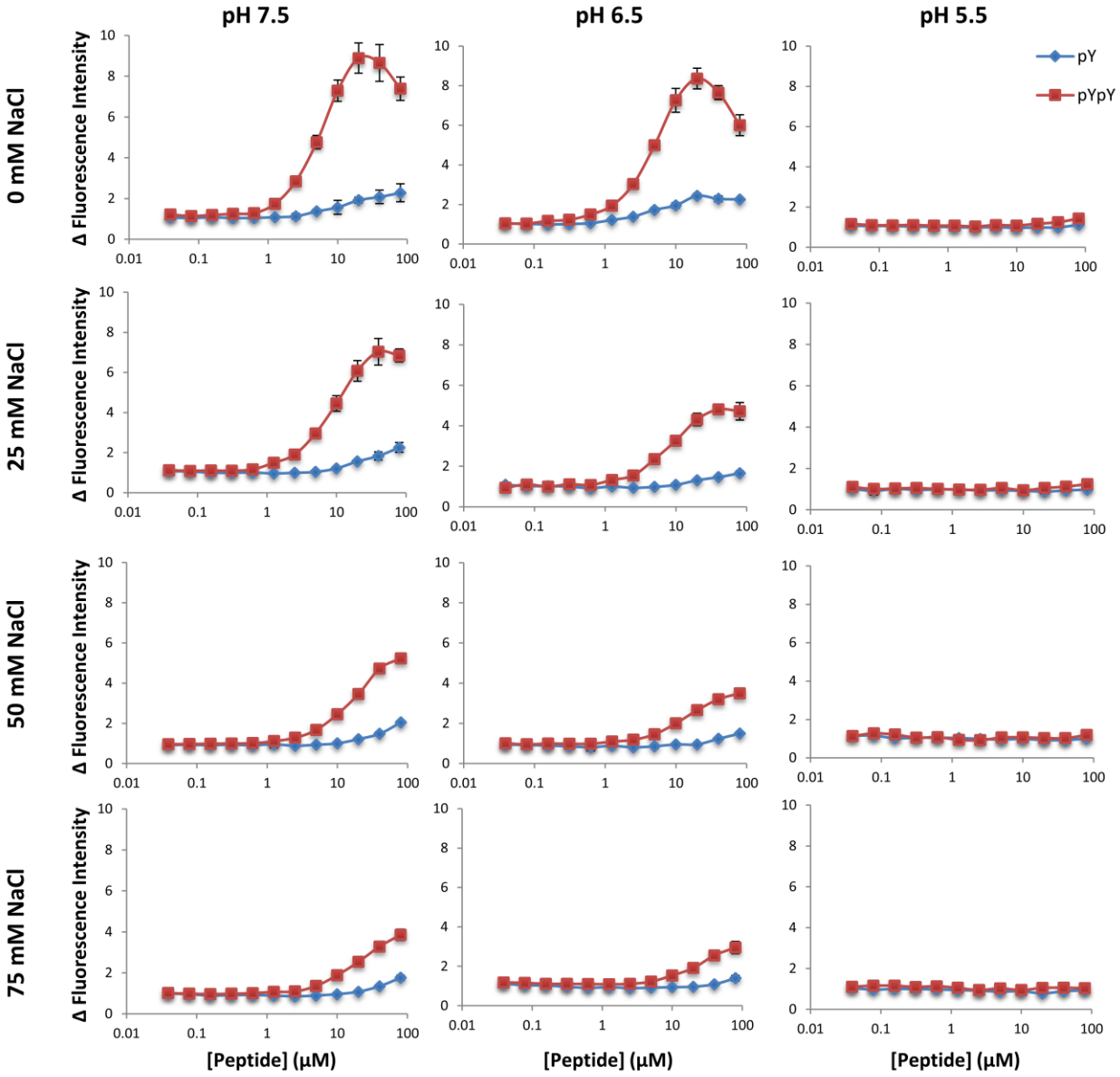


Figure S7 ProxyPhos (40 µM) monomer (376 nm) and excimer (476 nm) emission intensities in the presence of 0-100 % v/v (A) propylene glycol and (B) DMSO (pH 7.5, 50 mM HEPES, no NaCl; λ_{ex} = 350 nm)

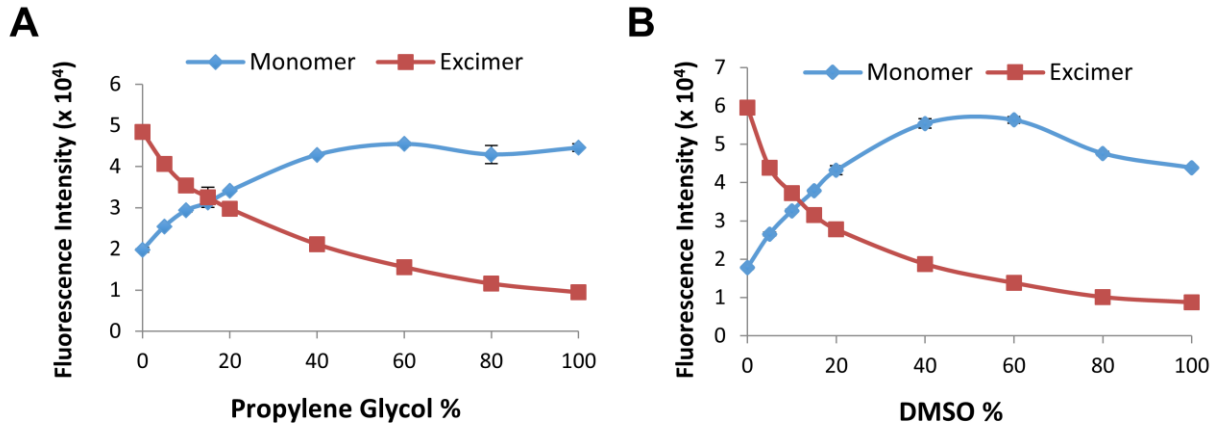


Figure S8 Titration of 40 µM ProxyPhos with variably phosphorylated proteins in 50 mM HEPES, pH 7.5, 10% DMSO buffer with increasing NaCl concentrations ($\lambda_{ex/em}$ = 350/476 nm)

