A cationic lanthanide complex binds selectively to phosphorylated tyrosine sites, aiding NMR analysis of the phosphorylated insulin receptor peptide fragment

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The binding of two cationic europium complexes to a differentially phosphorylated insulin receptor peptide has been studied by emission spectroscopy and ^{31}P NMR and ^{1}H NMR TOCSY methods. Analysis of the europium emission and NMR spectral data was consistent with the presence of species in slow exchange on the NMR and emission timescales, in agreement with selective binding of the lanthanide ion to the phospho-tyrosine site, allowing such complexes to be considered as prototypical chemoselective paramagnetic derivatising agents.

Introduction

There continues to be much interest in the quest for practicable methods of devising and signalling the chemoselective and reversible binding of anionic species in aqueous media. Key issues to be considered include achieving the required target binding affinity in the analyte of interest and realising how to minimise the impact of competitive binding of other anionic species. Several innovative approaches have been promulgated that usually respect the differing size/geometric requirements of the target anion, by engineering size and shape complementarity into the receptor.[1,2] Most often, this is addressed by devising positively charged receptors with appropriately positioned hydrogen bond donors or acceptors.[3,4] The approach that we, and others, have adopted is based upon the reversible binding of anions to a charged metal ion centre.[5–9] Anion displacement of one or two coordinated water molecules at a Ln(III) ion centre is signalled by modulation of the lanthanide emission spectral form, lifetime and circular polarisation. The affinity of the lanthanide receptor for a given anion may be controlled by varying the overall charge on the lanthanide complex or by perturbing the local electrostatic potential.[8] This may be effected by Ln ion permutation (*e.g.* Yb/Ln are most charge dense with highest intrinsic affinity) or by varying the nature of the encapsulating ligand.[7–9] For example, early work[5] revealed that inorganic phosphate (298 K, pH 7.4, 0.1 M NaCl) binds strongly to the cationic complex $[Eu\cdot\mathbf{1}]^{3+}$, with an affinity of the order of 10^5 M^{-1}, and that selectivity for this and related phosphorylated anions is conserved in the presence of protein and millimolar concentrations of hydrogen carbonate.[9] In systems where the anion binds reversibly to a metal centre, the order of anion affinity is also sensitive to the local steric demand imposed by the nature of the ligands encapsulating the metal ion. For example, europium complexes favouring chelation of citrate but inhibiting hydrogen carbonate binding are obtained by introducing a pyridyl-bound azaxanthone moiety.[10,11]

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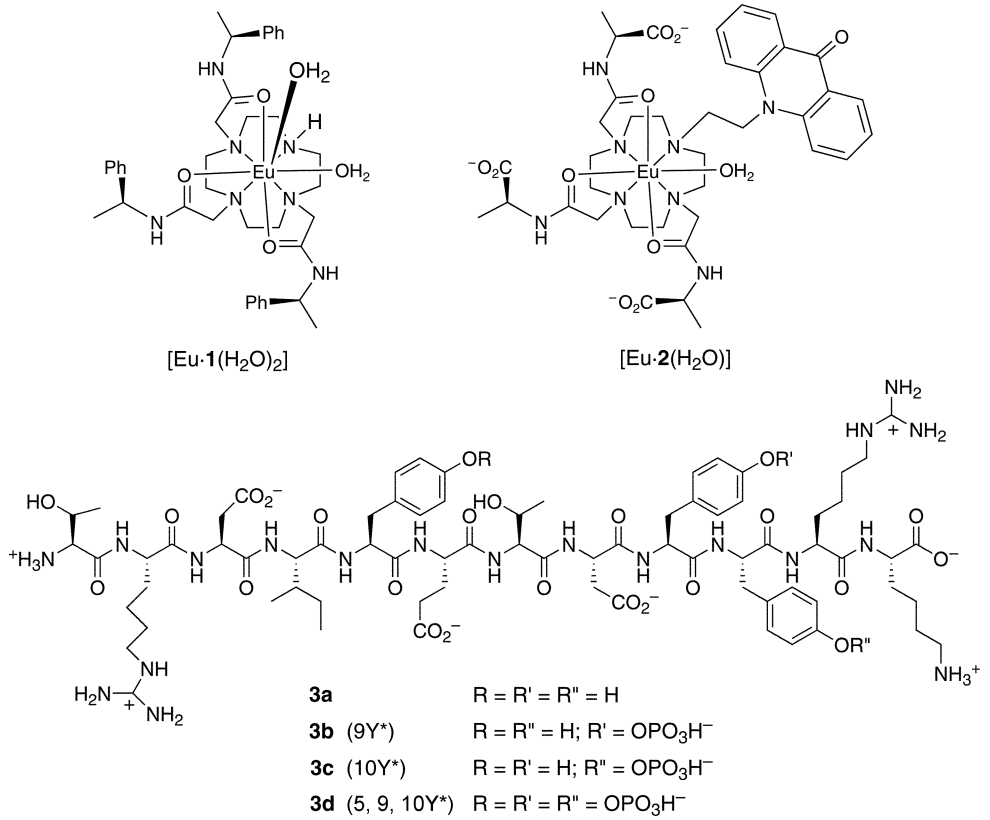
Recently, we have examined in detail the ^{1}H NMR and lanthanide emission spectral behaviour of $[Ln\cdot\mathbf{1}]^{3+}$ complexes (Ln = Eu, Tb, Yb, Tm) towards a variety of phosphorylated anions,[9] including *O*-phospho-tyrosine ('Tyr–OP') and serine ('Ser–OP') and some simple hexapeptides containing one phosphorylated residue. Using $[Eu\cdot\mathbf{1}]^{3+}$, a distinct binding preference for the phosphorylated site was observed, notwithstanding the presence of competitive binding sites at the peptide termini as well as side chain carboxylates in Asp or Glu residues. For the corresponding Yb and Tm complexes, phospho-anion binding was again observed, but a pH-dependent N-terminal chelation mode was the slightly preferred binding mode. A similar pattern of selectivity, favouring phospho-anion binding, was also evident from an analysis of the europium emission spectra of [Eu·**2**] (and related complexes with differing substituents in the ligand side chains), in the presence of various anionic species.[9,12]

