Electrochemical and Structural Coupling of the Naphthoquinone Amino Acid

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SUPPLEMENTARY INFORMATION

General Methods. All reactions for the synthesis of ^{15}N-labled naphthoquinone amino acid, Naq, were as before[1] with the use of ^{15}N-(diphenylmethylene)-glycine tert-butyl ester, which was prepared as described.[2] ^{15}N-glycine was purchased from Cambridge Isotope Laboratories, Inc. and Fmoc-^{15}N-Alanine was purchased from Sigma-Aldrich. HPLC grade solvents were purchased from Fisher Scientific. Peptide synthesis was carried out either a Pioneer Peptide Synthesis System or CEM Liberty Microwave Synthesiser using standard Fmoc chemistry (protected natural amino acids from Novabiochem) with HATU/HOAt (Genscript Corporation) as the coupling reagents (4-5.0 mol equivalents of activator/Fmoc-amino acid) on PAL-PEG-PS resin (Applied Biosciences). After cleavage and deprotection of the natural amino acids (Reagent R -- 90:5:3:2 TFA/Thioanisole/Ethane Dithiol/Anisole) under argon, the resin was filtered and washed with 10 mL TFA (3x). The TFA was removed *in vacuo*, and the crude peptide precipitated with cold methyl tert-butyl ether. HPLC purification of all peptides, before and after activation, was performed on a C18 preparative column using a gradient of water and acetonitrile, both with 0.1% TFA, for elution. The identities of all peptides, sequences in Supplementary Table 1, were confirmed with MALDI-TOF-MS, using either α-cyano-4-hydroxycinnamic acid or sinapic acid (Sigma-Aldrich) as the matrix. MALDI-TOF-MS determinations were performed on a PerSeptive Biosystem Voyager-DE RP. Kjedahl analysis was performed by Galbraith Laboratories, Inc. UV/*vis* spectra were acquired on an Agilent 8453 UV-Visible Spectrophotmeter or a Varian Cary-50 Spectrophotometer. A generic buffer, hereafter referred to as Common Buffer, composed of 20 mM cacodylate, 100 mM KCl, pH 6.9 was used for peptide stock solution, CD spectroscopy, CD titrations, NMR spectroscopy, and Kjedahl analysis.

Supplementary Table 1. Peptide Sequences

Peptide	**Sequence•**
P2Naq(ox)	Ac-DKDGDGYISAAEAQAQ-NH_2
P2Naq(red)	""
RCNaq	Ac-AEAQAE-NH_2
P2NaqExt	Ac-DKDGDGYISAAEAQAQAAAAEAAAAEAAAAE-NH_2
PolyNaqABC	EAAAAEAAAAEAQAAEAQAAEAQAAEAAAAEAAAAE-NH_2
PolyNaqAC	EAAAAEAAAAEAQAAEA A AAEAQAAEAAAAEAAAAE-NH_2
PolyNaqB	EAAAAEAAAAEA A AAEAQAAEA A AAEAAAAEAAAAE-NH_2

