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Design of a Protein Motif Responsive to Tyrosine Nitration and an Encoded Turn-Off Sensor of Tyrosine Nitration

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Abstract

Tyrosine nitration is a protein post-translational modification that is predominantly non-enzymatic and that is observed to be increased under conditions of nitrosative stress and in numerous disease states. A small protein motif (14–18 amino acids) responsive to tyrosine nitration has been developed. In this design, nitrotyrosine replaced the conserved Glu12 of an EF-Hand metal-binding motif. Thus, the non-nitrated peptide bound terbium weakly. In contrast, tyrosine nitration resulted in a 45-fold increase in terbium affinity. NMR spectroscopy indicated direct binding of nitrotyrosine to the metal and EF-Hand-like metal contacts in this designed peptide. Nitrotyrosine is an efficient quencher of fluorescence. In order to develop a sensor of tyrosine nitration, the initial design was modified to incorporate Glu residues at EF Hand positions 9 and 16 as additional metal-binding residues, to increase the terbium affinity of the peptide with unmodified tyrosine. This peptide with tyrosine at residue 12 bound terbium and effectively sensitized terbium luminescence. Tyrosine nitration resulted in a 180-fold increase in terbium affinity ($K_d = 1.6 \mu\text{M}$) and quenching of terbium luminescence. This sequence was incorporated as an encoded protein tag and applied as a turn-off fluorescent protein sensor of tyrosine nitration. The sensor was responsive to nitration by peroxynitrite, with fluorescence quenched on nitration. The greater terbium affinity upon tyrosine nitration resulted in high dynamic range and sensitivity to sub-stoichiometric nitration. An improved approach was also developed to the synthesis of peptides containing nitrotyrosine, via the *in situ* silyl protection of nitrotyrosine. This work represents the first designed, encodable protein motif that is responsive to tyrosine nitration.

Graphical Abstract

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Supporting Information Available

Peptide synthesis and characterization details, method and experimental setup for synthesis of peroxynitrite, fluorescence spectra and binding isotherms, experimental details for nitration of peptides and proteins with peroxynitrite, and NMR spectra and resonance assignments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

Tyrosine nitration (Figure 1) is a protein post-translational modification (PTM) that is most prominently observed under conditions of oxidative and nitrosative stress.^{1–3} Tyrosine nitration *in vivo* is believed to occur predominantly non-enzymatically, via the breakdown of peroxynitrite (ONOO^- , formed via the reaction of nitric oxide ($\bullet\text{NO}$) and superoxide ($\text{O}_2^{\bullet-}$)) into the $\bullet\text{NO}_2$ and $\bullet\text{OH}$ radicals (or from $\text{CO}_3^{\bullet-}$ via reaction with CO_2), which react with tyrosine in a stepwise manner.^{4–7} Tyrosine nitration is observed to be elevated from basal levels in key proteins important in heart disease, inflammatory diseases, diabetes, Alzheimer's disease, and Parkinson's disease, among other pathological states.^{1, 3, 5, 8–21} In amyotrophic lateral sclerosis (ALS), significant tyrosine nitration of superoxide dismutase (SOD) has been found, which results in reduced enzyme activity.^{22–24} In Parkinson's disease, a significant increase in tyrosine nitration of α -synuclein is observed in Lewy bodies, and tyrosine nitration increases α -synuclein aggregation *in vitro*.^{11, 13, 25–28} Increased tau nitration is also observed in the neurofibrillary tangles of Alzheimer's disease, and tyrosine nitration of the tau protein increases aggregation *in vitro*.^{12, 29–31} Alternately, a potential regulatory role for nitrotyrosine was observed in cardiac development of embryonic rats, associated with the regulation of myosin heavy chain isoforms.³² Most generally, increased levels of tyrosine nitration are associated with the inflammation response, where both nitric oxide and superoxide are central molecules.^{1, 6, 33–36}

Tyrosine nitration reduces the pK_a of the tyrosine phenol, from approximately 10 to approximately 7, due to the electron-withdrawing nature of the nitro group, which stabilizes the phenolate.^{1, 5} Thus, nitrotyrosine exists in two forms at physiological pH: the neutral form (protonated on the phenol) and the anionic form. Interestingly, the neutral form of nitrotyrosine exhibits greater hydrophobicity than tyrosine¹ (e.g. Figure S3), due to intramolecular hydrogen bonding of the phenol via the nitro group. In contrast, the anionic form of nitrotyrosine is significantly more polar than tyrosine. The Janus-like nature of nitrotyrosine, with two forms that have opposing chemical properties, and the similar populations of both the hydrophobic and polar forms of nitrotyrosine at physiological pH complicates understanding of the effects of nitrotyrosine on protein structure and function, as different effects are possible in the two protonation states, which might be preferred in different protein contexts. Tyrosine nitration in proteins can induce a loss of function, induce a toxic gain of function, or have no apparent effect, depending on the identities of the protein and specific tyrosine nitrated.^{1, 3, 5, 10, 11} Loss of function has been identified broadly within

diverse classes of enzymes, including glutathione reductase, prostaglandin synthase, ribonucleotide reductase, protein kinase, and cytochrome P450.^{24, 37–40} Gain of function due to tyrosine nitration has been observed in fibrinogen (which results in increased clotting), glutathione S-transferase 1, tau, α -synuclein, and Hsp90 (which induces apoptosis in motor neurons, even at low nitration stoichiometry), among an increasing number of examples.^{16, 19, 41–45} In addition, nitrated tyrosine can mimic phosphorylated tyrosine in binding to SH2 domains, suggesting the possibility of aberrant cell signaling induced by tyrosine nitration.^{9, 46, 47}

Despite the broad observation of tyrosine nitration across numerous disease states, central questions on tyrosine nitration remain. Some investigators have questioned the role of tyrosine nitration as a causative agent in disease, as opposed to being a biomarker for oxidative damage.⁴⁸ In addition, while some evidence has emerged of nitrotyrosine being a reversible post-translational modification, via the observation of denitrase activity in cell lysate fractions, no putative denitrase enzyme has been identified to date.^{49–51} Tyrosine nitration is typically identified either via anti-nitrotyrosine antibodies or via mass spectrometry-based proteomics.^{31, 52–59} In addition, nitrotyrosine may be incorporated into expressed proteins via engineered aminoacyl tRNA synthetases that are specific for nitrotyrosine or via expressed protein ligation.^{28, 60} As a step toward detecting the molecules that can induce tyrosine nitration, fluorescent protein and small molecule sensors have been developed for peroxynitrite, the presumptive nitrating agent for most instances of nitrotyrosine in proteins.^{61–64} However, to date, no direct, encodable fluorescent sensor of tyrosine nitration has been reported. Tyrosine nitration is often highly localized, and as such encodability to achieve subcellular targeting is an important feature of a sensor of tyrosine nitration.⁶⁵ In addition, tyrosine nitration is often a low stoichiometry event, emphasizing the importance of a nitration sensor that exhibits a high degree of responsiveness to tyrosine nitration and a large dynamic range.⁵⁶ Herein, we describe the development of a protein motif responsive to tyrosine nitration, whose structure is dependent on its tyrosine nitration state. We further demonstrate that this approach can be applied to develop an encoded turn-off fluorescent protein sensor of tyrosine nitration.

Experimental

Peptide Synthesis and Characterization.

Peptides were synthesized using Rink amide resin by standard Fmoc solid-phase peptide synthesis protocols. Fmoc-3-Nitrotyrosine was protected on the side chain after amide coupling using *t*-butyldimethylsilyl chloride or using an acetyl group (Scheme S1). All peptides were acetylated on the N-termini and contained C-terminal amides. Synthetic procedures and characterization data are in the Supporting Information.

Fluorescence Spectroscopy.

Fluorescence spectra were collected on a Photon Technology International fluorescence spectrometer model QM-3/2003 with a CW source and a Hamamatsu R928 photomultiplier tube. Fluorescence spectra were collected using a 495 nm highpass filter on the emission monochromator and with 10 nm slitwidths. Experiments were conducted with an excitation

wavelength of 280 nm. Spectra were normalized to the local minimum in terbium emission at 570 nm. Peptide solutions were prepared at room temperature by dilution of stock solutions into 5 mM HEPES buffer (pH 7.8) with 100 mM NaCl, with a final peptide concentration of 10 μ M unless otherwise noted. Tb³⁺ binding isotherms were acquired via addition of 2-fold serial dilutions of Tb³⁺ into the peptide solution. Dissociation constants and errors were determined by non-linear least-squares fitting of fluorescence as a function of metal (direct binding experiments) or peptide (competition binding experiments) concentration to equations for direct or competition binding experiments. Details are in the Supporting Information. Due to the potential for superstoichiometric metal binding after saturation and observed metal-induced precipitation at high metal concentrations, binding isotherms were not extended beyond the apparent beginning of saturation. Tb³⁺ solution concentrations were standardized with xylenol orange, as described in the Supporting Information.

NMR Spectroscopy.