With this background in mind, we set out to assess the utility of $[Eu\cdot\mathbf{1}]^{3+}$ and [Eu·**2**] with a more complex peptide. The system chosen for study was the insulin receptor fragment, 1154–1165, a dodecapeptide (Thr–Arg–Asp–Ile–Tyr–Glu–Thr–Asp–Tyr–Tyr–Arg–Lys) with three Tyr residues at sites 5, 9 and 10. Early ^{1}H NMR experiments had demonstrated that related peptides exhibited well-resolved proton NMR resonances with one predominant (random coil) conformer populated at room temperature[13,14] in solution. The series of peptides chosen for examination comprised the parent peptide **3a**, two monophosphorylated systems, **3b** (9Y*) and **3c** (10Y*), and the triphosphorylated system **3d** (5, 9, 10Y*). Each peptide was purified by reverse-phase HPLC to a $\geqslant$95% level of homogeneity.

Results and discussion

Emission spectral changes with [Eu·2]

The europium complex, [Eu·**2**], incorporates an acridone moiety that serves as a sensitiser for europium emission, following excitation at 408 nm.[12] To a solution of this europium complex (0.05 mM) was added a solution of various peptides or 'Tyr–OP'



(0.1 mM), maintaining the pH at 7.4 (±0.05) with MOPS buffer (0.1 M). With the non-phosphorylated peptide **3a**, the observed europium emission spectrum was identical to that obtained for the complex on its own, and quite distinctly different from that obtained following addition of 'Tyr–OP' (Fig. 1). Moreover, the emission spectra observed for [Eu·**2**] in the presence of **3c** (or **3b**) was very similar in form to that obtained with 'Tyr–OP' (Fig. 2), consistent with weak binding to the parent peptide **3a** and selective binding of the Eu to a phospho-anion site, in accord with earlier observations.[9] The similarity of the splitting pattern in the three bands of the magnetic-dipole allowed $\Delta J = 1$ manifold around 590 nm, and the similar form and relative intensity of the hypersensitive $\Delta J = 2$ and $\Delta J = 4$ transitions around 616 and 695 nm respectively are particularly apparent.