•In these sequences Q is the one letter character for Naq

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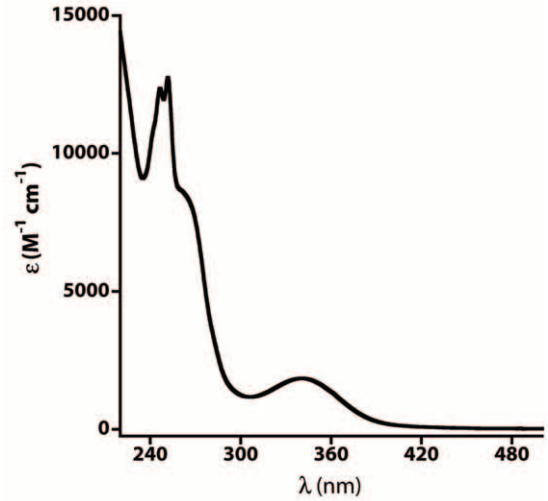
Naq(OMe/OMe) Deprotection. P2Naq(OMe/OMe) formed hard, transparent millimeter scale aggregates in water post-purification that only dissolved in 8 M GdnHCl or TFA. In a modification of the previously described procedure,[1] initial samples of P2Naq(OMe/OMe) were deprotected by use of cerium ammonium nitrate (CAN) in slight molar excess (>2 fold) in acidic (pH < 2 by TFA) aqueous GdnHCl solutions (peptide concentration was determined using a Naq(OMe/OMe) + Tyr ε_{301} 5065) at room temperature due to reduced solubility of GdnHCl on ice. However, because slightly higher selectivity (significantly curtailed oxidation of the tyrosine residue in P2Naq and P2NaqExt) and yields were observed using dichlorodicyanoquinone (DDQ), all peptides were ultimately deprotected using the following procedure: Naq(OMe/OMe) containing peptide was dissolved in 200 µL TFA and placed on ice. The concentration of the peptide in this solution was determined quickly by absorbance and exactly 1 equivalent of DDQ in MeCN (stock ~ 9 mg mL^{-1}) was added while shaking. The solution becomes dark upon DDQ addition and slowly lightens over the course of at least 30 minutes on ice. Dilution of the reaction with ddH_2O is followed immediately by HPLC purification, chromatographs in Supplementary Figure 1, and peptide identity confirmed by MALDI-TOF-MS, Supplementary Table 2. After deprotection, purification, and lyopholization, P2Naq, RCNaq, and P2NaqExt were dissolved in the Common Buffer, concentrations were determined by measured absorbance, and the solutions aliquoted and flash frozen.

Supplementary Figure 1. HPLC chromatographs of the purification of DDQ-based activation reactions on the indicated peptides.

Supplementary Table 2. MALDI-TOF Results of DDQ-Activated Peptides

Peptide	**Observed Mass (Amu)**	**Expected Mass (Amu)**
P2Naq	1780.2	1778.7 ($M+H^+$)
RCNaq (^{15}N-labeled at AQA, from Common Buffer)	801.7	799.25 ($M+K^+$)
P2NaqExt (^{15}N-labeled at AQA)	3025.2	3023.3 ($M+H^+$)
PolyNaqABC	3514.3	3508.5 ($M+H^+$)
PolyNaqAC	3356.9	3352.5 ($M+H^+$)
PolyNaqB	3199.4	3196.4 ($M+H^+$)

Determination of Molar Absorptivity of P2Naq. Kjedahl total nitrogen of solutions of oxidized P2Naq (deprotected as described above, dissolved in the Common Buffer) with known absorbance spectra were determined and the molar absorptivity spectrum calculated, Supplementary Figure 2. The molar absorptivity at 252 nm is 12700 M^{-1} cm^{-1}, and at 340 nm is 1840 M^{-1} cm^{-1}. The spectrum contains a small contribution from the tyrosine at 252 nm (316 M^{-1} cm^{-1}).[3] For reduced protein samples, a spectrum of the protein solution prior to reduction was used for protein concentration determination.