NMR spectra were recorded at 298 K on a Bruker Avance 600 MHz NMR spectrometer with a triple-resonance cryoprobe or a TXI probe, or a Bruker Avance III 600 MHz NMR spectrometer with a 5-mm Bruker SMART probe. Solutions contained 100 mM NaCl, La³⁺ or Ce³⁺ at the indicated concentrations, 10 or 20 μ M TSP-d₄, and 90% H₂O/10% D₂O (for 1-D, TOCSY, ROESY, or NOESY spectra) or 100% D₂O (for HSQC and HMBC experiments), with final peptide concentrations of 600 μ M, unless otherwise indicated. Samples were adjusted to pH 7.5 unless otherwise indicated. Water suppression was accomplished using excitation sculpting for 2-D experiments and using WATERGATE water suppression for 1-D experiments. Additional details are in the Supporting Information. Experiments were conducted with La³⁺, a diamagnetic lanthanide. Severe peak broadening occurs in the presence of paramagnetic metals, including Tb³⁺, precluding NMR analysis of the Tb³⁺ complex.^{66–68} Cerium (Ce³⁺) exhibits significantly less paramagnetic broadening than Tb³⁺ due to the presence of only one unpaired electron, versus six unpaired electrons in Tb³⁺. Notably, lanthanide-binding ligands, including peptides, exhibit a dependence of metal affinity on the size of the lanthanide, typically exhibiting the highest affinity at an intermediate lanthanide size, with weaker affinity observed with either smaller (right on the periodic table) or larger (left on the periodic table) lanthanides.^{69–72} Since peptides were optimized for Tb³⁺ affinity, experiments with La³⁺ and Ce³⁺ thus represent compromises compared to the optimal lanthanide affinities of sensor peptides, and are employed in order to obtain useful NMR data.

Design of the Fusion Protein MBP-pNO₂.

The sensor peptide sequence from pNO₂-E₉E₁₆Y₁₂ was encoded as a C-terminal tag to maltose-binding protein (MBP). Plasmids were generated via gene synthesis and cloning (GenScript) into a pMAL-C5E vector. The peptide sequence was added C-terminal to a short linker (sequence GPPGG), in order to minimize interactions of the sensor sequence with MBP. The poly-aspartate proteolysis site from the parent vector was excluded from the construct to avoid potential interference with terbium binding. Protein sequences are in the Supporting Information.

Protein Expression and Purification.

Chemically competent *E. coli* cells were transformed with a pMAL vector containing either unmodified MBP (MBP-c) or MBP with a C-terminal extension including the sensor sequence from the peptide pNO₂-E₉E₁₆Y12 (MBP-pNO₂). Proteins were expressed in 1 L Terrific Broth with ampicillin at 37 °C and induced with IPTG. Cells were harvested, resuspended, and lysed by sonication. Proteins were purified using amylose resin and concentrated by lyophilization and ultracentrifugation. Experimental details are in the Supporting Information.

Tyrosine Nitration.

Tyrosine nitration was accomplished by reaction with peroxynitrite. Peroxynitrite was synthesized using a procedure modified from references ^{73, 74}, via the reaction of NaNO₂ and H₂O₂. Unless noted otherwise, a solution of 50 μM peptide or protein in nitration buffer²⁹ (100 mM K₂HPO₄, 25 mM NaHCO₃, 0.2 mM EDTA, pH 7.4) was chilled on ice, peroxynitrite was added to a final concentration of 300 μM, and the solution was immediately vortexed for 30–60 seconds. Peptide with ¹⁵NO₂-labeled nitrotyrosine was prepared using Na¹⁵NO₂ (Cambridge Isotope Labs). Additional details, including the experimental setup used for the efficient generation of peroxynitrite in high yield, are in the Supporting Information.

Results and Discussion

The anionic form of nitrotyrosine contains three oxygens bearing negative charge, including the phenolate oxygen and the two nitro group oxygens. In the design of a protein motif that is specifically responsive to tyrosine nitration, we sought to take advantage of the substantial chemical difference between a neutral phenol and an anionic nitrophenolate. One potential basis for structural differences as a result of nitration is via the binding of the tyrosine side chain to an oxophilic metal. A nitrophenolate has three negatively charged oxygens and a geometry capable of bidentate interactions with a metal. In contrast, a neutral phenol inherently has more modest metal affinity and is only capable of monodentate interactions with the metal.

EF Hand proteins are central mediators of cellular signaling due to calcium.^{75–77} In addition to calcium, EF Hand proteins and peptides bind lanthanides (generically Ln(III)), including the luminescent lanthanide terbium (Tb³⁺).^{78–80} The highly oxophilic nature of lanthanides^{72, 81–90} makes them ideal as a basis for the design of protein motifs in which metal binding is dependent on tyrosine nitration.⁹¹ EF-Hand proteins bind Ca²⁺ and Tb³⁺ via 12-residue calcium-binding motifs, in which metal binding occurs through residues at positions 1, 3, 5, 7, 9, and 12 of the motif (Figure 2).^{76, 92–95} Residues 1, 3, and 5 are typically Asp or Asn, which bind the metal via one side chain oxygen. Residue 7 binds the metal via its main-chain carbonyl. In native and designed EF Hand motifs that exhibit terbium luminescence, a Trp at residue 7 both binds the metal via its main chain carbonyl and sensitizes terbium luminescence due to energy transfer from the indole side chain to the nearby peptide-bound terbium, resulting in terbium emission (observed as fluorescence) at 544 nm.^{80, 96, 97} Residue 9 in a canonical EF Hand typically binds the metal via a water-

mediated metal contact by the side chain (Asp, Ser, Thr). Finally, residue 12 is predominantly a Glu, which binds the metal via a bidentate interaction involving both carboxylate oxygens. A similar sequence motif was also recently identified in a native lanthanide-binding protein, lanmodulin, which contains anionic ligands at all 5 side-chain metal-coordinating residues in the EF-Hand-like structure.^{72, 89, 90}

The conserved, bidentate interaction with metal by Glu12 renders that position particularly favorable as a site of modification for PTM-responsive protein design. We have previously demonstrated that phosphoserine, phosphothreonine, phosphotyrosine, or glutathionylated cysteine can mimic Glu12, bind terbium, and exhibit robust terbium luminescence, including in the presence of physiological concentrations of Ca^{2+} and Mg^{2+} .^{98–101} In contrast, unmodified Ser, Thr, Tyr, or Cys poorly mimic Glu and exhibit substantially weaker terbium binding and terbium luminescence.

Here, we envisioned that nitrotyrosine, by nature of its anionic and oxygen-rich side chain, could function to mimic Glu12 in an EF-Hand motif (Figure 3). Indeed, nitrotyrosine inherently exhibits lanthanide affinity.⁹¹ Thus, incorporation of Tyr in place of Glu at residue 12 would result in modest lanthanide binding, as has been observed previously in the design of protein motifs responsive to tyrosine phosphorylation.^{86, 99, 102–104} In contrast, tyrosine nitration would result in significantly greater lanthanide affinity in the designed redox-responsive protein motif.

As proof of concept, we designed an initial peptide which contains an optimized N-terminal proto-terbium-binding motif (DKDADGW, residues 1–7), which is necessary but not sufficient for lanthanide binding.^{93, 94, 99–101} Residues 8–14 were included from a previous design of a protein motif responsive to tyrosine phosphorylation,⁹⁹ with residue 12 included as a site of tyrosine nitration, with the combined design elements yielding the peptide pNO₂-Y12.

Fmoc-3-Nitrotyrosine is commercially available as an amino acid without a hydroxyl side chain protecting group. While this unprotected amino acid has been used extensively in peptide synthesis, the presence of the nitrotyrosine phenolate under amide coupling conditions is likely to yield a substantial number of side products. In order to improve the synthesis of peptides containing nitrotyrosine, multiple protecting group strategies for the nitrotyrosine phenol were considered. Previously, nitrotyrosine has been protected using trityl, benzyl, or acetyl protecting groups.^{47, 105–107} However, the trityl group on nitrotyrosine has been observed to be labile under standard solid-phase peptide synthesis conditions.⁴⁷ Alternatively, the installation of a benzyl protecting group involved multiple solution-phase synthesis and purification steps and only modestly improved yields of longer peptides containing nitrotyrosine.¹⁰⁷

In order to allow the use of commercially available protected amino acids, we considered *in situ* strategies for protection of the nitrotyrosine side chain.¹⁰⁸ Fmoc-3-Nitrotyrosine was incorporated using standard Fmoc solid-phase peptide synthesis protocols, coupling using 2–3 equivalents each of Fmoc-3-nitrotyrosine and HBTU. After amide coupling, the side chain phenol was protected as an acetate ester^{105, 106} using acetic anhydride (Scheme S1) or as a

silyl ether using TBSCl (Scheme 1).¹⁰⁸ After *in situ* side chain protection, the remainder of the peptide was synthesized by standard solid-phase peptide synthesis. The silyl protecting group was stable to continued peptide synthesis, but was not stable to standard N-terminal acetylation with acetic anhydride. Therefore, N-terminal acetylation on the peptide with TBS-protected nitrotyrosine was accomplished by direct amide coupling using acetic acid and DIC (page S3). Global deprotection and cleavage from resin was accomplished by standard approaches with TFA. The peptide with acetyl protection then required an additional acetyl deprotection step with LiOH. In contrast, the TBS group is removed cleanly under standard TFA cleavage/deprotection conditions.

The purities of the peptides obtained via the acetyl and silyl protecting group strategies were compared (Figure S1). Notably, peptides synthesized using the acetyl protecting group exhibited a substantially larger number of impurities, including evidence of both acyl transfer and incomplete acetyl deprotection. In contrast, the peptide synthesized with a silyl protecting group exhibited a substantially cleaner HPLC chromatogram, indicating high chemical yield in synthesis and efficient silyl deprotection under standard TFA cleavage/deprotection conditions. Combining these factors with the use of a commercially available amino acid and the absence of a need to synthesize the protected amino acid using solution-phase chemistry, nitrotyrosine protection via *in situ* silyl protection both is highly practical and appears to yield peptides of higher yield than prior approaches, suggesting its general application for the synthesis of peptides containing nitrotyrosine.

