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Chemoselective signalling of selected phospho-anions using lanthanide luminescence†

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Selectivity in the binding of phosphorylated tyrosine residues to aqua–lanthanide complexes is signalled by changes in spectral form by luminescence emission and ^{1}H NMR spectroscopy.

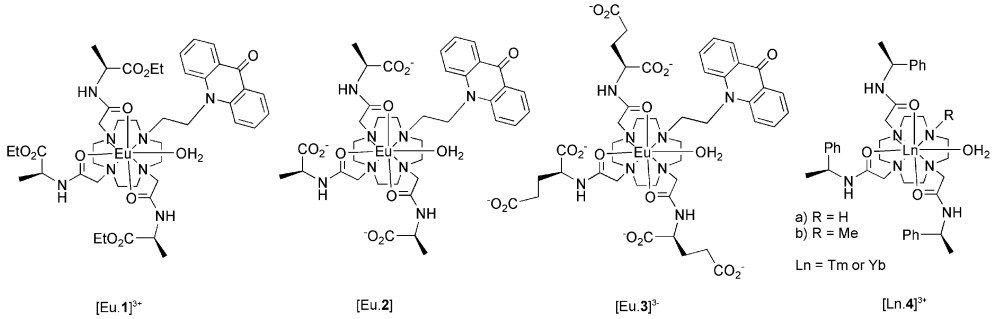
The oxyanions of pentavalent phosphorus constitute arguably the most important class of anion in the natural world.[1] Examples include ATP and cyclic AMP as signalling molecules, phosphate salts in bone (*e.g.* apatite) and the phosphorylated peptides and proteins that control many processes including cellular differentiation, development and proliferation.[2] Phosphorus-31 NMR spectroscopy has proved to be invaluable in defining the time dependence of the concentration of the abundant phosphorylated species in cells, each P(v) oxyanion being distinguished by its unique chemical shift. Chemical sensors have also been developed and afford enhanced sensitivity and spatial resolution. Examples include amperometric and potentiometric biosensors for analysis of inorganic phosphate based on phosphorylases[3] or phosphate binding protein[4] and colorimetric[5,6] or optical sensors,[7] based on absorption or emission intensity modulation. Non-invasive optical methods for directly monitoring the intracellular concentration and spatial distribution of phosphorylated species in real time are lacking. Therefore, we have begun to explore the complexation chemistry of the common phosphorylated anions, with the aim of devising luminescent intracellular probes for species such as phospho-tyrosine sites in proteins.

In recent work, it has been established that carbonate and inorganic phosphate bind reversibly to aqua–lanthanide complexes such as [Eu.**1–3**], with affinities of $> 7 \times 10^4$ M^{-1}. Selectivity for these two species is maintained in the presence of protein and endogenous cellular constituents.[8] The affinity of the anion for the lanthanide centre is modulated by permuting the Ln ion (Yb > Tm > Tb > Eu) or by modification of the ligand. Thus, controlled variation of the peripheral electrostatic gradient around the Ln ion centre allows not only further tuning of the desired affinity but an opportunity to complement the local electrostatic potential, *e.g.* on a protein surface.[2]

† Electronic supplementary information (ESI) available: Representative Job plot for [Eu.**2**] and AMP; binding isotherm for titration of [Eu.**2**] with Tyr–OP and Ser–OP in the presence of 5 mM HCO_3^-; ^{1}H NMR data for Yb/Tm complexes [Ln.**4**]$^{3+}$ with selected oxyanions, correlating Yb/Tm data, tabulating limiting chemical shift data, and giving representative partial NMR spectra (500 MHz, 295 K, D_2O, 4 mM complex, five-fold excess of anion). See http://www.rsc.org/suppdata/cc/b3/b313496m/

Here, we report preliminary observations regarding the binding of the Eu–acridone complexes to selected phosphates, the phosphorylated amino-acids of Ser, Thr and Tyr, a doubly phosphorylated hexapeptide, Gly–Ser–Pro–Tyr–Lys–Phe, and simple monophosphorylated analogues. The hexapeptide was selected as it represents a typical sequence in the important retinoblastoma protein that regulates passage through the G1 phase of the cell division cycle and is believed to be sequentially phosphorylated near the C-terminal Ser/Tyr sites.[9] In parallel, we have also examined by ^{1}H NMR spectroscopy the paramagnetically shifted spectra of the phosphate ternary adducts with the model Yb and Tm complexes, [Ln.**4**], the analysis of which allows information to be gained on the local coordination environment at the Ln centre, by comparison to a large number of structurally characterised analogues.[10]