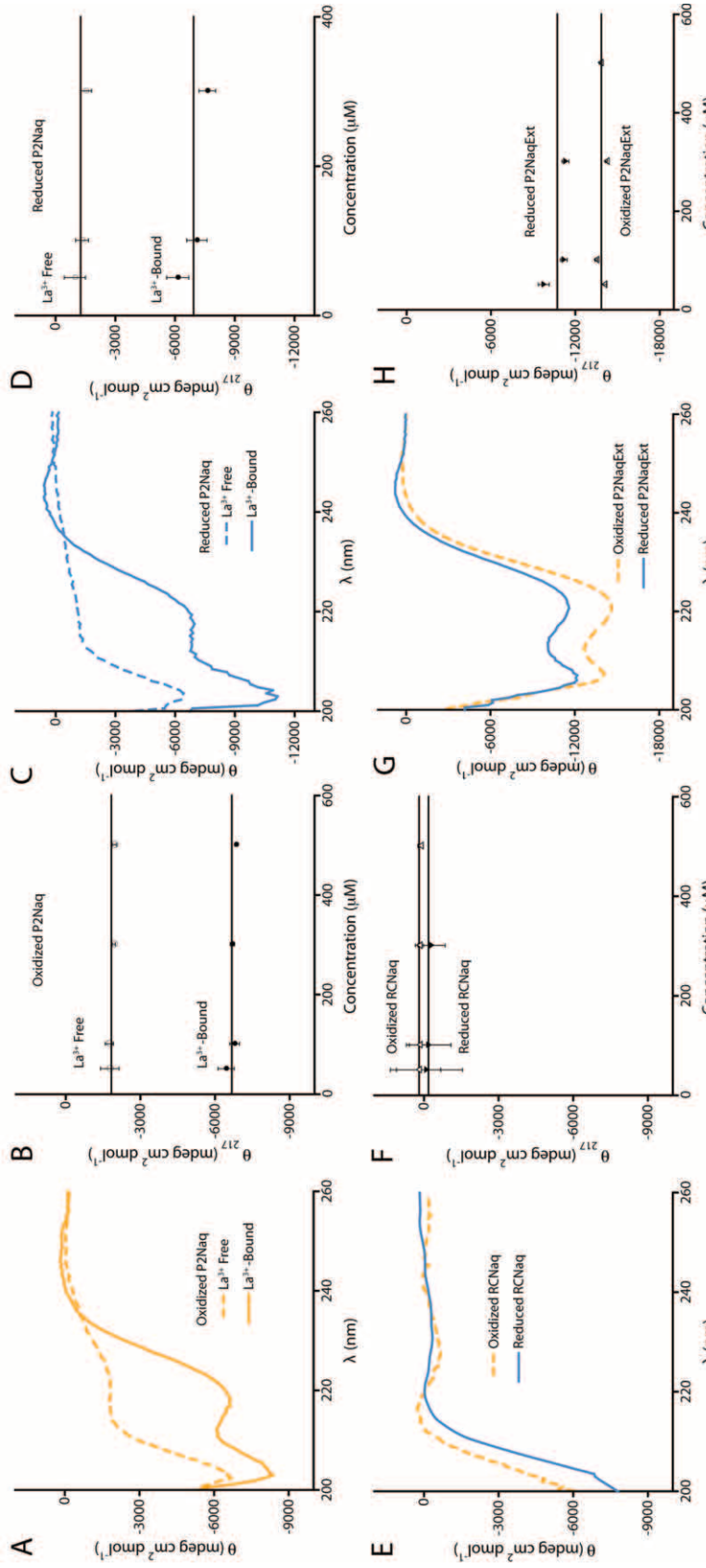


Supplementary Figure 2. Molar absorptivity of oxidized P2Naq in the Common Buffer as determined by Kjedahl total nitrogen analysis.

Reduction of Naq. The borane (particularly water stable ammonium borane) and borohydride reagents commonly used for the reduction of quinones interfere with lanthanide binding due to the formation of insoluble lanthanide borate salts. In addition, common redox mediator dyes used for maintenance of redox poises are generally not spectroscopically silent and tend to interfere with spectroscopic characterization. Enzymatic systems capable of low potential reduction, such as xanthine oxidase, proved to interfere either spectroscopically or by substrate binding to lanthanide. A relatively spectroscopically silent, non-interfering reducing system was realized by using colloidal platinum nanoparticles with a polyvinylpyrrolidinone (PVP) protective polymer in conjunction with hydrogen gas. The PVP polymer serves

to prevent platinum nanoparticle aggregation. A small amount of benzyl viologen indicator was added to ensure the system remained reduced, which is readily observed by a blue-purple color of the benzyl viologen radical (H_2 $E_{m,\ pH\ 6.9}$ -414 mV; benzyl viologen E_m -311 mV; expected Naq $E_{m,\ pH\ 6.9}$ ≈27 mV). Platinum nanoparticles with a 40 kD PVP protective polymer (40:1 monomer/platinum ratio) were prepared using standard conditions,[4] and dialyzed extensively against pure water to remove any free platinum that may have interfered with lanthanide binding. Platinum concentration in the colloidal platinum stock was calculated from the concentration of platinum in the preparation reaction. While there has been a previous report where a natural hydrogenase was used for the same purpose, [5] as far as we are aware, this is the first report of using easily prepared platinum nanoparticles for the reduction of a protein and maintenance of a low potential redox poise.