Peptides containing tyrosine, nitrotyrosine, or phosphorylated tyrosine at residue 12 were examined by fluorescence spectroscopy. The peptide containing nitrotyrosine (pNO₂-Y^{NO₂}12) exhibited a reduction in terbium luminescence compared to the peptide containing either unmodified tyrosine (pNO₂-Y12) or phosphotyrosine (pNO₂-Y^{OPO₃²⁻}12) (Figure 4). Notably, the phosphorylated peptide pNO₂-Y^{OPO₃²⁻}12 exhibited significantly greater terbium affinity compared to the peptide with unmodified tyrosine (pNO₂-Y12) (Figure 4, Table 1). Nitrotyrosine is a highly efficient quencher of fluorescence.^{109–111} The observation of a loss in terbium luminescence of the nitrated peptide compared to the peptide with unmodified tyrosine thus suggested metal binding by pNO₂-Y^{NO₂}12.

In order to quantify the terbium affinities of peptides containing nitrotyrosine, competition binding experiments were conducted using the phosphorylated peptide pNO₂-Y^{OPO₃²⁻}12, which binds terbium with 19-fold greater affinity than the peptide with unmodified tyrosine (Figure 4b, Table 1). The competition experiments revealed that the nitrated peptide pNO₂-Y^{NO₂}12 exhibited a substantial increase in terbium affinity compared to the peptide with unmodified tyrosine (Table 1, Figure 5, Figure S8). In addition, the nitrated peptide had modestly greater terbium affinity than the phosphorylated peptide. The competition experiments also indicated the substantial inherent terbium affinity of nitrotyrosine,⁹¹ via analysis of the terbium affinity of the model peptide Ac-T(3-NO₂-Tyr)PN-NH₂,¹¹² which lacks other anionic groups that would promote lanthanide binding (Table 1, Figure S7).

These experiments indicated that nitrotyrosine at residue 12 significantly increased terbium affinity in an EF-Hand-derived peptide, suggesting a mode of binding similar to that in an EF Hand, with nitrotyrosine mimicking the conserved Glu12. In addition, NMR experiments

on pNO₂-Y^{NO2}12 with the diamagnetic lanthanide lanthanum(III) (La³⁺) indicated significant chemical shift changes of the peptide upon binding La³⁺ (Figures S11, S12, S15–S18). Most significantly, incubation of the peptide pNO₂-Y^{NO2}12 with the lanthanide cerium(III) (Ce³⁺) provided direct evidence of the metal being in close proximity both to the aspartates and to the nitrotyrosine side chain (Figures S14–S15), whereby the unpaired electron in cerium results in broadening or loss of signals of the side chains of these residues due to paramagnetic shifts.

However, the nitrophenolate group could potentially increase terbium affinity through longer-range electrostatic interactions that do not involve direct metal binding. In order to obtain direct evidence of metal binding via the nitro group of nitrotyrosine in the peptide pNO₂-Y^{NO2}12, a variant of the peptide was synthesized with the nitro group isotopically labeled with ¹⁵N. ¹⁵N-NO₂-labeled pNO₂-Y^{NO2}12 was synthesized via reaction of pNO₂-Y12 with ¹⁵N-peroxynitrite, prepared from ¹⁵N-sodium nitrite.⁵⁵ This peptide was analyzed by NMR spectroscopy, in the absence and presence of La³⁺ (Figure 6). H² of the nitrotyrosine ring exhibits ³J coupling to the nitrogen of the nitro group (³J ~ 2 Hz), allowing for indirect detection of the ¹⁵N resonance via ¹H-¹⁵N HMBC spectroscopy. These data revealed a large 1.3 ppm change in ¹⁵N chemical shift of the nitro group upon metal binding, consistent with the direct involvement of the nitro group in metal binding.

The work above describes the development of a small, encodable protein motif whose structure and metal binding depend on tyrosine nitration. In order to develop a fluorescent sensor of tyrosine nitration, however, a significant change in fluorescence would need to be observed as a result of tyrosine nitration. Thus, the peptide pNO₂-Y^{NO2}12 was suboptimal as a sensor of tyrosine nitration, due to the weak terbium affinity and terbium luminescence of the non-nitrated form of the peptide, pNO₂-Y12. In order to develop an effective sensor of tyrosine nitration, with maximum dynamic range, the protein motif would ideally exhibit increased affinity and terbium luminescence of the non-nitrated peptide. In that case, tyrosine nitration would result in a switch from one metal-bound structure that exhibits substantial terbium luminescence to an alternative metal-bound structure of the nitrated peptide that quenches terbium luminescence.

In the design of a non-nitrated peptide with increased terbium affinity, two positions were considered for the introduction of an additional chelating group. Glu residues at position 9 of an EF Hand can significantly increase terbium affinity, as has been observed in multiple EF-Hand peptide contexts, including Imperiali's design of optimized lanthanide-binding tags.^{80, 96, 97} In addition, in some EF Hand proteins, residue 16 is a Glu which can replace the Glu at residue 12. Therefore, peptides were synthesized (Figure 2) with either Glu9 or Glu16 in combination with Tyr12, and the peptides analyzed for terbium affinity and terbium luminescence in both non-nitrated and nitrated forms (Figure 5bc, Figures S5–S6 and S9–S10). The data revealed (Table 1) that both modifications resulted in increased terbium affinity of the nitrated peptide compared to pNO₂-Y^{NO2}12. In addition, analysis of the non-nitrated peptides indicated that the peptide with Glu at residue 9 exhibited increased terbium affinity compared to the peptide lacking Glu9.

In order to further optimize the design, peptides were examined in which both Glu9 and Glu16 were incorporated (pNO₂-E₉E₁₆Y12). Direct terbium binding experiments indicated that the non-nitrated peptide exhibited increased terbium affinity over designs lacking one or both Glu residues (Figure 4b, Table 1). In addition, competition terbium binding experiments (Figure 5d, Table 1, Figure S11) indicated that the nitrated peptide pNO₂-E₉E₁₆Y^{NO2}12 exhibited the highest terbium affinity of all nitrated peptides examined herein, as well as the largest differentiation in metal binding between the non-nitrated and nitrated forms of the peptide (180-fold greater terbium affinity for the nitrated peptide).

The optimized nitrated peptide pNO₂-E₉E₁₆Y^{NO2}12 was further examined by NMR spectroscopy, in order to identify whether the mode of metal binding was still similar to that in an EF-Hand protein. Titration of pNO₂-E₉E₁₆Y^{NO2}12 with the diamagnetic lanthanide La³⁺ (Figure 7) indicated chemical shift changes in the resonances of all nitrotyrosine aromatic hydrogens as a function of metal concentration. Substantial changes in ¹³C chemical shift were also observed on addition of La³⁺ for the nitrotyrosine aromatic carbons (Figure S26). In addition, significant changes were observed in the chemical shifts of the Asp C β hydrogens (Figure S20–S22), as expected if these residues are binding the metal in a manner similar to that in an EF Hand.

Further evidence for an EF-Hand-like structure of the metal-bound peptide pNO₂-E₉E₁₆Y^{NO2}12 was obtained via analysis of the NMR spectra of the peptide in the presence and absence of Ce³⁺. Paramagnetism via the single unpaired electron in Ce³⁺ provides evidence of hydrogens in close proximity to the metal. These data (Figure 8) indicated the loss of resonances throughout the EF-Hand loop. In contrast, resonances associated with Glu16 were still observed in the presence of Ce³⁺, consistent with the design principles. Collectively, the terbium luminescence and NMR spectroscopy data indicate that the peptide pNO₂-E₉E₁₆Y^{NO2}12 binds metal via an EF-Hand-like structure, employing N-terminal Asp residues and the nitrotyrosine at residue 12 as the metal binding elements, as designed.

In order to examine the application of the designed protein motif to detect tyrosine nitration, the sequence of the peptide pNO₂-E₉E₁₆Y12 was incorporated as a C-terminal tag on maltose-binding protein (MBP), as proof of principle for the employment of this sequence as an encodable sensor of tyrosine nitration. This protein (MBP-pNO₂), plus a control protein lacking the nitration-responsive sequence (MBP-c), were expressed in *E. coli*. Tyrosine nitration is dependent on the tyrosine residues being solvent exposed. In addition, the extent of tyrosine nitration in proteins is increased by the presence of nearby acidic residues.¹¹³ Therefore, the incorporation of the sensor sequence in a solvent-exposed loop combined with the nearby carboxylates from the pNO₂-E₉E₁₆Y12 sequence was expected to promote tyrosine nitration within the expressed protein construct containing the designed sequence.