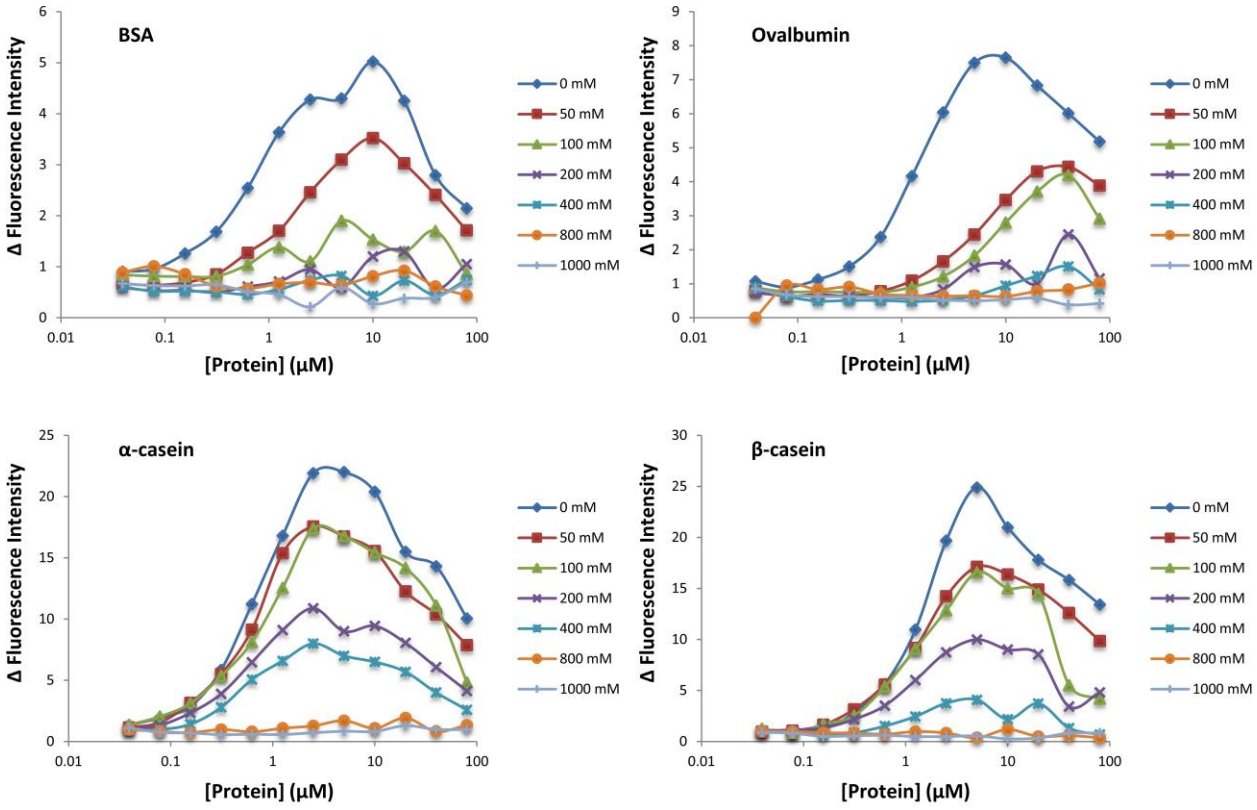


Figure S9 Titration of 40 µM ProxyPhos with variably phosphorylated proteins in 50 mM HEPES, pH 7.5, 10% DMSO buffer with increasing NaCl concentrations ($\lambda_{ex/em}$ = 350/476 nm); alternative representation of data presented in Figure S8

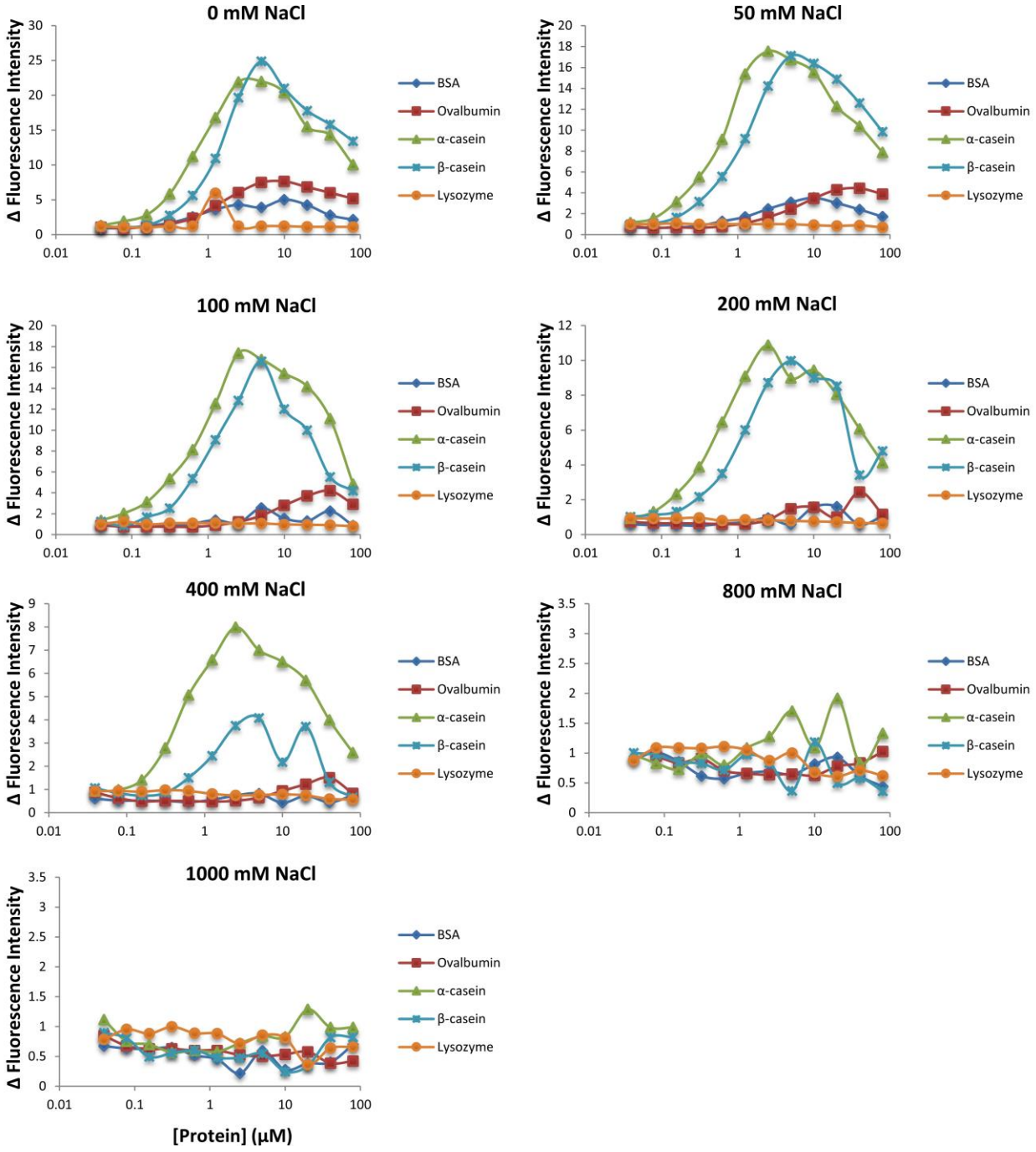


Figure S10 Titration of 40 µM ProxyPhos with control proteins in buffers (50 mM HEPES/Acetate, 10% DMSO) of different pH values ($\lambda_{ex/em}$ = 350/476 nm)