CD Spectroscopy. Circular dichroism spectra were acquired on an Aviv 410 CD Spectrophotometer. Automated titrations were performed with a Hamilton Company automated diluter controlled by a macro written in house for the Aviv CD Spectrophotometer. CD spectra of all peptides were taken at 25 °C in the Common Buffer (20 mM cacodylate, 100 mM KCl, pH 6.9). There was no observed concentration dependence of the CD spectra of the peptides at various concentrations, determined spectroscopically prior to data collection, Supplementary Figure 3. All CD samples containing $LaCl_3$ were at saturating lanthanide concentrations (either 2 mM, oxidized samples, or 10 mM, reduced samples). Reported spectra are averages of the mean residue molar ellipticity across all concentrations tested. Reduced protein samples were prepared in pear shaped round bottoms containing peptide solutions and benzyl viologen (100 μM). Hydrogen gas was flowed over the solution for at least 30 minutes with stirring before addition of colloidal platinum (10 μM platinum). Solutions would turn blue-purple upon benzyl viologen reduction, at which point the solution would be transferred via cannula into a CD cuvette with a positive pressure of hydrogen gas maintained during data collection.



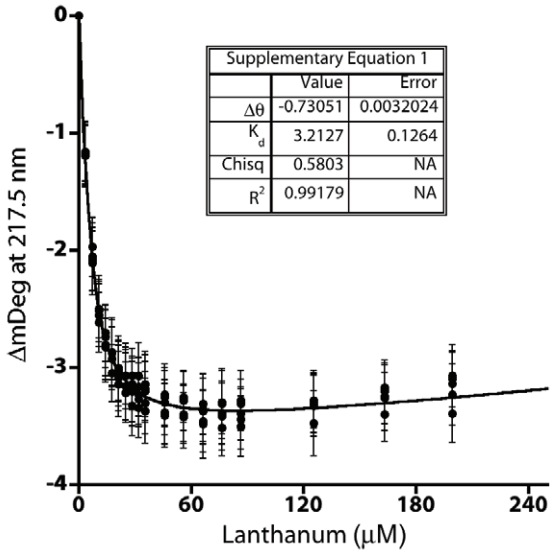
Supplementary Figure 3. CD spectra of lanthanide bound (solid) and free (dashed), oxidized (orange) P2Naq (A) and reduced (blue) P2Naq (C); and oxidized (orange) and reduced (blue), RCNaq (E) and P2NaqExt (G). Concentration dependence of the CD signal at 217 nm are shown with experimental errors and a line drawn through the average for lanthanide bound and free, oxidized P2Naq (B) and reduced P2Naq (D); and oxidized and reduced, RCNaq (F) and P2NaqExt (H).

Lanthanide binding titrations of P2Naq by CD. Lanthanide titrations were automated to reduce experimental variability with a Hamilton dilutor controlled by a macro written for the Aviv 410 CD spectrophotometer. Minimal internal diameter (0.25 mm) steel tubing was used for all liquid flow paths to minimize measurement errors introduced by diffusion of the lanthanide containing titrant into the experimental sample. Samples were incubated for 2 minutes after each aliquot addition with stirring at 25 °C. All titrations were measured in a 3 mL, custom quartz cuvette that had an elongated neck and a septum cap that was perforated for introduction of the steel titrant line. Lanthanide stock solutions were made in the Common Buffer, aliquoted, and flash frozen immediately to avoid precipitation of lanthanide carbonate salts upon prolong exposure to atmospheric CO_2. Lanthanide concentration was determined by a spectrophotometric end point titration using xylenol orange and a calibrated EDTA solution.[6] The reported P2Ala[7, 8] was used to test the binding affinity measurements for accuracy under both oxidized and reduced conditions.