MBP-pNO₂ and MBP-c were examined for responsiveness to tyrosine nitration. The proteins (50 μ M) were incubated in the absence of peroxynitrite and with 150 μ M or 450 μ M added peroxynitrite, and the terbium luminescence subsequently measured as a function of added peroxynitrite (Figure 9). MBP-pNO₂ exhibited robust terbium luminescence in the absence of peroxynitrite and a substantial reduction in terbium luminescence that corresponded with the concentration of added peroxynitrite. Notably, under conditions of

limiting terbium, MBP-pNO₂ exhibited a greater reduction in terbium luminescence than the extent of nitration (0.22 ± 0.02 and 0.62 ± 0.08 tyrosines nitrated per protein at 150 μ M and 450 μ M peroxyntirite, respectively) because of the significantly greater terbium affinity of the nitrated protein sequence compared to that of the non-nitrated sequence (due to the linked equilibria that promote metal binding to the nitrated over the non-nitrated protein, discussed in the Supporting Information, Figure S4 and pages S12–S13). These data indicate high sensitivity of this protein design even to low stoichiometry of tyrosine nitration, which is commonly observed in cellular studies and *in vivo*. In contrast, the control protein MBP-c exhibited weak terbium luminescence both in the absence and presence of added peroxyntirite, with only a small overall change in terbium luminescence. MBP-c contains 15 tyrosine residues, many solvent-exposed, but exhibited a significantly lower extent of nitration than MBP-pNO₂, even with 450 μ M added peroxyntirite (0.41 ± 0.06 tyrosines nitrated per protein). Collectively, these data indicate that terbium binding and terbium luminescence in the MBP fusion protein were dependent on the added sequence from pNO₂-E₉E₁₆Y12, and that specific nitration of the tyrosine in the added EF-Hand loop led to the quenching of terbium luminescence, with the extent of reduction in fluorescence dependent on the concentration of added peroxyntirite and thus the extent of tyrosine nitration. These results, within an encoded protein, suggest the future application of this approach to a range of outstanding questions in tyrosine nitration.

Conclusion

We have developed a small protein motif (14–18 amino acids) whose structure is dependent on tyrosine nitration. In the design, nitrotyrosine replaces Glu12 as a critical residue of an EF-Hand motif. Lanthanide binding is responsive to tyrosine nitration. NMR spectroscopy provided evidence consistent with the peptides adopting metal coordination similar to that of a canonical EF Hand, including binding via side chain aspartates and direct metal binding via the nitrophenolate of the nitrotyrosine. This design approach was applied to develop an encoded turn-off fluorescence-based sensor of tyrosine nitration. The turn-off sensor employed glutamate residues at EF Hand positions 9 and 16. These glutamates increased the terbium affinity of the EF-Hand motif, resulting in terbium luminescence of the non-nitrated peptide motif. Tyrosine nitration resulted in up to a 180-fold increase in terbium affinity of the nitrated peptide, which quenched terbium luminescence. The encoded protein motif exhibited fluorescence that was quenched as a function of the concentration of added peroxyntirite. In addition, in this work, a new silyl protecting group strategy was developed to improve the synthesis of peptides containing nitrotyrosine. Tyrosine nitration has been observed in a diverse range of diseases and normal physiological states, with substantial open questions about the extent, importance, and reversibility of tyrosine nitration. The design of a protein motif dependent on tyrosine nitration that was developed herein provides a novel approach to address key challenges in understanding tyrosine nitration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

- [1]. Ferrer-Sueta G, Campolo N, Trujillo M, Bartesaghi S, Carballa S, Romero N, Alvarez B, and Radi R (2018) Biochemistry of Peroxynitrite and Protein Tyrosine Nitration, *Chem. Rev* 118, 462–1408.
- [2]. Bottari SP (2015) Protein tyrosine nitration: A signaling mechanism conserved from yeast to man, *Proteomics* 15, 185–187. [PubMed: 25529898]
- [3]. Aicardo A, Martinez DM, Campolo N, Bartesaghi S, and Radi R (2016) Biochemistry of Nitric Oxide and Peroxynitrite: Sources, Targets and Biological Implications, In *Biochemistry of Oxidative Stress: Physiopathology and Clinical Aspects*, pp 49–77.
- [4]. Gunaydin H, and Houk KN (2009) Mechanisms of Peroxynitrite-Mediated Nitration of Tyrosine, *Chem. Res. Toxicol* 22, 894–898. [PubMed: 19374346]
- [5]. Houee-Levin C, Bobrowski K, Horakova L, Karademir B, Schoneich C, Davies MJ, and Spickett CM (2015) Exploring oxidative modifications of tyrosine: An update on mechanisms of formation, advances in analysis and biological consequences, *Free Radic. Res* 49, 347–373. [PubMed: 25812585]
- [6]. Szabo C, Ischiropoulos H, and Radi R (2007) Peroxynitrite: biochemistry, pathophysiology and development of therapeutics, *Nat. Rev. Drug Discov* 6, 662–680. [PubMed: 17667957]
- [7]. Ischiropoulos H (2009) Protein tyrosine nitration-An update, *Arch. Biochem. Biophys* 484, 117–121. [PubMed: 19007743]
- [8]. Peluffo G, and Radi R (2007) Biochemistry of protein tyrosine nitration in cardiovascular pathology, *Cardiovasc. Res* 75, 291–302. [PubMed: 17544386]
- [9]. Shi WQ, Cai H, Xu DD, Su XY, Lei P, Zhao YF, and Li YM (2007) Tyrosine phosphorylation/dephosphorylation regulates peroxynitrite-mediated peptide nitration, *Regul. Pept* 144, 1–5. [PubMed: 17669515]
- [10]. Moldogazieva NT, Mokhosoev IM, Feldman NB, and Lutsenko SV (2018) ROS and RNS signalling: adaptive redox switches through oxidative/nitrosative protein modifications, *Free Radic. Res* 52, 507–543. [PubMed: 29589770]
- [11]. He YX, Yu ZW, and Chen SD (2019) Alpha-Synuclein Nitration and Its Implications in Parkinson's Disease, *ACS Chem. Neurosci* 10, 777–782. [PubMed: 30183251]
- [12]. Horiguchi T, Uryu K, Giasson BI, Ischiropoulos H, Lightfoot R, Bellmann C, Richter-Landsberg C, Lee VMY, and Trojanowski JQ (2003) Nitration of tau protein is linked to neurodegeneration in tauopathies, *Am. J. Pathol* 163, 1021–1031. [PubMed: 12937143]
- [13]. Hodara R, Norris EH, Giasson BI, Mishizen-Eberz AJ, Lynch DR, Lee VMY, and Ischiropoulos H (2004) Functional consequences of alpha-synuclein tyrosine nitration - Diminished binding to lipid vesicles and increased fibril formation, *J. Biol. Chem* 279, 47746–47753. [PubMed: 15364911]
- [14]. Vadseth C, Souza JM, Thomson L, Seagraves A, Nagaswami C, Scheiner T, Torbet J, Vilaire G, Bennett JS, Murciano JC, Muzykantov V, Penn MS, Hazen SL, Weisel JW, and Ischiropoulos H (2004) Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species, *J. Biol. Chem* 279, 8820–8826. [PubMed: 14681238]
- [15]. Mishizen-Eberz AJ, Norris EH, Giasson BI, Hodara R, Ischiropoulos H, Lee VMY, Trojanowski JQ, and Lynch DR (2005) Cleavage of alpha-synuclein by calpain: Potential role in degradation of fibrillized and nitrated species of alpha-synuclein, *Biochemistry* 44, 7818–7829. [PubMed: 15909996]
- [16]. Parastatidis I, Thomson L, Burke A, Chernysh I, Nagaswami C, Visser J, Stamer S, Liebler DC, Koliakos G, Heijnen HFG, FitzGerald GA, Weisel JW, and Ischiropoulos H (2008) Fibrinogen beta-Chain Tyrosine Nitration Is a Prothrombotic Risk Factor, *J. Biol. Chem* 283, 33846–33853. [PubMed: 18818200]