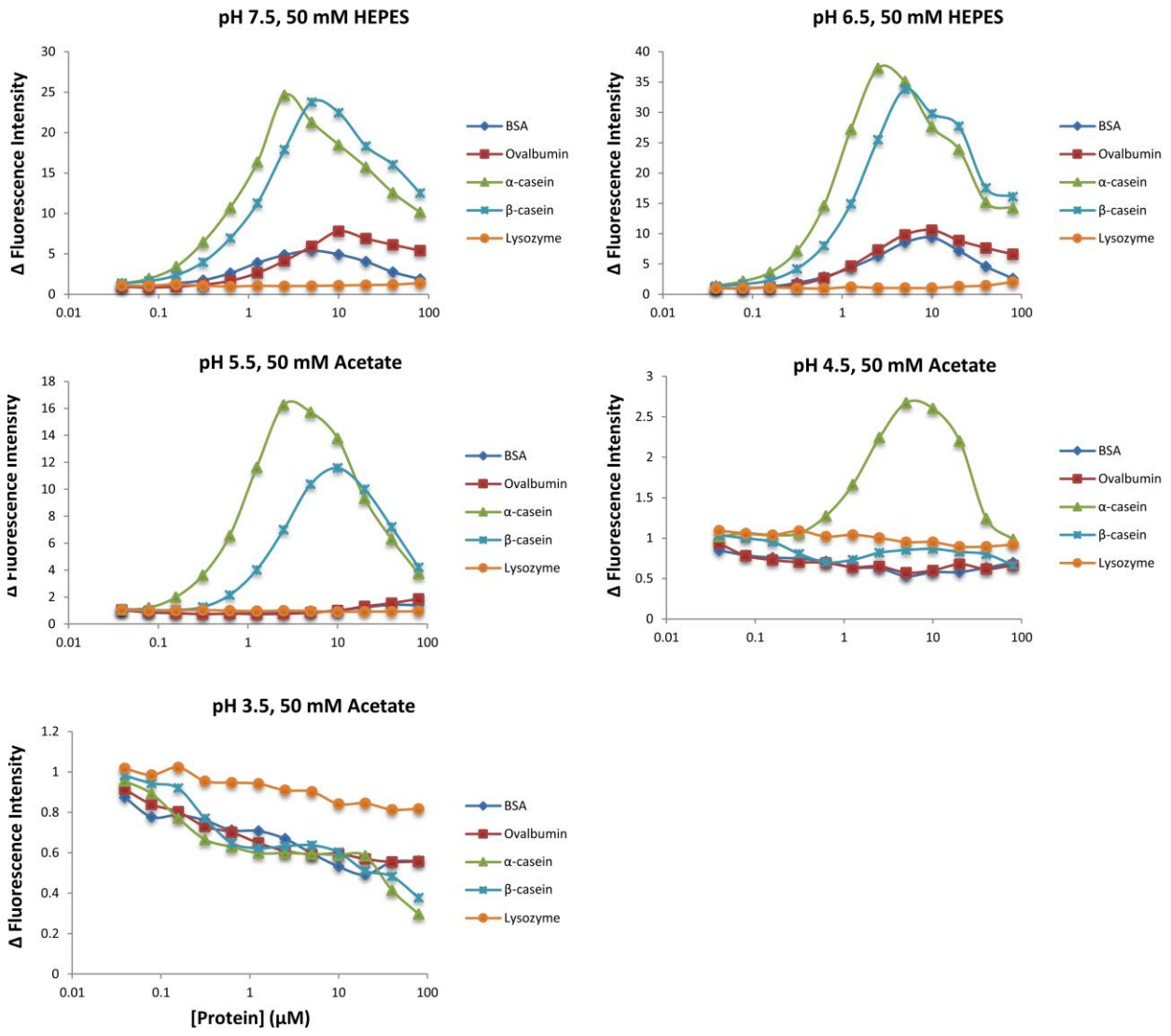


Figure S11 Signal selectivity of 40 μM ProxyPhos for α-casein over ovalbumin (ΔΔFI) in buffers of variable pH and salt concentrations ($\lambda_{ex/em}$ = 350/476 nm). 50 mM HEPES, 10% DMSO was used for pH 6.5 and 7.5; 50 mM Acetate, 10% DMSO was used for pH 5.5

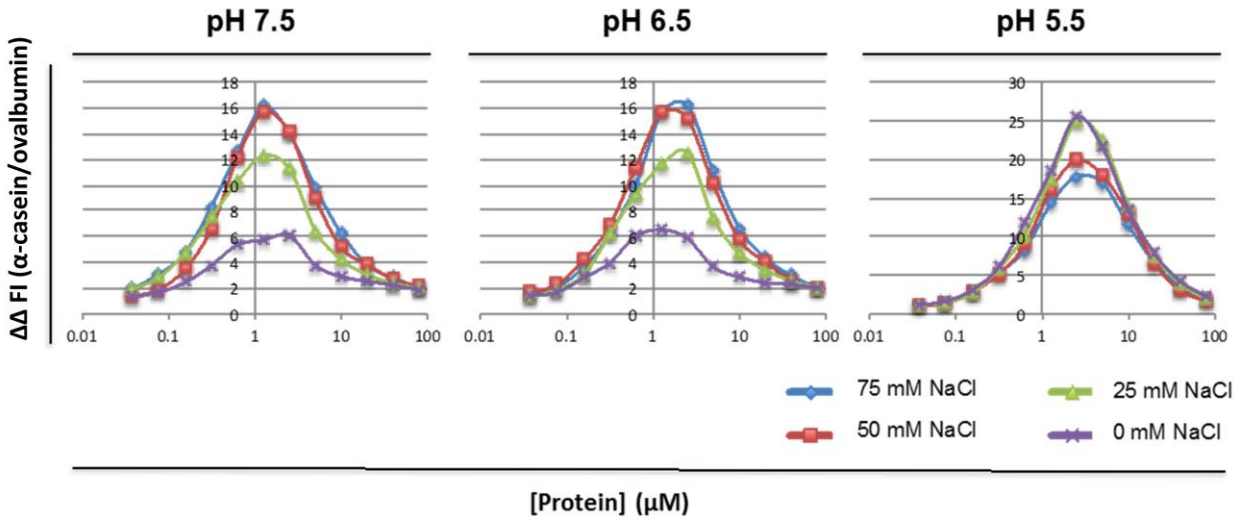


Figure S12 Signal selectivity of 40 μM ProxyPhos (ΔΔFI) for α-casein over ovalbumin, BSA or β-casein in buffers (50 mM HEPES, pH 7.5) of variable NaCl and DMSO/PG concentrations ($\lambda_{ex/em}$ = 350/476 nm)