Emission spectra of ternary phosphate complexes of [Eu.**1–3**] were measured at 295 K and pH 7.4 (0.1 M MOPS) in the presence of at least a 10-fold excess of added phosphate (0.1 mM complex). Under these conditions, independent titration revealed formation of 1:1 adducts (Job plot and binding isotherm, ESI†), so that the spectra given (Fig. 1) relate to species which are ≥90% bound. An independent measurement of the hydration state, *q*, of each ternary adduct was made by determining the radiative rate constants for decay of the Eu excited state in H_2O and D_2O, applying a corrected empirical formula to assess the vibrational quenching contributions of coordinated or proximate OH and NH oscillators,[11] (Table 1). Addition of Ser–OP, Tyr–OP (Thr–OP, data not shown), HPO_4^{2-} and glucose-6-phosphate to [Eu.**1**] gave subtly different emission spectra (Fig. 1) with Tyr–OP giving the lowest $\Delta J = 2/\Delta J = 1$ intensity ratio and a distinctive $\Delta J = 1$ manifold. In each case, rate data were consistent with formation of mono-aqua species with phosphate acting as a monodentate ligand.[8] Cyclic-AMP showed no evidence of binding, whereas addition of AMP^{2-} gave a similar spectrum to glucose-6-phosphate/HPO_4^{2-}. The hexapeptide phosphorylated at both Tyr and Ser and the monophosphorylated Tyr–OP analogue (replacing Ser for Ala), gave rise to identical emission spectra (Fig. 2) when added to [Eu.**2**]. The analogous monophosphorylated Ser* peptide on addition to [Eu.**2**] gave a profile identical to that obtained independently with Ser–OP alone (Fig. 1). That selective O-phosphate ligation occurs in the presence of peptide/protein was confirmed by the absence of change (over 48 h) in the emission spectra of [Eu.**1–3**] with S*Y*-peptide and Tyr–OP, on addition of an 0.4 mM solution of human serum albumin. Moreover, the selectivity for Tyr–OP over Ser–OP was indicated



[Eu.**1**]$^{3+}$ [Eu.**2**] [Eu.**3**]$^{3-}$ [Ln.**4**]$^{3+}$

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by the apparent absence of change in the emission spectrum of [Eu.**2**/**3**] and Tyr–OP (5 equiv.) in the presence of up to 50 equivalents of added Ser–OP. The relative affinity of Ser–OP and Tyr–OP for the zwitterionic complex [Eu.**2**] was measured in the presence of 5 mM $NaHCO_3$, and gave apparent affinity constants (295 K, pH 7.4, 0.1 M MOPS) log*K* of 2.7 and 4.2, respectively (ESI†).

X-Ray and NMR studies have shown that amino-acids chelate to Yb/Tm complexes (*e.g.* [Yb.**4**]), in contrast to the Eu/Tb analogues which bind with a much lower affinity at ambient pH *via* monodentate carboxylate coordination – with the amino group remaining protonated.[8,10] Thus, the Yb/Tm analogues may bind to phosphorylated amino-acids and peptides either *via* phosphate coordination or chelation to the amino-acid/peptide N-terminus. Proton NMR spectra for [Yb/Tm.**4**] with added anionic species revealed distinctive paramagnetically shifted spectra. The mean shift of the H-4 macrocyclic ring proton lies to highest frequency in every case and is determined purely by the pseudo-contact contribution.[10] Its mean value and the shift pattern for the four non-equivalent resonances observed is *different* for each ternary adduct. Thus, phosphate adducts of [Yb.**4a**] with HPO_4^{2-}/AMP/glucose-6-phosphate/Tyr–OP/Ser–OP/Thr–OP were in slow exchange on the NMR timescale and gave similar spectra with a mean shift of 91 (±5) ppm and 268 (±15) ppm for the Tm analogue, values which did not change on addition of human serum albumin (0.35 mM). Competitive amino-acid chelation was observed with Tyr–OP and

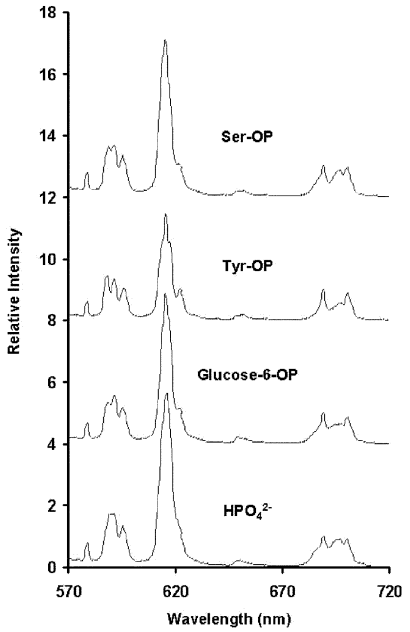

Fig. 1 Europium emission spectra for [Eu.**1**] in the presence of a 10-fold excess of added anions (λ_{exc} = 410 nm, 0.1 mM complex, 295 K, pH 7.4, 0.1 M MOPS).