- [17]. Martinez M, Cuker A, Mills A, Lightfoot R, Fan YY, Tang WHW, Hazen SL, and Ischiropoulos H (2012) Nitrated fibrinogen is a biomarker of oxidative stress in venous thromboembolism, *Free Radic. Biol. Med* 53, 230–236. [PubMed: 22580301]
- [18]. Thomson L, Tenopoulou M, Lightfoot R, Tsika E, Parastatidis I, Martinez M, Greco TM, Doulias PT, Wu YP, Tang WHW, Hazen SL, and Ischiropoulos H (2012) Immunoglobulins Against Tyrosine-Nitrated Epitopes in Coronary Artery Disease, *Circulation* 126, 2392–+. [PubMed: 23081989]
- [19]. Martinez M, Weisel JW, and Ischiropoulos H (2013) Functional impact of oxidative posttranslational modifications on fibrinogen and fibrin clots, *Free Radic. Biol. Med* 65, 411–418. [PubMed: 23851017]
- [20]. Feeney MB, and Schoneich C (2012) Tyrosine Modifications in Aging, *Antioxid. Redox Signal* 17, 1571–1579. [PubMed: 22424390]
- [21]. Moldogazieva NT, Lutsenko SV, and Terentiev AA (2018) Reactive Oxygen and Nitrogen Species-Induced Protein Modifications: Implication in Carcinogenesis and Anticancer Therapy, *Cancer Res* 78, 6040–6047. [PubMed: 30327380]
- [22]. Drechsel DA, Estevez AG, Barbeito L, and Beckman JS (2012) Nitric Oxide-Mediated Oxidative Damage and the Progressive Demise of Motor Neurons in ALS, *Neurotox. Res* 22, 251–264. [PubMed: 22488161]
- [23]. Surmeli NB, Litterman NK, Miller AF, and Groves JT (2010) Peroxynitrite Mediates Active Site Tyrosine Nitration in Manganese Superoxide Dismutase. Evidence of a Role for the Carbonate Radical Anion, *J. Am. Chem. Soc* 132, 17174–17185. [PubMed: 21080654]
- [24]. Demicheli V, Moreno DM, Jara GE, Lima A, Carballal S, Rios N, Batthyany C, Ferrer-Sueta G, Quijano C, Estrin DA, Marti MA, and Radi R (2016) Mechanism of the Reaction of Human Manganese Superoxide Dismutase with Peroxynitrite: Nitration of Critical Tyrosine 34, *Biochemistry* 55, 3403–3417. [PubMed: 27227512]
- [25]. Gao HM, Kotzbauer PT, Uryu K, Leight S, Trojanowski JQ, and Lee VMY (2008) Neuroinflammation and oxidation/nitration of alpha-synuclein linked to dopaminergic neurodegeneration, *J. Neurosci* 28, 7687–7698. [PubMed: 18650345]
- [26]. Danielson SR, Held JM, Schilling B, Oo M, Gibson BW, and Andersen JK (2009) Preferentially Increased Nitration of alpha-Synuclein at Tyrosine-39 in a Cellular Oxidative Model of Parkinson's Disease, *Anal. Chem* 81, 7823–7828. [PubMed: 19697948]
- [27]. Yu ZW, Xu XH, Xiang ZH, Zhou JF, Zhang ZH, Hu C, and He C (2010) Nitrated alpha-Synuclein Induces the Loss of Dopaminergic Neurons in the Substantia Nigra of Rats, *PLoS One* 5.
- [28]. Burai R, Ait-Bouziad N, Chiki A, and Lashuel HA (2015) Elucidating the Role of Site-Specific Nitration of alpha-Synuclein in the Pathogenesis of Parkinson's Disease via Protein Semisynthesis and Mutagenesis, *J. Am. Chem. Soc* 137, 5041–5052. [PubMed: 25768729]
- [29]. Reynolds MR, Berry RW, and Binder LI (2005) Site-specific nitration and oxidative dityrosine bridging of the tau protein by peroxynitrite: Implications for Alzheimer's disease, *Biochemistry* 44, 1690–1700. [PubMed: 15683253]
- [30]. Reynolds MR, Reyes JF, Fu YF, Bigio EH, Guillozet-Bongaarts AL, Berry RW, and Binder LI (2006) Tau nitration occurs at tyrosine 29 in the fibrillar lesions of Alzheimer's disease and other tauopathies, *J. Neurosci* 26, 10636–10645. [PubMed: 17050703]
- [31]. Vana L, Kanaan NM, Hakala K, Weintraub ST, and Binder LI (2011) Peroxynitrite-Induced Nitrative and Oxidative Modifications Alter Tau Filament Formation, *Biochemistry* 50, 1203–1212. [PubMed: 21210655]
- [32]. Viera L, Radmilovich M, Vargas MR, Dennys CN, Wilson L, Barnes S, Franco MC, Beckman JS, and Estevez AG (2013) Temporal patterns of tyrosine nitration in embryo heart development, *Free Radic. Biol. Med* 55, 101–108. [PubMed: 23195686]
- [33]. Radi R (2018) Oxygen radicals, nitric oxide, and peroxynitrite: Redox pathways in molecular medicine, *Proc. Natl. Acad. Sci. U.S.A* 115, 5839–5848. [PubMed: 29802228]
- [34]. Adams L, Franco MC, and Estevez AG (2015) Reactive nitrogen species in cellular signaling, *Exp. Biol. Med* 240, 711–717.

- [35]. Bogdan C (2015) Nitric oxide synthase in innate and adaptive immunity: an update, *Trends Immunol* 36, 161–178. [PubMed: 25687683]
- [36]. Pacher P, Beckman JS, and Liaudet L (2007) Nitric Oxide and Peroxynitrite in Health and Disease, *Physiol. Rev* 87, 315–424. [PubMed: 17237348]
- [37]. Batthyany C, Souza JM, Duran R, Cassina A, Cervenansky C, and Radi R (2005) Time course and site(s) of cytochrome c tyrosine nitration by peroxynitrite, *Biochemistry* 44, 8038–8046. [PubMed: 15924423]
- [38]. Aggarwal S, Gross CM, Rafikov R, Kumar S, Fineman JR, Ludewig B, Jonigk D, and Black SM (2014) Nitration of Tyrosine 247 Inhibits Protein Kinase G-1 alpha Activity by Attenuating Cyclic Guanosine Monophosphate Binding, *J. Biol. Chem* 289, 7948–7961. [PubMed: 24469460]
- [39]. Garcia-Heredia JM, Diaz-Moreno I, Nieto PM, Orzaez M, Kocanis S, Teixeira M, Perez-Paya E, Diaz-Quintana A, and De la Rosa MA (2010) Nitration of tyrosine 74 prevents human cytochrome c to play a key role in apoptosis signaling by blocking caspase-9 activation, *BBA-Bioenergetics* 1797, 981–993. [PubMed: 20227384]
- [40]. Yang JJ, Zhang RH, Lu GM, Shen Y, Peng L, Zhu C, Cui M, Wang WD, Arnaboldi P, Tang M, Gupta M, Qi CF, Jayaraman P, Zhu HF, Jiang B, Chen SH, He JCJ, Ting AT, Zhou MM, Kuchroo VK, Morse HC, Ozato K, Sikora AG, and Xiong HB (2013) T cell-derived inducible nitric oxide synthase switches off T(H)17 cell differentiation, *J. Exp. Med* 210, 1447–1462. [PubMed: 23797094]
- [41]. Ji YB, Neverova I, Van Eyk JE, and Bennett BM (2006) Nitration of tyrosine 92 mediates the activation of rat microsomal glutathione S-transferase by peroxynitrite, *J. Biol. Chem* 281, 1986–1991. [PubMed: 16314419]
- [42]. Zaragoza R, Torres L, Garcia C, Eroles P, Corrales F, Bosch A, Lluch A, Garcia-Trevijano ER, and Vina JR (2009) Nitration of cathepsin D enhances its proteolytic activity during mammary gland remodelling after lactation, *Biochem. J* 419, 279–288. [PubMed: 19125694]
- [43]. Franco MC, Ye YZ, Refakis CA, Feldman JL, Stokes AL, Basso M, de Mera R, Sparrow NA, Calingasan NY, Kiaei M, Rhoads TW, Ma TC, Grumet M, Barnes S, Beal MF, Beckman JS, Mehl R, and Estevez AG (2013) Nitration of Hsp90 induces cell death, *Proc. Natl. Acad. Sci. U.S.A* 110, E1102–E1111. [PubMed: 23487751]
- [44]. Randall LM, Manta B, Hugo M, Gil M, Batthyany C, Trujillo M, Poole LB, and Denicola A (2014) Nitration Transforms a Sensitive Peroxiredoxin 2 into a More Active and Robust Peroxidase, *J. Biol. Chem* 289, 15536–15543. [PubMed: 24719319]
- [45]. Kolodziejczyk-Czepas J, Ponczek MB, and Nowak P (2015) Peroxynitrite and fibrinolytic system-The effects of peroxynitrite on t-PA-induced plasmin activity, *Int. J. Biol. Macromol* 81, 212–219. [PubMed: 26234576]
- [46]. Mallozzi C, Di Stasi AMM, and Minetti M (2001) Nitrotyrosine mimics phosphotyrosine binding to the SH2 domain of the src family tyrosine kinase lyn, *FEBS Lett* 503, 189–195. [PubMed: 11513880]
- [47]. Song YL, Peach ML, Roller PP, Qiu S, Wang SM, and Long YQ (2006) Discovery of a novel nonphosphorylated pentapeptide motif displaying high affinity for Grb2-SH2 domain by the utilization of 3 '-substituted tyrosine derivatives, *J. Med. Chem* 49, 1585–1596. [PubMed: 16509576]
- [48]. Souza JM, Peluffo G, and Radi R (2008) Protein tyrosine nitration - Functional alteration or just a biomarker?, *Free Radical Biology and Medicine* 45, 357–366. [PubMed: 18460345]
- [49]. Irie Y, Saeki M, Kamisaki Y, Martin E, and Murad F (2003) Histone H1.2 is a substrate for denitrase, an activity that reduces nitrotyrosine immunoreactivity in proteins, *Proc. Natl. Acad. Sci. U.S.A* 100, 5634–5639. [PubMed: 12719531]
- [50]. Smallwood HS, Lourette NM, Boschek CB, Bigelow DJ, Smith RD, Pasa-Tolic L, and Squier TC (2007) Identification of a denitrase activity against calmodulin in activated macrophages using high-field liquid chromatography - FTICR mass spectrometry, *Biochemistry* 46, 10498–10505. [PubMed: 17711305]