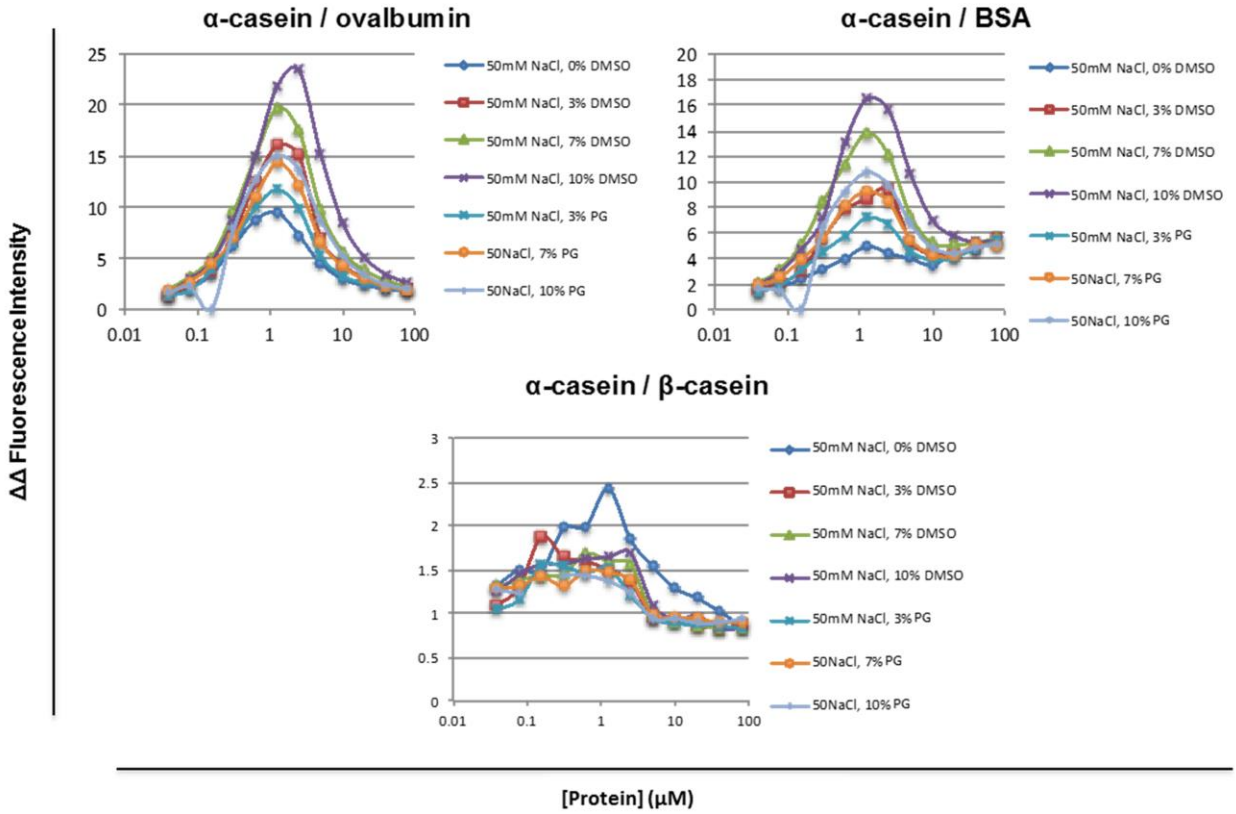
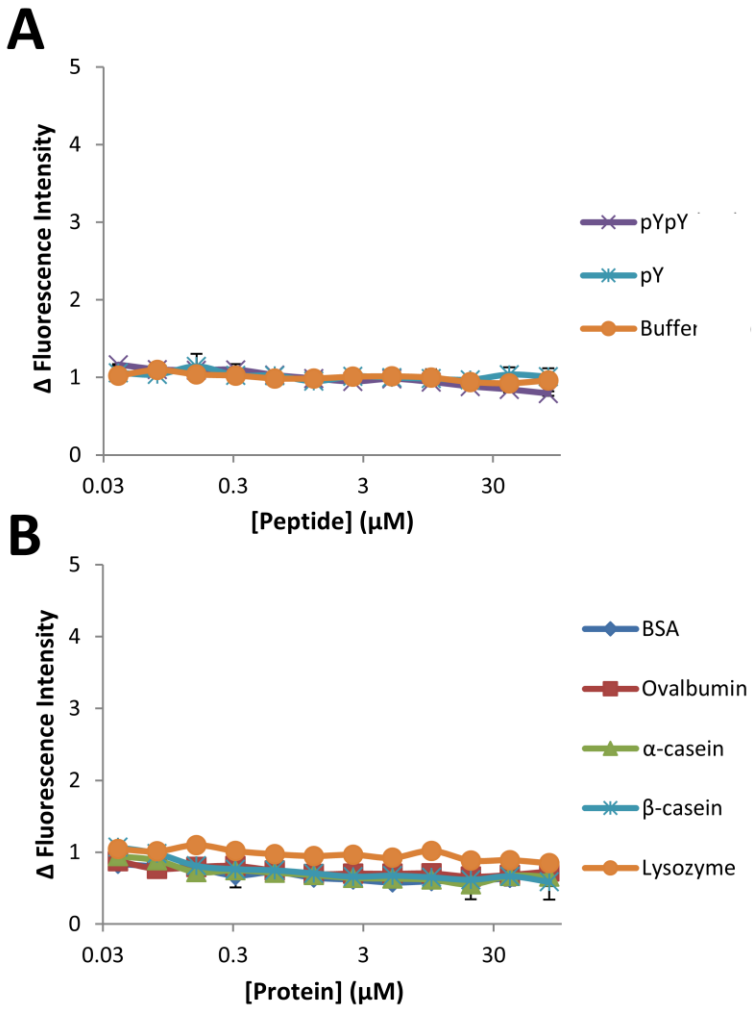


Figure S13 Titration of 40 μM unmetallated ProxyPhos with (A) increasing concentrations of pY and pYpY peptide (50 mM HEPES, pH 7.5, 10% DMSO) and (B) control proteins (50 mM HEPES, pH 7.5, 20% DMSO, 75 mM NaCl; $\lambda_{ex/em}$ = 350/476 nm)



The following computational images are gas phase structures, PM3 (MM corrected).