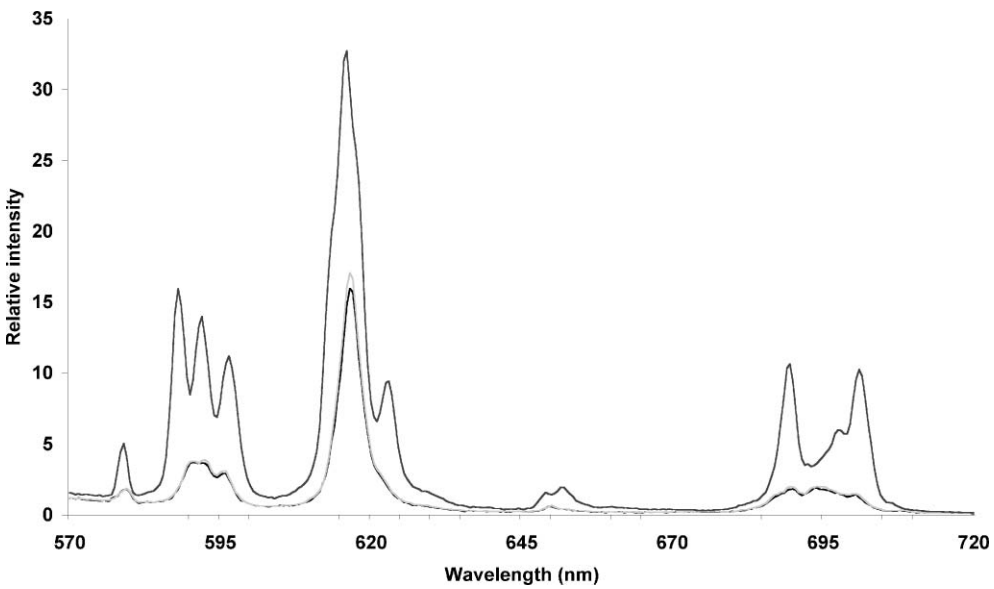


Fig. 1 Europium emission spectra for [Eu·**2**] (50 μM) (lower, black) and [Eu·**2**] + **3a** (50 μM/100 μM) (lower, grey), and for [Eu·**2**] + phospho-tyrosine (50 μM/100 μM) (upper, black) (pH 7.4, 0.1 M MOPS buffer, λ_{exc} 408 nm).

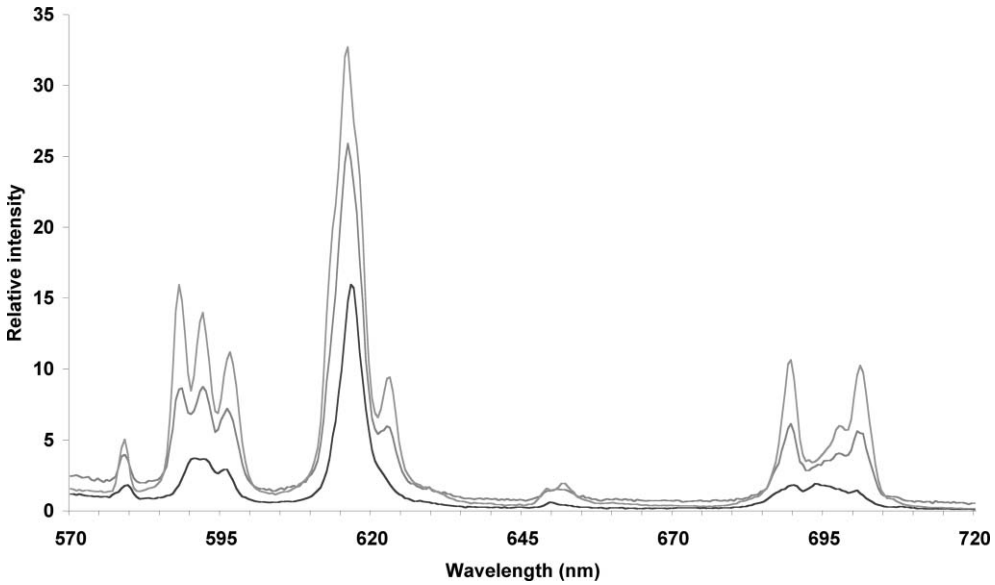


Fig. 2 Europium emission spectra for [Eu·**2**] (50 μM) (lower), [Eu·**2**] + **3c** (50 μM/100 μM) (centre), and [Eu·**2**] + phospho-tyrosine (50 μM/100 μM) (upper) (pH 7.4, 0.1 M MOPS, λ_{exc} 408 nm).