- [51]. Deeb RS, Nuriel T, Cheung C, Summers B, Lamon BD, Gross SS, and Hajjar DP (2013) Characterization of a cellular denitrase activity that reverses nitration of cyclooxygenase, *Am. J. Physiol. Heart Circ. Physiol* 305, H687–H698. [PubMed: 23792683]
- [52]. Butt YKC, and Lo SCL (2008) Detecting nitrated proteins by proteomic technologies, In *Nitric Oxide, Part F: Oxidative and Nitrosative Stress in Redox Regulation of Cell Signaling*, pp 17–31.
- [53]. Reynolds MR, Berry RW, and Binder LI (2005) Site-specific nitration differentially influences tau assembly in vitro, *Biochemistry* 44, 13997–14009. [PubMed: 16229489]
- [54]. Varadarajan N, Pogson M, Georgiou G, and Verson BL (2009) Proteases That Can Distinguish among Different Post-translational Forms of Tyrosine Engineered Using Multicolor Flow Cytometry, *J. Am. Chem. Soc* 131, 18186–18190. [PubMed: 19924991]
- [55]. Seeley KW, Fertig AR, Dufresne CP, Pinho JPC, and Stevens SM (2014) Evaluation of a Method for Nitrotyrosine Site Identification and Relative Quantitation Using a Stable Isotope-Labeled Nitrated Spike-In Standard and High Resolution Fourier Transform MS and MS/MS Analysis, *Int. J. Mol. Sci* 15, 6265–6285. [PubMed: 24736779]
- [56]. Valim LR, Davies JA, Jensen KT, Guo R, Willison KR, Spickett CM, Pitt AR, and Klug DR (2014) Identification and Relative Quantification of Tyrosine Nitration in a Model Peptide Using Two-Dimensional Infrared Spectroscopy, *J. Phys. Chem. B* 118, 12855–12864. [PubMed: 25347525]
- [57]. Yeo WS, Kim YJ, Kabir MH, Kang JW, and Kim KP (2015) Mass Spectrometric Analysis of Protein Tyrosine Nitration in Aging and Neurodegenerative Diseases, *Mass Spectrom. Rev* 34, 166–183. [PubMed: 24889964]
- [58]. Batthyany C, Bartesaghi S, Mastrogiovanni M, Lima A, Demicheli V, and Radi R (2017) Tyrosine-Nitrated Proteins: Proteomic and Bioanalytical Aspects, *Antioxid. Redox Signal* 26, 313–U383. [PubMed: 27324931]
- [59]. Tsikas D (2017) What we-authors, reviewers and editors of scientific work-can learn from the analytical history of biological 3-nitrotyrosine, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci* 1058, 68–72.
- [60]. Neumann H, Hazen JL, Weinstein J, Mehl RA, and Chin JW (2008) Genetically encoding protein oxidative damage, *J. Am. Chem. Soc* 130, 4028–4033. [PubMed: 18321101]
- [61]. Chen ZJ, Ren W, Wright QE, and Ai HW (2013) Genetically Encoded Fluorescent Probe for the Selective Detection of Peroxynitrite, *J. Am. Chem. Soc* 135, 14940–14943. [PubMed: 24059533]
- [62]. Sun X, Xu Q, Kim G, Flower SE, Lowe JP, Yoon J, Fossey JS, Qian X, Bull SD, and James TD (2014) A water-soluble boronate-based fluorescent probe for the selective detection of peroxynitrite and imaging in living cells, *Chem. Sci* 5, 3368–3373.
- [63]. Cheng D, Pan Y, Wang L, Zeng ZB, Yuan L, Zhang XB, and Chang YT (2017) Selective Visualization of the Endogenous Peroxynitrite in an Inflamed Mouse Model by a Mitochondria-Targetable Two-Photon Ratiometric Fluorescent Probe, *J. Am. Chem. Soc* 139, 285–292. [PubMed: 27996249]
- [64]. Jia XT, Chen QQ, Yang YF, Tang Y, Wang R, Xu YF, Zhu WP, and Qian XH (2016) FRET-Based Mito-Specific Fluorescent Probe for Ratiometric Detection and Imaging of Endogenous Peroxynitrite: Dyad of Cy3 and Cy5, *J. Am. Chem. Soc* 138, 10778–10781. [PubMed: 27517310]
- [65]. Heijnen HFG, van Donselaar E, Slot JW, Fries DM, Blachard-Fillion B, Hodara R, Lightfoot R, Polydoro M, Spielberg D, Thomson L, Regan EA, Crapo J, and Ischiropoulos H (2006) Subcellular localization of tyrosine-nitrated proteins is dictated by reactive oxygen species generating enzymes and by proximity to nitric oxide synthase, *Free Radic. Biol. Med* 40, 1903–1913. [PubMed: 16716892]
- [66]. Marinetti TD, Snyder GH, and Sykes BD (1976) Nuclear Magnetic-Resonance Determination of Intramolecular Distances in Bovine Pancreatic Trypsin-Inhibitor Using Nitrotyrosine Chelation of Lanthanides, *Biochemistry* 15, 4600–4608. [PubMed: 9977]
- [67]. Allegrozzi M, Bertini I, Janik MBL, Lee YM, Lin GH, and Luchinat C (2000) Lanthanide-induced pseudocontact shifts for solution structure refinements of macromolecules in shells up to 40 angstrom from the metal ion, *J. Am. Chem. Soc* 122, 4154–4161.

- [68]. Martin LJ, Hahnke MJ, Nitz M, Wohnert J, Silvaggi NR, Allen KN, Schwalbe H, and Imperiali B (2007) Double-lanthanide-binding tags: Design, photophysical properties, and NMR applications, *J. Am. Chem. Soc* 129, 7106–7113. [PubMed: 17497862]
- [69]. Bertini I, Gelis I, Katsaros N, Luchinat C, and Provenzano A (2003) Tuning the affinity for lanthanides of calcium binding proteins, *Biochemistry* 42, 8011–8021. [PubMed: 12834353]
- [70]. am Ende CW, Meng HY, Ye M, Pandey AK, and Zondlo NJ (2010) Design of Lanthanide Fingers: Compact Lanthanide-Binding Metalloproteins, *ChemBioChem* 11, 1738–1747. [PubMed: 20623571]
- [71]. Cheisson T, and Schelter EJ (2019) Rare earth elements: Mendeleev's bane, modern marvels, *Science* 363, 489–493. [PubMed: 30705185]
- [72]. Cotruvo JA, Featherston ER, Mattocks JA, Ho JV, and Laremore TN (2018) Lanmodulin: A Highly Selective Lanthanide-Binding Protein from a Lanthanide-Utilizing Bacterium, *J. Am. Chem. Soc* 140, 15056–15061. [PubMed: 30351021]
- [73]. Beckman JS, Chen J, Ischiropoulos H, and Crow JP (1994) Oxidative Chemistry of Peroxynitrite, *Methods Enzymol* 233, 229–240. [PubMed: 8015460]
- [74]. Reitman I (1984) Ghostbusters
- [75]. Ikura M (1996) Calcium binding and conformational response in EF-hand proteins, *Trends Biochem. Sci* 21, 14–17. [PubMed: 8848832]
- [76]. Nelson MR, and Chazin WJ (1998) Structures of EF-hand Ca^{2+} -binding proteins: Diversity in the organization, packing and response to Ca^{2+} binding, *Biometals* 11, 297–318. [PubMed: 10191495]
- [77]. Lewit-Bentley A, and Rety S (2000) EF-hand calcium-binding proteins, *Curr. Opin. Struct. Biol* 10, 637–643. [PubMed: 11114499]
- [78]. Horrocks WD, and Sudnick DR (1981) Lanthanide ion luminescence probes of the structure of biological macromolecules, *Acc. Chem. Res* 14, 384–392.
- [79]. Richardson FS (1982) Terbium(III) and Europium(III) Ions as Luminescent Probes and Stains for Biomolecular Systems, *Chem. Rev* 82, 541–552.
- [80]. Macmanus JP, Hogue CW, Marsden BJ, Sikorska M, and Szabo AG (1990) Terbium Luminescence in Synthetic Peptide Loops from Calcium-Binding Proteins with Different Energy Donors, *J. Biol. Chem* 265, 10358–10366. [PubMed: 2355005]
- [81]. Selvin PR (2002) Principles and biophysical applications of lanthanide-based probes, *Ann. Rev. Biophys. Biomol. Struct* 31, 275–302. [PubMed: 11988471]
- [82]. Bunzli JCG, and Piguet C (2005) Taking advantage of luminescent lanthanide ions, *Chem. Soc. Rev* 34, 1048–1077. [PubMed: 16284671]
- [83]. Gunnlaugsson T, Glynn M, Tocci GM, Kruger PE, and Pfeffer FM (2006) Anion recognition and sensing in organic and aqueous media using luminescent and colorimetric sensors, *Coord. Chem. Rev* 250, 3094–3117.
- [84]. dos Santos CMG, Fernandez PB, Plush SE, Leonard JP, and Gunnlaugsson T (2007) Lanthanide luminescent anion sensing: evidence of multiple anion recognition through hydrogen bonding and metal ion coordination, *Chem. Comm*, 3389–3391. [PubMed: 18019507]
- [85]. Massue J, Quinn SJ, and Gunnlaugsson T (2008) Lanthanide luminescent displacement assays: The sensing of phosphate anions using Eu(III)-cyclen-conjugated gold nanoparticles in aqueous solution, *J. Am. Chem. Soc* 130, 6900–6901. [PubMed: 18461933]
- [86]. Pazos E, and Vázquez ME (2014) Advances in lanthanide-based luminescent peptide probes for monitoring the activity of kinase and phosphatase, *Biotechnol. J* 9, 241–252. [PubMed: 24323746]
- [87]. Shuvaev S, Starck M, and Parker D (2017) Responsive, Water-Soluble Europium(III) Luminescent Probes, *Chem. Eur. J* 23, 9974–9989. [PubMed: 28471496]
- [88]. Hewitt SH, and Butler SJ (2018) Application of lanthanide luminescence in probing enzyme activity, *Chem. Commun* 54, 6635–6647.
- [89]. Mattocks JA, Ho JV, and Cotruvo JA (2019) A Selective, Protein-Based Fluorescent Sensor with Picomolar Affinity for Rare Earth Elements, *J. Am. Chem. Soc* 141, 2857–2861. [PubMed: 30726674]