Figure S14 PM3 Optimized structure for ApSApSA.

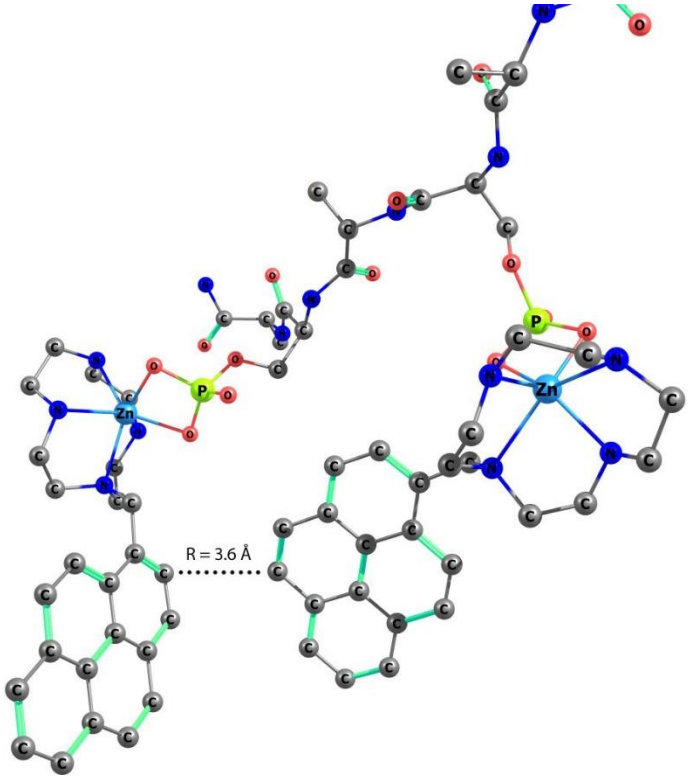


Figure S15 PM3 Optimized structure for ApSA4pSA.

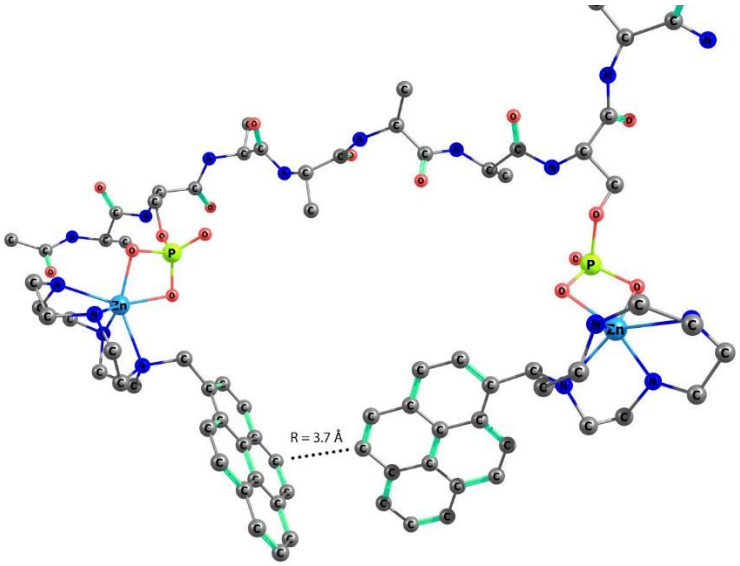


Figure S16 PM3 Optimized structure for ApSA5pSA.

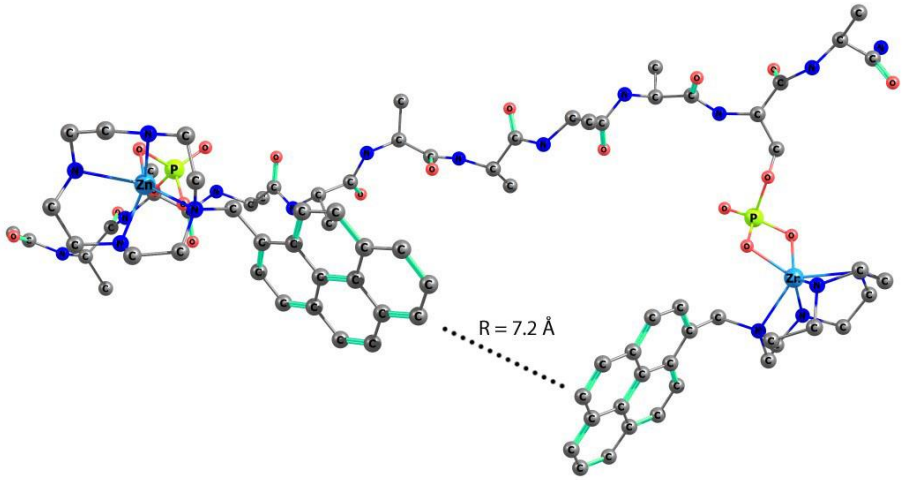


Figure S17 PM3 Optimized structure for ApSA11pSA (Helical).

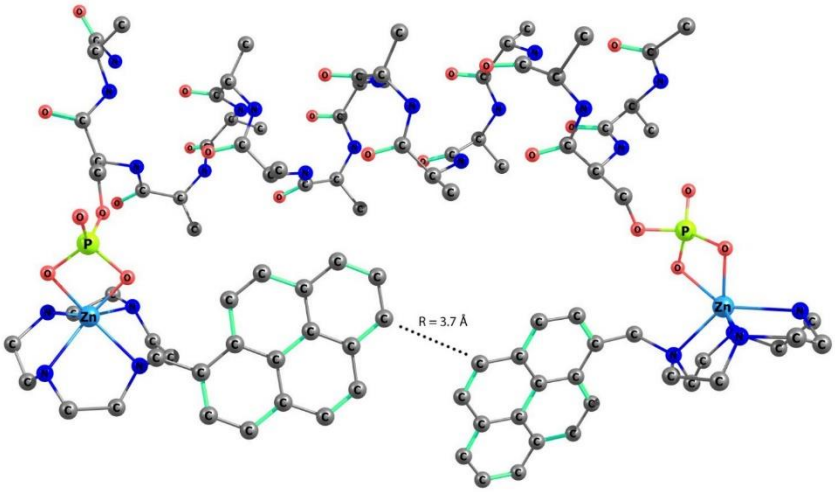


Figure S18 PM3 Optimized structure for ApSA12pSA (Helical).