^{1}H NMR TOCSY and ^{31}P NMR analyses

Earlier work on phospho-anion binding with $[Eu\cdot\mathbf{1}]^{3+}$ and its congeners suggested that the free and Eu-bound species would be in slow exchange on the NMR timescale at 500 MHz, and hence the europium complex in this instance may be considered to behave as a chemoselective derivatising agent, rather than as a shift or relaxation agent.[15] The binding of $[Eu\cdot\mathbf{1}]^{3+}$ to the phospho-anion sites on the peptide was anticipated to induce shifts in the resonances of the nearby amino-acid NMR-active nuclei, without creating too much line-broadening, and hence allow or confirm the identification of residues close to the binding site. Indeed, the relatively low magnetic anisotropy exhibited by the Eu(III) ion[16,17] restricts the range over which the paramagnetic shift is measurable to <10 Å, significantly less than the peptide dimensions (around 40–50 Å). Such a study may be contrasted with related earlier work using positively or negatively charged gadolinium complexes to probe any preferential binding sites upon a protein surface. The strong relaxation effect of the Gd(III) ion was used in an attempt to determine distances between any preferred site of Gd complex binding and residues within the protein. Only a simple charge complementarity effect was observed, with some evidence for preferential Asp or Glu interactions to the cationic system.[15]

Two-dimensional proton NMR TOCSY spectra of the peptides **3a**–**3d** were examined in 95% H_2O–D_2O using the 'Watergate' water suppression pulse sequence,[18] allowing *J*-connectivities of amide protons for individual peptide bands to be examined, thereby facilitating the identification of each amino-acid residue.[19] Attempts were also made to examine 2-D ROESY and NOESY spectra, but despite using a variety of spin-mixing and delay times, high quality spectra were not obtained. Similar problems of weak ROESY correlation peaks were also encountered recently in the attempted analysis of a shorter fragment of the insulin receptor peptide.[13] However, the analysis of the ^{1}H NMR TOCSY spectra proceeded smoothly. For each peptide examined, only one set of resonances was observed, consistent with the presence of one major conformer in solution. For example, in the NMR analysis of **3c** (Fig. 3), resonances corresponding to each of the amino-acid residues were distinguished, facilitated by the unique TOCSY pattern of resonances observed in the amide proton region. Each of the Arg, Asp and Thr residues could be distinguished and Thr-1 was readily assigned, as it lacks an amide proton. Tyrosine-5 was also clearly distinguished in each peptide examined, although Tyr-9 and Tyr-10 were only present as distinct resonances in **3b**. The 'positional' assignment of Arg-2 and -11, Asp-3 and -8 and Tyr-9 and -10 could not be made

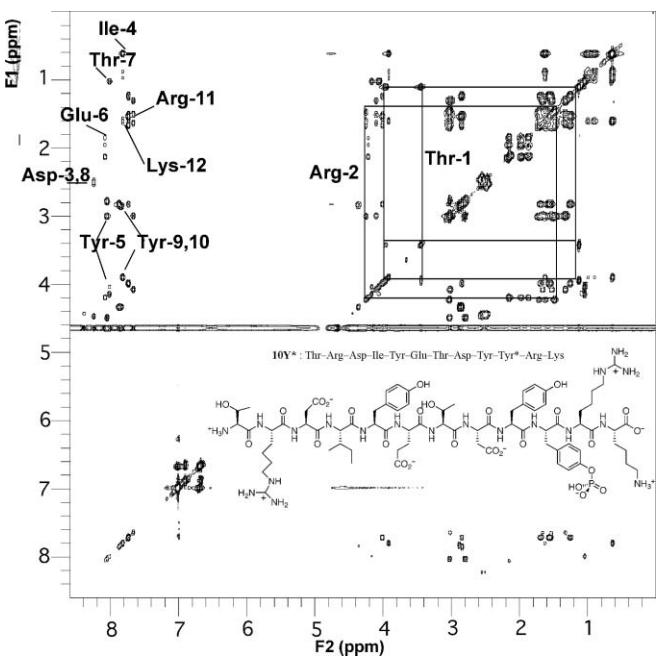


Fig. 3 ^{1}H NMR TOCSY spectrum for the Tyr*-10 phosphorylated peptide, **3c**, showing assignments (500 MHz, 95% H_2O–D_2O, 295 K, 'Watergate' H_2O suppression).

unequivocally on the basis of the individual TOCSY spectral data alone for each peptide examined. However, for the three phosphorylated peptides examined, the general order of chemical shifts for the resonances of each amino-acid residue was observed to be virtually identical, notwithstanding their differing degree and site of phosphorylation. The most significant changes in the positions of the resonances occurred in the amide region for residues adjacent to the Tyr sites. For example, comparison of the chemical shift for the amide proton for Ile-4 in **3c** (7.82 ppm) and **3d** (7.74 ppm) revealed a shift to lower frequency upon phosphorylation of Tyr-5, and the Ile-4 amide resonance resonated to lowest frequency for **3d** only. Similar analyses of small chemical shift differences for residues adjacent to tyrosine sites in the differentially phosphorylated peptides allowed the tentative assignment of Arg-11 and hence Arg-2 and, of course, Tyr-5 itself (Fig. 3).