Oxidized CD Titrations. P2Naq concentrations in the oxidized samples were at 5 μM, determined spectroscopically prior to titration. The titrant for oxidized P2Naq samples contained 1.08 mM lanthanum chloride in the Common Buffer. For all of the oxidized titrations CD signals were measured at 217.5 nm, 222 nm and 260 nm. It was discovered that significant changes in the 260 nm signal indicated that the sample cuvette had moved during the course of the titration and this proved a helpful diagnostic. Since 217.5 nm was the wavelength of the most intense signal of the lanthanide saturated P2Naq CD spectrum, data at that wavelength from five titrations were fit simultaneously with one-site, tight binding Supplementary Equation 1 with a floating y-axis intercept, since that was the wavelength of the most intense signal of the lanthanide saturated P2Naq CD spectrum.

$$\Delta CD_{signal} = \Delta\theta \cdot \frac{A - \sqrt{A^2 - 4[P2Naq]_{tot}[La^{3+}]_{tot}}}{2} \qquad \textbf{Suppl. Eq. 1}$$

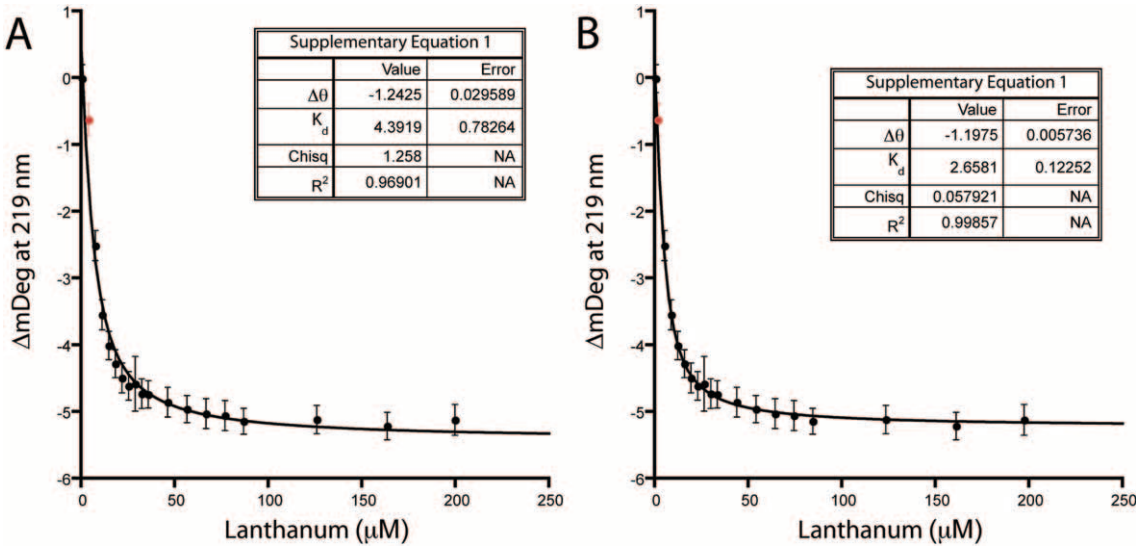
where ΔCD_{signal} is the measured change of CD signal at a chosen wavelength, $\Delta\theta$ is the change in the molar CD signal between the lanthanide bound and metal free P2Naq, A is (K_d + $[P2Naq]_{tot}$ + $[La^{3+}]_{tot}$), K_d is the dissociation constant, $[La^{3+}]_{tot}$ is the total concentration of lanthanide ion, and $[P2Naq]_{tot}$ is the total concentration of P2Naq. Sample dilution due to addition of titrant volume was accounted for in data fitting. The K_d was determined to be 3.2 μM ± 0.1 μM (error from fit), Supplementary Figure 4.



Supplementary Figure 4. Five identical CD lanthanide binding titrations of oxidized P2Naq measured at 217.5 nm simultaneously fit to Supplementary Equation 1 with a floating y-axis intercept. Error bars represent error reported by the spectrophotometer.