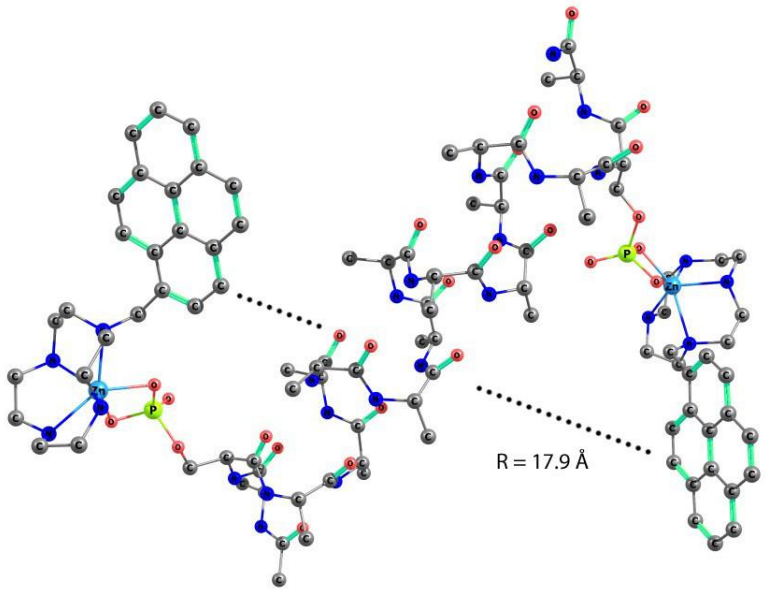


Figure S19 PM3 Optimized structure for ApYApYA.

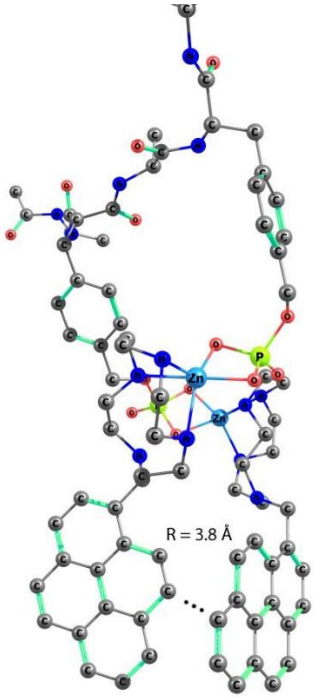


Figure S20 PM3 Optimized structure for ApYA10pYA.

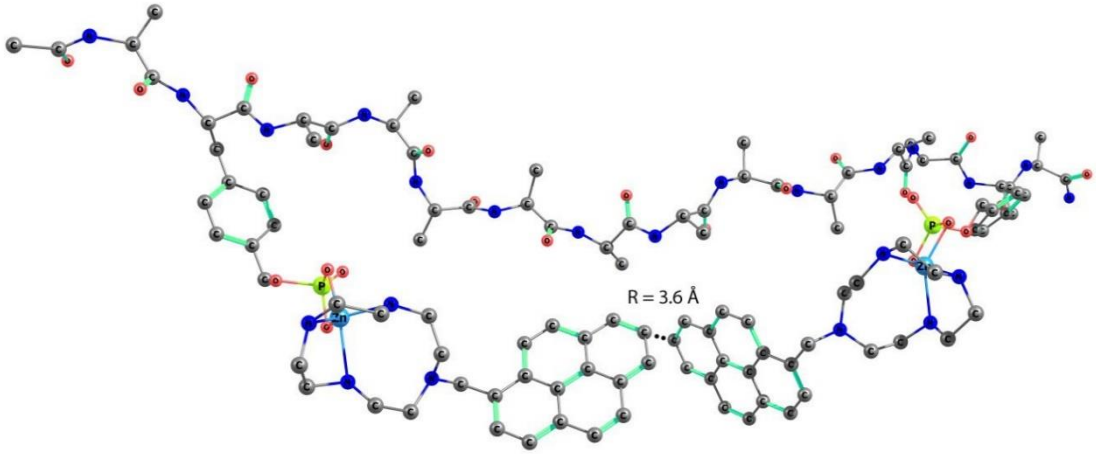


Figure S21 Optimized structure for ApYA11pYA

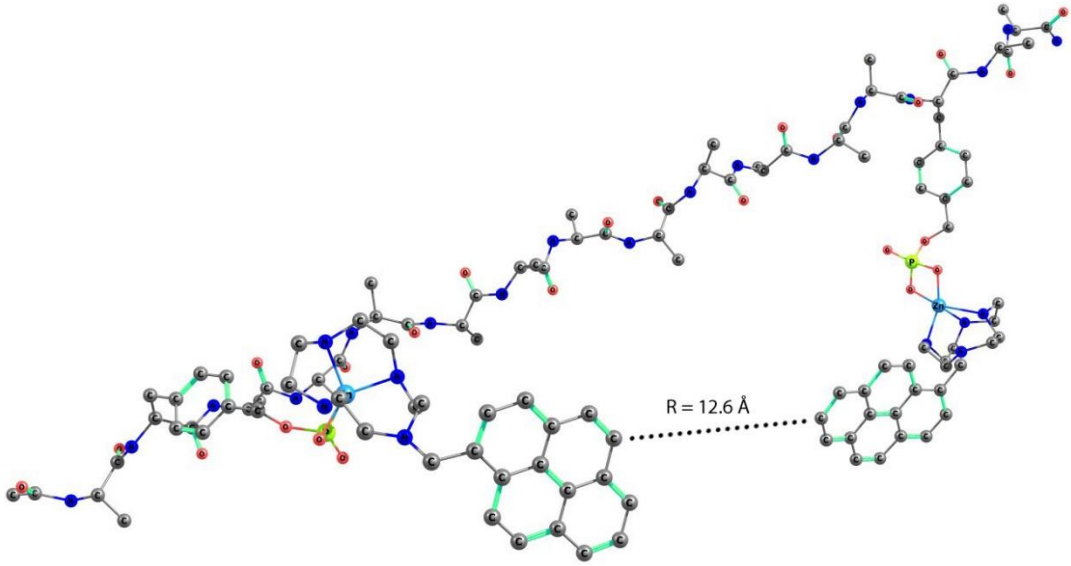


Figure S22 PM3 Optimized structure for ApYA19pYA (Helical).