- [90]. Cook EC, Featherston ER, Showalter SA, and Cotruvo JA (2019) Structural Basis for Rare Earth Element Recognition by *Methylobacterium extorquens* Lanmodulin, *Biochemistry* 58, 120–125. [PubMed: 30352145]
- [91]. Marinetti TD, Snyder GH, and Sykes BD (1975) Lanthanide Interactions with Nitrotyrosine - Specific Binding-Site for Nuclear Magnetic-Resonance Shift Probes in Proteins, *J. Am. Chem. Soc* 97, 6562–6570. [PubMed: 1184871]
- [92]. Chattopadhyaya R, Meador WE, Means AR, and Quioco FA (1992) Calmodulin Structure Refined at 1.7 Angstrom Resolution, *J. Mol. Biol* 228, 1177–1192. [PubMed: 1474585]
- [93]. Rigden DJ, and Galperin MY (2004) The DxDxDG motif for calcium binding: Multiple structural contexts and implications for evolution, *J. Mol. Biol* 343, 971–984. [PubMed: 15476814]
- [94]. Rigden DJ, Woodhead DD, Wong PWH, and Galperin MY (2011) New Structural and Functional Contexts of the DxD[DN]xDG Linear Motif: Insights into Evolution of Calcium-Binding Proteins, *PLoS One* 6, e21507. [PubMed: 21720552]
- [95]. Zhou YB, Xue SH, and Yang JJ (2013) Calciomics: integrative studies of Ca²⁺-binding proteins and their interactomes in biological systems, *Metallomics* 5, 29–42. [PubMed: 23235533]
- [96]. Nitz M, Franz KJ, Maglathlin RL, and Imperiali B (2003) A powerful combinatorial screen to identify high-affinity terbium(III)-binding peptides, *ChemBioChem* 4, 272–276. [PubMed: 12672106]
- [97]. Nitz M, Sherawat M, Franz KJ, Peisach E, Allen KN, and Imperiali B (2004) Structural origin of the high affinity of a chemically evolved lanthanide-binding peptide, *Angew. Chem., Int. Ed* 43, 3682–3685.
- [98]. Balakrishnan S, and Zondlo NJ (2006) Design of a Protein Kinase-Inducible Domain, *J. Am. Chem. Soc* 128, 5590–5591. [PubMed: 16637598]
- [99]. Zondlo SC, Gao F, and Zondlo NJ (2010) Design of an Encodable Tyrosine Kinase-Inducible Domain: Detection of Tyrosine Kinase Activity by Terbium Luminescence, *J. Am. Chem. Soc* 132, 5619–5621. [PubMed: 20361796]
- [100]. Scheuermann MJ, Forbes CR, and Zondlo NJ (2018) Redox-Responsive Protein Design: Design of a Small Protein Motif Dependent on Glutathionylation, *Biochemistry* 57, 6956–6963. [PubMed: 30511831]
- [101]. Gao F, Thornley BS, Tressler CM, Naduthambi D, and Zondlo NJ (2019) Phosphorylation-Dependent Protein Design: Design of a Minimal Protein Kinase-Inducible Domain, *Org. Biomol. Chem* accepted article.
- [102]. Lipchik AM, Perez M, Bolton S, Dumrongprechachan V, Ouellette SB, Cui W, and Parker LL (2015) KINATEST-ID: A Pipeline To Develop Phosphorylation-Dependent Terbium Sensitizing Kinase Assays, *J. Am. Chem. Soc* 137, 2484–2494. [PubMed: 25689372]
- [103]. Tremblay MS, Zhu Q, Marti AA, Dyer J, Halim M, Jockusch S, Turro NJ, and Sames D (2006) Phosphorylation state-responsive lanthanide peptide conjugates: A luminescence switch based on reversible complex reorganization, *Org. Lett* 8, 2723–2726. [PubMed: 16774241]
- [104]. Liu LL, and Franz KJ (2005) Phosphorylation of an α -synuclein fragment enhances metal binding, *J. Am. Chem. Soc* 127, 9662–9663. [PubMed: 15998051]
- [105]. Singh S, Khaytin I, Botsko S, Crossley G, Plank DK, Lefievre Y, Giuffrida F, and Pennington MW (2002) Addition of o-aminobenzoic acid during Fmoc solid phase synthesis of a fluorogenic substrate containing 3-nitrotyrosine, *Lett. Pept. Sci* 9, 221–225.
- [106]. Alexander JP, Ryan TJ, Ballou DP, and Coward JK (2008) gamma-glutamyl hydrolase: Kinetic characterization of isopeptide hydrolysis using fluorogenic substrates, *Biochemistry* 47, 1228–1239. [PubMed: 18171026]
- [107]. Niederhafner P, Safarik M, Brichtova E, and Sebestik J (2016) Rapid acidolysis of benzyl group as a suitable approach for syntheses of peptides naturally produced by oxidative stress and containing 3-nitrotyrosine, *Amino Acids* 48, 1087–1098. [PubMed: 26767371]
- [108]. Pandey AK, Naduthambi D, Thomas KM, and Zondlo NJ (2013) Proline Editing: A General and Practical Approach to the Synthesis of Functionally and Structurally Diverse Peptides. Analysis of Steric versus Stereoelectronic Effects of 4-Substituted Prolines on Conformation within Peptides., *J. Am. Chem. Soc* 135, 4333–4363. [PubMed: 23402492]

- [109]. Duus JO, Meldal M, and Winkler JR (1998) Fluorescence Energy-Transfer Probes of Conformation in Peptides: The 2-Aminobenzamidine-Nitrotyrosine Pair, *J. Phys. Chem. B* 102, 6413–6418.
- [110]. De Filippis V, Draghi A, Frasson R, Grandi C, Musi V, Fontana A, and Pastore A (2007) O-Nitrotyrosine and p-iodophenylalanine as spectroscopic probes for structural characterization of SH3 complexes, *Protein Sci* 16, 1257–1265. [PubMed: 17567746]
- [111]. De Filippis V, Frasson R, and Fontana A (2006) 3-Nitrotyrosine as a spectroscopic probe for investigating protein-protein interactions, *Protein Sci* 15, 976–986. [PubMed: 16641485]
- [112]. Zondlo NJ (2013) Aromatic-Proline Interactions: Electronically Tunable CH/ π Interactions, *Acc. Chem. Res* 46, 1039–1049. [PubMed: 23148796]
- [113]. Souza JM, Daikhin E, Yudkoff M, Raman CS, and Ischiropoulos H (1999) Factors Determining the Selectivity of Protein Tyrosine Nitration, *Arch. Biochem. Biophys* 371, 169–178. [PubMed: 10545203]

Figure 1.

Nitration of tyrosine can occur via peroxynitrite or other nitrosative agents. Tyrosine nitration reduces the pK_a of the phenol to approximately 7.^{1,5} Thus, at physiological pH, nitrotyrosine exists in two forms: a neutral form (left) which is more hydrophobic than tyrosine¹ due to internal phenol hydrogen bonding; and a significantly more polar anionic form (right). The presence of anionic nitrotyrosine is readily identified via its absorbance of visible light and its yellow color in solution.

Figure 2. Peptide sequences.

(a) Structure of an EF hand from calmodulin (CaM, pdb 1c1l, residues 93–106, 1.7 Å resolution). (b) Sequences of CaM EF hand and designed peptides based on that motif. Residues in cyan bind metal directly via their side chains in an EF Hand. Residue 7 in an EF Hand binds the metal via its main chain carbonyl. Trp7 (magenta) acts as an energy transfer donor to sensitize terbium emission. Residue 9 binds metal via a water-mediated contact (Ser, Asp, or Asn) or directly (Glu). The conserved Glu12 binds the metal via a bidentate interaction, using both carboxylate oxygens. In the design of proteins with metal binding dependent on nitration, Glu12 is replaced by Tyr. Tyrosines were either unmodified (blue), nitrated (red), or phosphorylated (green).

Figure 3. Design of a nitration-dependent protein switch.

(a) Nitrotyrosine has multiple anionic oxygen atoms that can bind terbium, with the possibility to present the oxygens with charge and geometry similar to glutamate. (b) Nitration of a tyrosine at position 12 of an EF hand to induce lanthanide binding via the nitro and/or phenolate groups. Tyrosine nitration reduces the phenol pK_a from 10 to 7, promoting binding via the phenolate. Alternatively, nitrotyrosine could bind metal via a bidentate interaction using the two oxygens of the nitro group.

Figure 4. Terbium luminescence spectra and terbium binding isotherms of the peptides pNO₂-Y12, pNO₂-Y^{NO2}12, pNO₂-Y^{OPO3²⁻}12, and pNO₂-E₉E₁₆Y12.

(a) Fluorescence emission spectra of 10 μ M pNO₂-Y12 (blue circles), pNO₂-Y^{NO2}12 (red squares), pNO₂-Y^{OPO3²⁻}12 (green triangles), or pNO₂-E₉E₁₆Y12 (black inverted triangles) in the presence of 250 μ M Tb³⁺. (b) Terbium binding isotherms of 10 μ M pNO₂-Y12 (blue circles), pNO₂-Y^{NO2}12 (red squares), pNO₂-Y^{OPO3²⁻}12 (green triangles), or pNO₂-E₉E₁₆Y12 (black inverted triangles); the fluorescence emission of Tb³⁺ in buffer (magenta diamonds) is also indicated. Fluorescence emission at 544 nm is shown. Fluorescence experiments were conducted in an aqueous solution, titrating peptides with Tb³⁺ via a 2-fold serial dilution, with 5 mM HEPES and 100 mM NaCl at pH 7.8. Fluorescence spectra were acquired using an excitation wavelength of 280 nm, with emission recorded over the range of 520–580 nm. Fluorescence emission was normalized to 300,000 counts at the local minimum of 570 nm. Fluorescence emission spectra are background subtracted. Terbium binding isotherms are not background corrected. Error bars indicate standard error. Data represent the average of at least 3 independent trials.