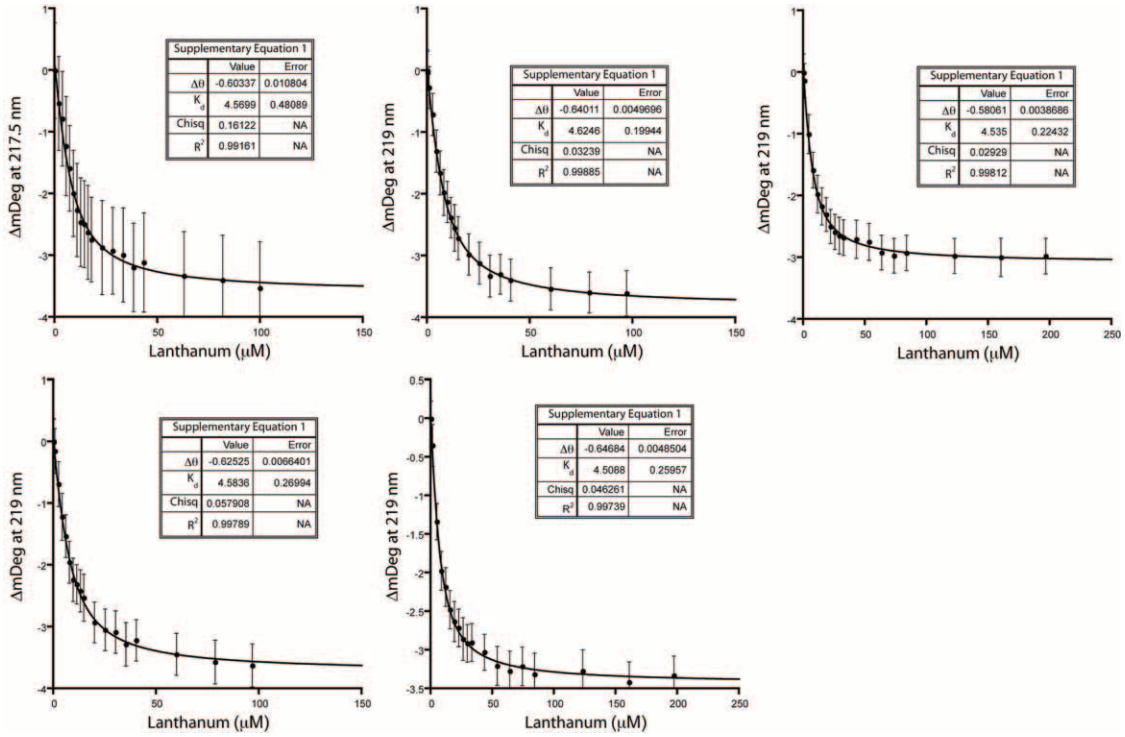
Reduced CD Titrations. Reduced titrations were performed similarly to the oxidized titrations, however for ease of sample handling peptide concentrations were kept constant in both the sample cuvette and the titrant. Hydrogen gas was used to purge sample and titrant for at least 30 minutes prior to the addition of the platinum nanoparticle catalyst. Freeze-pump-thaw cycles had been initially used to degas the solutions, however this added unnecessary complexity and decreased accuracy of volume measurements due to evaporative and transfer losses.

Reduced sample conditions (50 μM benzyl viologen, platinum nanoparticles at 10 μM platinum) were tested with the previously reported P2Ala[7, 8] peptide in the absence and presence of hydrogen gas positive pressure. In the absence of positive pressure, the titration behaved as expected, giving a measured K_d within error of the literature value, 2.4 μM ± 0.2 μM. However, it was observed that under positive pressure, the titration demonstrated an unexpected, but consistent behavior showing a smaller change than expected in the CD signal during the first aliquot(s) of lanthanide, Supplementary Figure 5A, resulting in an inaccurate measure of the binding constant, 4.4 μM ± 0.8 μM. The same behavior was observed with the experimental reduced P2Naq CD titrations. This apparent delay of lanthanide addition was attributed to the compression of either a bubble or the plastic in the titrant line when under pressure. The total titrated lanthanide concentration was corrected by considering that the first partial addition should cause a linear response in the CD signal with respect to lanthanide identical to the first full aliquot. Correcting the P2Ala data using this approach resulted in a measure of the binding affinity, 2.6 μM ± 0.1 μM, consistent with the reported value, Supplementary Figure 5B. Since reduced P2Naq CD titration data demonstrated similar delays in lanthanide addition, the same data correction was used for those titrations.