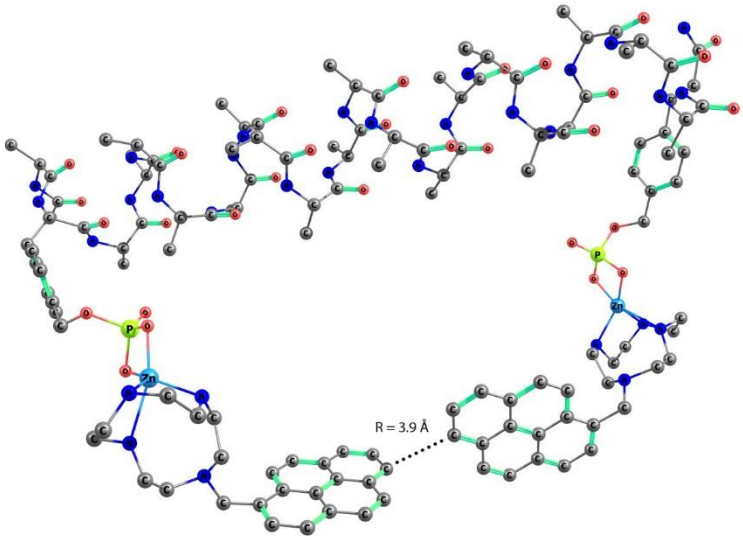


Figure S23 PM3 Optimized structure for ApYA20pYA (Helical).

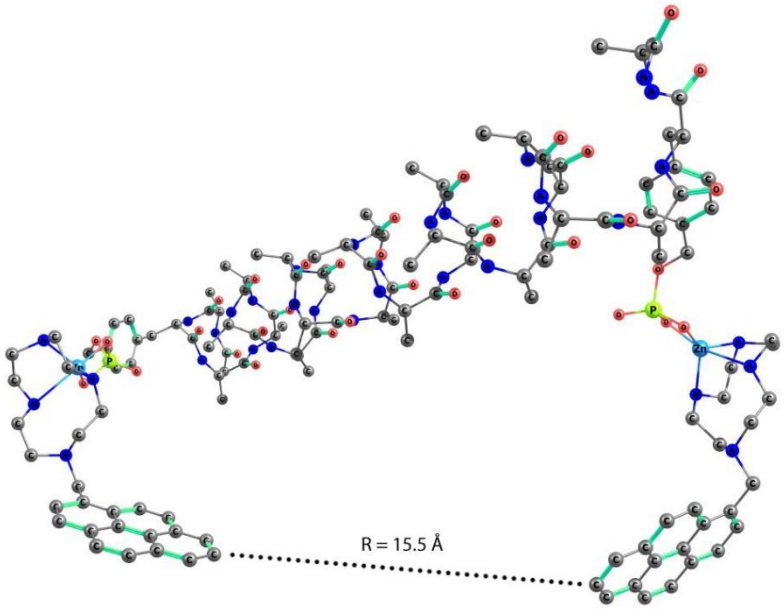


Figure S24 Titration of 40 µM ProxyPhos ($\lambda_{ex/em}$ = 350/476 nm) with pYpY and DADpYDLS peptides in different buffer solutions (all combination buffers contain 10% DMSO).

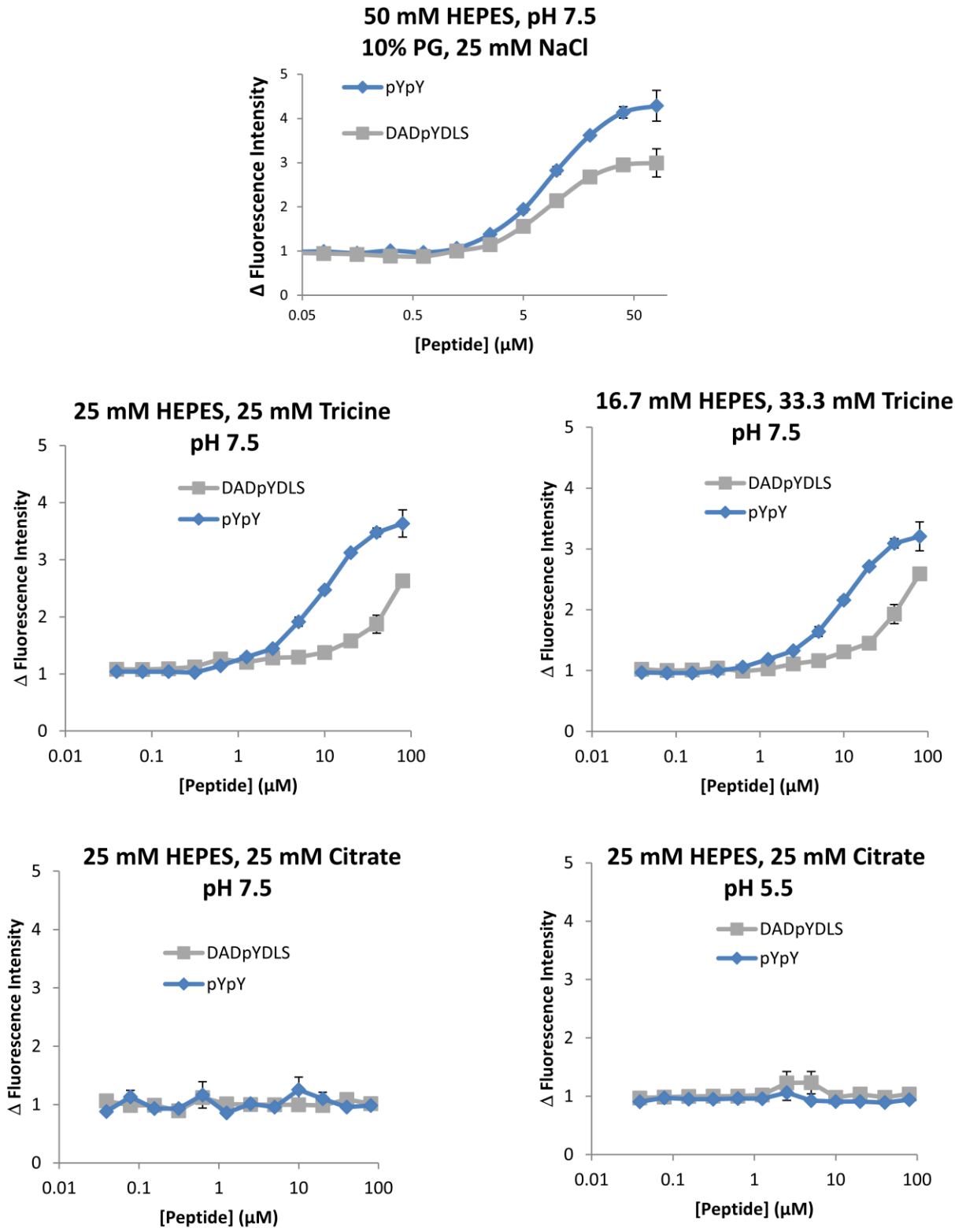


Figure S25 Titration of 40 µM ProxyPhos with different phosphorylated peptides and proteins before and after treatment with alkaline phosphatase (50 mM HEPES, pH 7.5, 10% DMSO, 10 µM $MgCl_2$; $\lambda_{ex/em}$ = 350/476 nm)