Figure 5. Determination of terbium binding affinities of nitrated peptides by competition binding experiments.

Dissociation constants of the nitrotyrosine-containing peptides (a) pNO₂-Y^{NO2}12, (b) pNO₂-E₉Y^{NO2}12, (c) pNO₂-E₁₆Y^{NO2}12, and (d) pNO₂-E₉E₁₆Y^{NO2}12 in complex with Tb³⁺ were determined by competition binding experiments versus the peptide pNO₂-Y^{OPO3²⁻}12. The peptide pNO₂-Y^{OPO3²⁻}12 (10 μM for a-c, 5 μM for d) was mixed with 1 equivalent of Tb³⁺, in the presence of luminescence-quenching nitrated peptides via a 2-fold serial dilution to determine the dissociation constants for peptide•Tb³⁺ complexes of pNO₂-Y^{NO2}12, pNO₂-E₉Y^{NO2}12, pNO₂-E₁₆Y^{NO2}12, or pNO₂-E₉E₁₆Y^{NO2}12. (e) Schematic representation of the equilibrium of Tb³⁺ binding during competition experiments. The phosphorylated peptide (green) sensitizes terbium emission while bound to metal, while the nitrated peptides quench fluorescence. Reversed competition experiments, using a constant concentration of each nitrated peptide with titration of the phosphorylated peptide, yielded similar *K_d* values (Figures S7–S10) to those in the experiments in this Figure.

Figure 6. ^1H - ^{15}N HMBC spectra of the peptide pNO₂-Y^{NO₂}12 in the absence and presence of La^{3+} .

^1H - ^{15}N HMBC spectra of pNO₂-Y^{NO₂}12 labeled with ^{15}N on the nitro group were acquired in the absence of metal (red) or in the presence of 1 equivalent of La^{3+} (blue). The ^{15}N resonance is correlated with H² (the hydrogen adjacent to the nitro group, shown) on the nitrotyrosine ring. Addition of La^{3+} to the apo peptide caused a significant change in the chemical environment of the nitrogen of the nitro group ($\delta = 1.3$ ppm), consistent with direct metal binding by one or both oxygen atoms of the nitro group. Specific ^{15}N labeling of the nitro group was accomplished by nitration of the peptide with ^{15}N -peroxynitrite (Figure S3), generated from $\text{Na}^{15}\text{NO}_2$, as described in the Supporting Information (Figure S2). Spectra were acquired using delays based on a ^1H - ^{15}N coupling constant of 20 Hz. The observed ^1H - ^{15}N coupling constant was 3 Hz. Spectra were acquired at 298 K with 300 μM peptide in 5 mM deuterated PIPES buffer pH 7.5 with 10 mM NaCl in D_2O . The spectrum in the presence of metal was acquired with 1 equivalent (300 μM) La^{3+} .

Figure 7. ^1H NMR spectra of the peptide $\text{pNO}_2\text{-E}_9\text{E}_{16}\text{Y}^{\text{NO}_2}\text{12}$ in the presence of increasing concentrations of La^{3+} .

The aromatic region of the ^1H NMR spectra of the peptide $\text{pNO}_2\text{-E}_9\text{E}_{16}\text{Y}^{\text{NO}_2}\text{12}$ in the presence of 0 (red), 0.2, 0.4, 0.6, 0.8, or 1 (purple) equivalents of La^{3+} show changes consistent with EF-hand-like binding of the peptide to the metal. Nitrotyrosine aromatic hydrogens and the amide hydrogen of Ile8 show significant broadening and chemical shift changes, while the tryptophan aromatic hydrogens (unlabeled peaks) are not significantly affected by addition of metal. Spectra were acquired with 300 μM peptide (0–0.8 equivalents La^{3+}) or with 600 μM peptide (1 equivalent La^{3+}) in a solution with 5 mM deuterated acetate, pH 7.5, in 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$ with 100 mM NaCl, at 298 K using WATERGATE water suppression.

Figure 8. TOCSY spectra of the peptide pNO₂-E₉E₁₆Y^{NO₂}12 in the absence and presence of Ce³⁺.

Superposition of TOCSY NMR spectra of pNO₂-E₉E₁₆Y^{NO₂}12 in the presence (blue) and absence (red) of the paramagnetic metal Ce³⁺. Resonances from groups near the metal are not observed due to paramagnetic broadening. (a) The fingerprint region of the spectra shows significant loss of signals upon the addition of metal, specifically the loss of signals associated with the first 14 residues of the peptide. Inset: the apo peptide (red) is expected to adopt a random coil conformational ensemble, and resonances are observed for all residues; the metal-bound peptide is expected to adopt an EF-hand-like structure (structure shown based on calmodulin, pdb 1c1l) with residues that were observed in the presence of Ce³⁺ indicated in blue, and residues that were not observed in the presence of Ce³⁺ indicated in gray. (b) Nitrotyrosine and tryptophan H_N-H_β correlations were not observed in the presence of Ce³⁺, while K₁₈ exhibited an H_N-H_ε correlation in the absence or presence of metal. (c) Correlations between the amide hydrogen and the β and γ hydrogens of E₁₆ were observed in the presence of metal, along with resonances of the flanking K and A residues. Spectral changes suggest binding of metal via a structure analogous to a canonical EF-hand. Residues surrounding the metal in a canonical EF-hand-like structure were not observed, while residues more distant from the metal in the expected structure were observed. Spectra were acquired at 298 K with 1 mM peptide in 5 mM deuterated PIPES buffer pH 7.5, with 10 mM

NaCl in 10% D₂O/H₂O. The spectrum in the presence of metal was acquired with 1 mM Ce³⁺.

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Figure 9. Fluorescent detection of tyrosine nitration in the MBP-pNO₂ fusion protein.

(a) Maltose-binding protein (MBP) fusion proteins with pNO₂-E₉E₁₆Y₁₂ (MBP-pNO₂) or a control sequence (MBP-c) were employed to determine the efficacy of nitration sensing using the sequence as an encoded tag on proteins. (b) Fluorescence emission spectra of MBP-pNO₂, after reaction with 0 (blue circles), 150 (green diamonds), or 450 (red squares) μM peroxynitrite. (c) Fluorescence emission spectra of MBP-c, after reaction with 0 (open blue circles), 150 (open green diamonds), or 450 (open red squares) μM peroxynitrite. (d) Superposition of the fluorescence emission spectra of both MBP-pNO₂ and MBP-c after nitration. (e) Fluorescence emission at 544 nm versus added ONOO⁻ for both proteins. MBP-pNO₂ (blue circles) shows a strong inverse correlation between the fluorescence emission and the concentration of peroxynitrite added. In contrast, MBP-c (cyan diamonds) shows a weak correlation with peroxynitrite added and significantly lower starting terbium luminescence. Proteins were nitrated using peroxynitrite in nitration buffer (100 mM potassium phosphate, 25 mM NaHCO₃, 0.2 mM EDTA, pH 7.4); non-nitrated protein samples were instead incubated with spent peroxynitrite (quenched by prior addition of DTT). Proteins were dialyzed to remove nitration byproducts and phosphate, then mixed with 100 μM Tb³⁺ in fluorescence buffer (5 mM HEPES, 100 mM NaCl, pH 7.8). Fluorescence spectra were acquired using an excitation wavelength of 280 nm, with fluorescence emission recorded over a range of 520–580 nm. Emission was normalized to

300,000 counts at the local minimum of 570 nm. Emission data are background subtracted. Error bars indicate standard error. Data represent the average of at least 3 independent trials.

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Scheme 1.

Synthesis of peptides with nitrotyrosine via *in situ* silyl protection of the nitrotyrosine phenol.

Table 1.
Dissociation constants of peptide•Tb³⁺ complexes.

Dissociation constants for the peptide•Tb³⁺ complexes of peptides with unmodified tyrosine, nitrotyrosine, and phosphotyrosine. K_d values were determined by fluorescence spectroscopy (Figures 3 and 4, Supporting Figures S4–S10) according to equation 1 and equation 2 (pages S10–S11). Error indicates the calculated error of the non-linear least squares fit of the data to the appropriate binding equation. ^a G is the free energy of the peptide•terbium complex, calculated as $G = -RT\ln(1/K_d)$. ^b G indicates the relative free energies of the terbium complex of the tyrosine-modified peptide relative to terbium complex of the peptide with tyrosine,

$$G = G_{\text{modified peptide}} - G_{\text{unmodified peptide}}$$

peptide	$K_d, \mu\text{M}$	error, μM	$G, \text{kcal mol}^{-1}{}^a$	$G, \text{kcal mol}^{-1}{}^b$
pNO ₂ -Y12	720	30	-4.3	
pNO ₂ -Y ^{NO₂} 12	16	2	-6.5	-2.2
pNO ₂ -Y ^{OPO₃²⁻} 12	37	5	-6.0	-1.7
pNO ₂ -E ₉ Y12	420	60	-4.6	
pNO ₂ -E ₉ Y ^{NO₂} 12	5.4	0.8	-7.2	-2.6
pNO ₂ -E ₁₆ Y12	890	110	-4.2	
pNO ₂ -E ₁₆ Y ^{NO₂} 12	9.7	1.1	-6.8	-2.6
pNO ₂ -E ₉ E ₁₆ Y12	300	30	-4.8	
pNO ₂ -E ₉ E ₁₆ Y ^{NO₂} 12	1.6	0.5	-7.9	-3.1
TY ^{NO₂} pN	70	26	-5.7	