Incremental addition of up to 0.5 equiv. of the europium complex, $[Eu{\cdot}\mathbf{1}(H_2O)_2]^{3+}Cl_3$ to the mono-phosphorylated peptides **3b** and **3c** led to formation of a new species in slow exchange on the NMR timescale with the peptide. A broad, shifted ^{31}P NMR resonance at -136 ppm ($\omega_{1/2} \approx 200$ Hz) grew in intensity as the Eu complex was added, and was observed at the same time as the resonance for the native peptide at *ca.* 0.5 ppm (298 K, H_2O, 162 MHz, pH 7.4). This shifted ^{31}P NMR resonance is characteristic of a phosphate-bound ternary adduct, and an identical resonance was observed for the adduct of $[Eu{\cdot}\mathbf{1}(H_2O)_2]^{3+}$ with phospho-tyrosine. Furthermore, analysis of the paramagnetically shifted ligand resonances for $[Eu{\cdot}\mathbf{1}]^{3+} + \mathbf{3c}$, over the range +40 to -30 ppm, gave the same distinct spectral pattern and mean chemical shifts, as has been reported for a variety of ternary phospho-anion adducts.[9] For example, the four most shifted, constitutionally heterotopic axial ring protons of the [12]-ring macrocycle resonated at 26.5, 21.1, 18.0 and 15.5 ppm (mean $\delta^{ax}{}_{H_4} = 20.3$ ppm). This is a very similar value (± 0.8 ppm) to that observed for adducts with phospho-tyrosine or glucose-6-phosphate.[9,20] It has previously been established that in nine-coordinate lanthanide complexes adopting a capped square-antiprismatic geometry, the nature and polarisability of the axial ligand donor determines the second-order crystal field coefficient, B^2_o, that predominantly defines the paramagnetic dipolar NMR shift.[7,21,22]

When $[Eu{\cdot}\mathbf{1}(H_2O)_2]Cl_3$ was added (up to 0.75 equiv.) to the non-phosphorylated peptide, **3a**, the ^1H NMR resonances due to the macrocyclic ligand in the europium complex broadened slightly but did not shift significantly. The observed paramagnetically shifted spectrum simply resembled that of the starting aqua complex, corroborating the europium emission spectral observations carried out separately.

The ^1H NMR TOCSY spectra of the phosphorylated peptides **3b**–**3d** were repeated in the presence of 0.25, 0.5 and 0.75 equiv. of $[Eu{\cdot}\mathbf{1}]^{3+}$. Given that the ternary adducts of the europium complex were in slow exchange in the ^{31}P NMR analyses, it was anticipated that a new set of resonances would be evident, in addition to those of the unbound peptide. Except for **3d**, addition of more than 0.5 equiv. of $[Eu{\cdot}\mathbf{1}]^{3+}$ generally led to significant exchange and paramagnetic broadening of the H_2O signal, so that water suppression was very poor. The presence of the phospho-Tyr-bound europium complex led to the shifting of some, but not all, of the amino-acid residues. For example, with **3c** (phosphorylated at Tyr-10), addition of 0.5 equiv. of $[Eu{\cdot}\mathbf{1}]^{3+}$ exhibited the smallest number of shifted resonances in the phospho-Tyr adduct (Fig. 4). Inspection of the spectrum revealed a new set of shifted resonances for Lys-12 and one of the Arg residues. The other set of Arg residues showed no shift, confirming the assignment of Arg-11 tentatively made earlier, due to its proximity to Tyr-10. The only other distinctive shifts occurred for two of the tyrosine residues – presumably Tyr-9 and -10 which possess very similar chemical shifts in **3c**. It is tempting to speculate that the most shifted set of resonances relate to Tyr-10 itself, although it must be noted that the angular dependence $[3\cos^2\theta - 1/r^3]$ of the induced paramagnetic shift can be very significant.