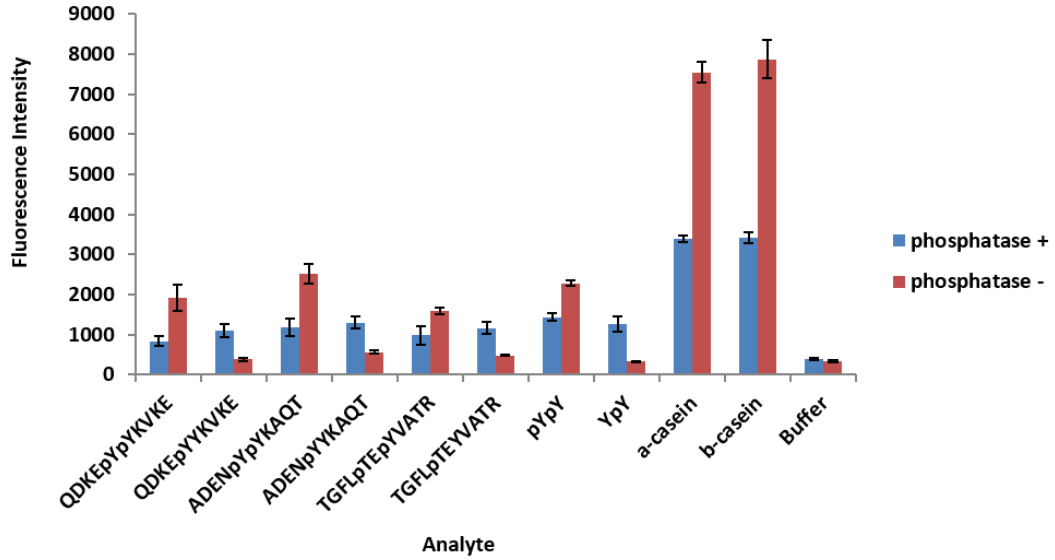
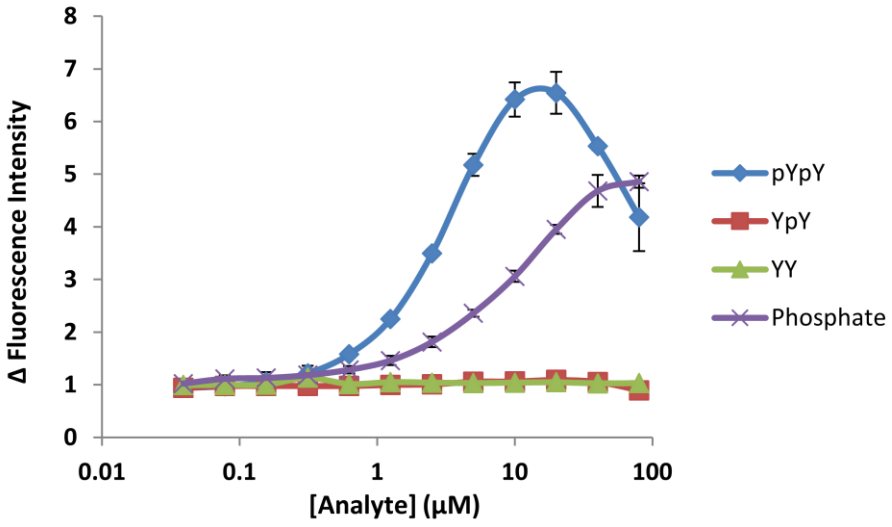


Figure S26 Titration of 40 µM ProxyPhos with different control analytes including inorganic phosphate (50 mM HEPES, pH 7.5, 10% DMSO; $\lambda_{ex/em}$ = 350/476 nm)



Supplementary tables

Table S1 Isoelectric Points of Proteins

Protein	Isoelectric point (pI)
BSA	5.6
Ovalbumin	5.19
β-casein	5.13
α-casein	4.91
Lysozyme	11.35

Iso-electric points were found using ExPASy in conjunction with UniProt database.

Table S2 Predicted inter-pyrene distances in various peptides

Spacer Length	Shortest pyrene-pyrene C-C distance (Å)			
	pS		pY	
	Linear	Helix	Linear	Helix
0	3.7		4.2	
1	3.6		3.8	
2	3.8		3.6	
3	3.6			
4	3.7			
5	7.2			
6	13.0			
7	11.0			
8			3.6	
9		3.9	3.4	
10		3.6	3.6	
11		3.7	12.6	
12		17.9	12.5	
13		6.3	16.7	
14		3.6		
15		10.4		
16				
17				3.7
18				3.6
19				3.9
20				15.5
21				7.1
22				7.0

Supplementary Notes

Supplementary Note 1 List of full peptide sequences

All peptides were purchased from CanPeptide at 95% purity as lyophilized powder.

YpY - Ac-AYpYAA-NH_2

YY - Ac-AYYAA-NH_2

pYpY - Ac-ApYpYAA-NH_2

pYAApY - Ac-ApYAApYA-NH_2,

pYAAApY = Ac-pYAAApY-NH_2

pYAAAApY = Ac-pYAAAApY-NH_2

pYAAAAApY = Ac-pYAAAAApY-NH_2.

pSpS = Ac-ApSpSAA-NH_2

SpS = Ac-ASpSAA-NH_2

pSApS = Ac-ApSApSAA-NH_2

pTAY = Ac-ApTAYAA-NH_2

pTApY = Ac-ApTApYAA-NH_2

DADpYDLS = NH_2-DADpYDLS-COOH

QDpYDLS = NH_2-QDpYDLS-COOH

QDKEpYpYKVKE = Ac-QDKEpYpYKVKE-NH2

QDKEpYYKVKE = Ac-QDKEpYYKVKE-NH2

ADENpYpYKAQT = Ac-ADENpYpYKAQT-NH2

ADENpYYKAQT = Ac-ADENpYYKAQT-NH2

TGFLpTEpYVATR = Ac-TGFLpTEpYVATR-NH2

TGFLpTEYVATR = Ac-TGFLpTEYVATR-NH2

Supplementary Note 2 ProxyPhos Assay Application Note

ProxyPhos is stable as a powder at -20 °C for at least two years and as a DMSO stock for at least 3 months at -80 °C. **The assay is sensitive to variations in ionic strength/salt concentration, pH, and hydrophobic content, presence of other metals and metal chelates.** Slight differences between Δ FI values may arise between different batches of buffers. It is advisable to use appropriate positive (e.g. pYpY/α-casein) and negative (pY/ovalbumin) for the peptide/protein assays, respectively, in order to normalize the data between different buffer batches.