Supplementary Figure 5. An example P2Ala CD lanthanide titration under reducing conditions with positive pressure hydrogen as was used for reduced P2Naq, see text for details. Error bars represent error reported by the spectrophotometer (A) Data fit with Supplementary Equation 1 with a floating y-axis intercept as collected, note the first aliquot (red point) CD signal is not within error of the fit causing a significant perturbation in the measured K_d, 4.4 μM ± 0.8 μM, from the literature value, 2.4 μM ± 0.2 μM. (B) Data fit with Supplementary Equation 1 after correcting the aliquot lanthanide concentrations to account for partial addition of first aliquot, as described in the text. The fit is greatly improved and the measured K_d, 2.6 μM ± 0.1 μM is consistent with the literature value.

Reduced P2Naq titrations CD signals were measured at 260 nm and 219.5 nm, with one exception measured at 217.5 nm, noted in Supplementary Figure 6. The change in experimental wavelengths was due to increased noise at the lower wavelengths introduced by the inclusion of benzyl viologen. In all samples benzyl viologen was kept at 50 μM, while colloidal platinum varied between 1 μM platinum and 10 μM platinum, and peptide concentration, determined by absorbance of the oxidized sample prior to addition of benzyl viologen, was between 5 μM and 6 μM. Data, after the correction described above, were fit with Supplementary Equation 1 with a floating y-axis intercept. The average K_d of five titrations was determined to be 4.6 μM ± 0.1 μM (error propagated from fitting error of each measurement, $\sigma = 0.04$ μM).



Supplementary Figure 6. Five non-identical CD lanthanide binding titrations of reduced P2Naq measured at 217.5 nm or 219 nm fit to Supplementary Equation 1 after correction of lanthanide concentrations as described. Error bars represent error reported by the spectrophotometer.

NMR Spectroscopy. All NMR experiments were performed on a Bruker 500 MHz Advance III Spectrometer equipped with a cold probe. All data were collected at 25 °C, calibrated with methanol. ^{15}N, ^{13}C, and ^{1}H chemical shifts were referenced using DSS following IUPAC recommendations.[9] For peptides analyzed by NMR, ^{15}N-backbone labels were incorporated at the Ala-Naq-Ala amino acid sequence, see General Methods section. NMR samples contained peptides at concentrations between 1 mM and 2.6 mM as determined by absorbance spectroscopy. Peptide solutions were made with dried peptide powder dissolved in the Common Buffer with 10% D_2O and adjusted to pH 6.9 ± 0.1 as measured by a pH microelectrode. Lanthanide-bound samples had approximately 10 mM $LaCl_3$ added prior to pH adjustment. Peptides were characterized using ^{15}N-^{1}H HSQC, natural abundance ^{13}C-^{1}H HSQC (RCNaq and P2Naq only), ^{15}N-filtered NOESY, and ^{15}N-filtered HNHA 2D experiments.[10, 11]

Reduced NMR Sample Preparation. All reduced samples contained colloidal platinum at 11 μM platinum and 100 μM benzyl viologen. Sample reduction was performed as described for reduced CD samples: peptide solutions containing benzyl viologen were stirred in a round bottom under hydrogen gas for at least 15 minutes before the introduction of the colloidal platinum catalyst. Reduction took ~5 minutes as indicated by the solutions developing a blue-purple color due to the reduction of benzyl viologen. After reduction, the solutions were quickly cannulated into a clean NMR tube equipped with a rubber septum that had been purged with hydrogen gas. Upon completion of sample transfer, the cannula and vent lines were carefully removed, and

the rubber septum coated with a 5-minute epoxy to create a gas tight seal. The epoxy was allowed to completely set (~1 hr) before NMR data was collected. All reduced samples prepared this way maintained the blue-purple color of reduced benzyl viologen throughout the NMR experiments indicating that the reduced redox state of the peptides was maintained. Additionally, a mixed redox state sample of lanthanide bound P2Naq was generated by transferring a sample to an NMR tube prior to complete reduction.