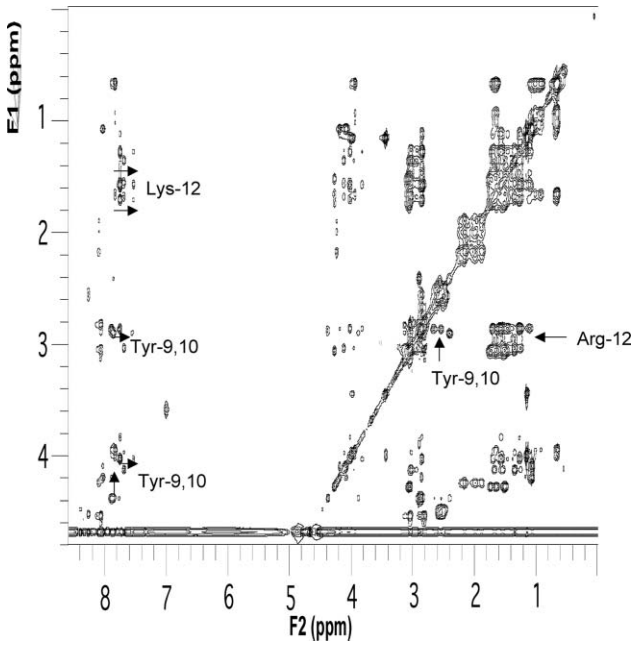


Fig. 4 ^1H NMR TOCSY spectrum for **3c** in the presence of 0.5 equiv. of $[Eu{\cdot}\mathbf{1}]Cl_3$; the arrows highlight the appearance of a second set of shifted resonances for selected amino-acids (500 MHz, 95% H_2O–D_2O, 295 K, 'Watergate' H_2O suppression).

Parallel experiments with $[Eu{\cdot}\mathbf{1}]^{3+}$/**3b** reveal new sets of shifted resonances for Lys-12, Arg-11 and for Tyr*-9 and Tyr-10. Additional shifted resonances were also observed for Thr-7, and less clearly due to line-broadening for Asp-8 (distinguishing it from Asp-3). The observation of shifted resonances for residues 7 and 8 (not seen with the Tyr-10 mono-phosphorylated peptides, **3c**) allows a means of distinguishing between the binding of the Eu probe to residues 9 or 10.

In analyses with the tri-phosphorylated peptide **3d**, addition of up to 0.75 equiv. of $[Eu{\cdot}\mathbf{1}]^{3+}$ per peptide was possible without compromising spectral quality too much and causing a lot of line-broadening. The spectrum obtained (Fig. 5) revealed new sets of shifted resonances that were not observed with either **3b** or **3c**, consistent with binding of the Eu complex to the Tyr*-5 site. Intense shifted resonances were observed for the N-terminal Thr residue and Ile-4 residue, in accord with this interpretation. Significantly shifted resonances were also observed for the C-terminal Lys residue (slightly more intense as the shifted set of signals associated with Tyr-5 binding), in accord with binding to either Tyr*-9 or Tyr*-10. Comparison of the appearance of

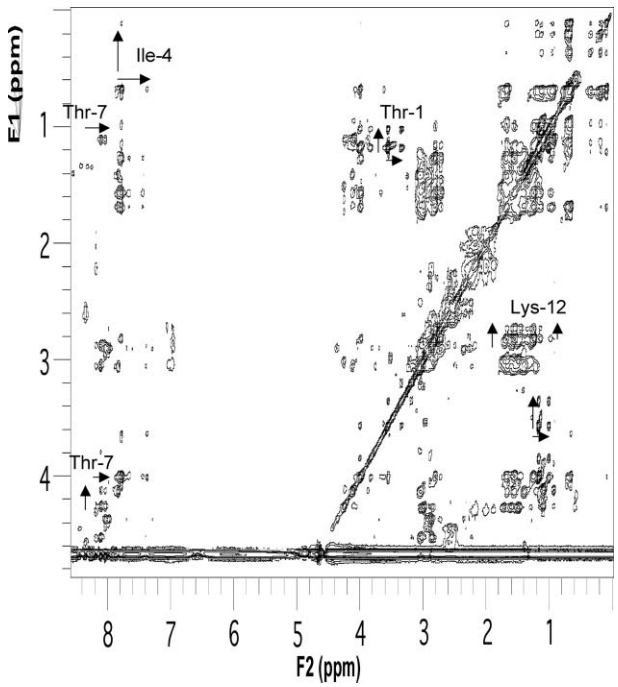


Fig. 5 ^{1}H NMR TOCSY spectrum for the tri-phosphorylated peptide, **3d**, following addition of 0.75 equiv. of [Eu·**1**]Cl_3; arrows indicate the appearance of additional resonances for the indicated residues (500 MHz, 95% H_2O–D_2O, 'Watergate' H_2O suppression, 295 K).

the shifted resonances for the C-terminal lysine in **3d** with the corresponding shifts observed with **3b** and **3c** suggests that these shifts are primarily caused by binding to the Tyr*-9 residue.