Table 1 Radiative rate constants (*k*/ms^{-1}, ±10%) for depopulation of the Eu $^5D_1$ excited state for [Eu.**1**] in H_2O and D_2O (295 K, pH 7.4) and derived hydration states, *q*, in the presence of a 10-fold excess of the anion; values in parentheses refer to [Eu.**2**]

Added anion	$k(H_2O)$/ms^{-1}		$k(D_2O)$/ms^{-1}		*q* (±0.2)	
Aqua (triflate)	2.16	(2.33)	1.03	(0.80)	0.8	(1.2)
HPO_4^{2-}	1.71	(1.66)	0.68	(0.87)	0.6	(0.7)
Glucose-6-phosphate^{2-}	1.65	(1.46)	0.67	(0.54)	0.6	(0.7)
Tyr-OP^{2-}	1.77	(1.69)	0.65	(0.53)	0.8	(0.8)
Ser-OP^{2-}	1.78	(1.64)	0.69	(0.59)	0.7	(0.7)
AMP^{2-}	1.26	(1.26)	0.68	(0.56)	0.3	(0.3)
ADP^{3-}	0.99	(1.10)	0.61	(0.54)	0	(0.1)
ATP^{4-}	0.89	(1.05)	0.57	(0.55)	0	(0.0)
Peptide S*	1.68	(2.29)	0.84	(0.79)	0.4	(1.2)
Peptide Y*	1.70	(1.75)	0.81	(0.62)	0.5	(0.8)
Peptide S*Y*	1.70	(1.86)	0.79	(0.59)	0.5	(0.9)

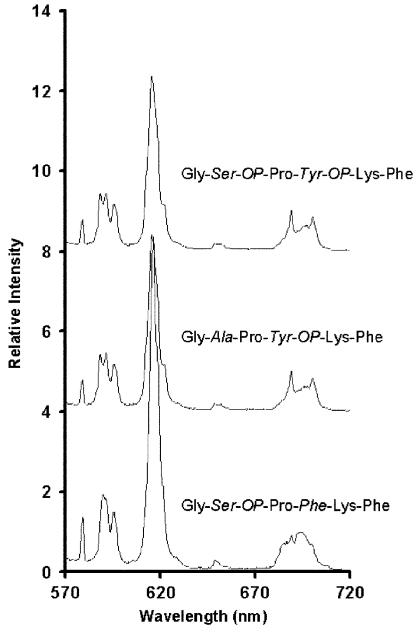

Fig. 2 Europium emission spectra for [Eu.**2**] in the presence of 2 equivalents of the differentially phosphorylated hexapeptide Gly–Ser–Pro–Tyr–Lys–Phe (λ_{exc} = 410 nm, 0.1 mM complex, 295 K, pH 7.4, 0.1 M MOPS).

Ser–OP (ESI†), but was absent in the N-acylated derivatives. Addition of one equivalent of the diphosphorylated hexapeptide to [Tm.**4b**]$^{3+}$gave an NMR spectrum consistent with selective Tyr–OP binding (*via* phosphate group) with no evidence for binding to the peptide N-terminus nor the Ser–OP site. Furthermore, addition of a 10-fold excess of Ser–OP to a solution containing [Yb.**4a**] and Tyr–OP in 1:5 ratio gave no significant spectral change in the shifted resonances, consistent with the chemoselective binding of Tyr–OP observed by Eu emission spectroscopy. The selectivity over Ser–OP may be related to the lesser hydration of the unbound Tyr–OP anion and hence a lower desolvation energy term in the overall free energy of binding.

In summary, NMR and emission spectral results reveal the chemoselective ligation of phosphorylated tyrosine in competitive media, both for the free amino acid and in a model hexapeptide. This is signalled by a change in the spectral form of the Eu ternary complex, distinctively different from that observed with say, added HCO_3^- or protein.

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