Summary and conclusion

The simple Eu complex, $[Eu\cdot\mathbf{1}(H_2O)_2]^{3+}$, serves as a chemoselective paramagnetic probe that targets phospho-tyrosine sites in phosphorylated peptides. Earlier work with some hexapeptides had established its selectivity profile for phospho-tyrosine residues over phospho-Ser/Thr sites ($\geqslant 30:1$).[9] The studies defined herein allow its utility as a spectral probe to be evaluated further. Addition of the europium complex to a phosphorylated peptide results in the formation of a phosphate-bound ternary adduct with the peptide. Nuclei in amino-acid residues that are close in space to the phosphate binding site undergo dipolar coupling with the unpaired electrons on the Eu(III) ion. The resultant paramagnetic shift is proportional to the distance (r^{-3}) between the Eu ion and the given nucleus, and also the angle, θ, between the nucleus and the principal magnetic axis [$3\cos^2\theta - 1$]. Given the modest magnetic anisotropy of the Eu(III) ion, the distance within which paramagnetic shifts observed is quite small (<10 Å), leading to a diminution of the paramagnetic shift [$3\cos^2\theta - 1/r^{-3}$] for more distant amino-acid residues. Indeed, a distinct 'cut-off' point can be defined, beyond which the paramagnetically induced shift is too small to be observed. For example, with the mono-phosphorylated peptides **3b** and **3c**, although the europium ion is only one amino-acid residue closer to the N-terminus for **3b** compared to **3c**, shifted resonances for Thr-7 are observed only in the former case. Using the tri-phosphorylated peptide **3d**, there was some evidence in support of a modest preference for the binding of the cationic Eu complex to Tyr*-9. This residue is flanked by the anionic residues Tyr*-10 and Asp-8, so that Coulombic attraction may slightly determine this preference, and this tendency may be enhanced by the presence of positively charged side chains at Arg-11 and Lys-12, disfavouring longer range encounter with $[Eu\cdot\mathbf{1}]^{3+}$.

This simple europium complex may exhibit only a modest preference between the phosphorylated Tyr sites within this insulin receptor peptide sequence, but its intrinsic chemoselectivity – in the presence of an N-terminal binding site (chelation of the N-terminal amine and any side chain donor or peptide carbonyl) and various anionic sites (Asp, C-terminus) – should not be overlooked. Indeed, replacement of Eu for Tb or Dy should allow much larger range paramagnetically induced shifts to be induced (>30 Å for Dy), as defined by Luchinat and Bertini.[15,17]

Experimental

UV/visible absorption spectra were recorded using a Perkin Elmer Lamda 900 UV/vis/IR spectrometer. Emission spectra were recorded at 295 K using an Instruments SA Fluorolog 3–11 spectrometer handling data using DataMax v.2.1 for Windows.

Luminescence spectra of the europium(III) complexes were recorded using a 420 nm cut-off filter, following indirect excitation of the europium(III) ion, either *via* the phenyl groups in the ligand at 264 nm or at 408 nm for acridone sensitiser in [Eu·**2**]. An integration time of 0.5 s, an increment of 0.5 nm and excitation and emission slit widths of 10 and 1 nm, respectively, were employed throughout.

^{1}H NMR spectra were recorded at 499.78 MHz on a Varian Inova-500 spectrometer. For paramagnetically shifted spectra, *tert*-butanol was added as an internal reference ($\delta = 0$ ppm). Solvent suppression was achieved with the 'Watergate' pulse sequence.

Peptides **3a**–**3d** were obtained from Peptide Protein Research Ltd., and their homogeneity as assessed by RP-HPLC was judged to be $\geqslant$95%. The synthesis and characterisation of [Eu·**1**]Cl_3 and [Eu·**2**] has been reported elsewhere.[5,12]

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