Chemical Shift Measurements. The ^{15}N-^{1}H HSQC data was used to make assignments, using alanine chemical shift indices and the mixed redox state sample, which greatly simplified the assignment process. $^{1}H^{\alpha}$ chemical shifts of the labeled amino acids were directly measured from the 2D ^{15}N-filtered HNHA. ^{13}C-^{1}H HSQC data proved unusable to determine $^{13}C^{\alpha}$ chemical shift of Naq since the $^{1}H^{\alpha}$ chemical shift of Naq was degenerate with the water resonance in the natural abundance ^{13}C-^{1}H HSQC even in a sample in buffer prepared with D_2O. The measured $^{1}H^{\alpha}$ chemical shifts are tabulated in Supplementary Table 3.

Supplementary Table 3. $^{1}H^{\alpha}$ Chemical Shifts of the Ala-Naq-Ala Amino Acid Sequence.

Peptide	**Antecedent Ala H^{α}**	**Naq H^{α}**	**Subsequent Ala H^{α}**
Lanthanide Bound			
Oxidized P2Naq	4.053	4.659	4.328
Reduced P2Naq	4.055	4.607	4.245
Ligand Free			
Oxidized P2Naq	4.135	4.698	4.29
Reduced P2Naq	4.142	4.642	4.253
Random Coil Control			
Oxidized RCNaq	4.202	4.746	4.316
Reduced RCNaq	4.215	4.694	4.268
Helical Control			
Oxidized P2NaqExt	4.054	4.528	4.163
Reduced P2NaqExt	4.007	4.399	4.069

Quantitative J-Correlation Measurements. The 2D ^{15}N-filtered HNHA experiment was used to measure the scalar (J_{HAHN}) coupling constant between the amide hydrogen and the H^{α} of the labeled amino acids.[11] Briefly, peak volumes were measured by Gaussian integration in Sparky[12] and used in Supplementary Equation 2.[11]

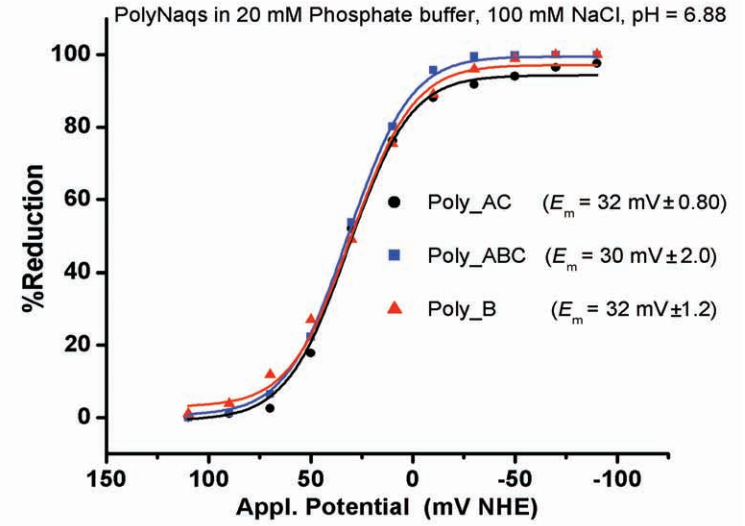
$$\frac{S_{cross}}{S_{diagonal}} = -\tan^2(2\pi J_{HAHN}\zeta) \qquad \textbf{Suppl. Eq. 2}$$

where S_{cross} and $S_{diagonal}$ are the measured peak volumes of the amide correlated cross (H^{α} - H^{N}) and diagonal (H^{N} - H^{N}) peaks, respectively, and ζ is a pulse delay in which dephasing due to J-coupling occurs (13.05 ms). The measured scalar (J_{HAHN}) coupling constants are tabulated in Supplementary Table 4.

Supplementary Table 4. Backbone J_{HAHN} scalar coupling constants. Values in grey were obtained from degenerate diagonal peaks and are only included for completeness.

Peptide	Antecedent Ala J_{HAHN} (Hz)	Naq J_{HAHN} (Hz)	Subsequent Ala J_{HAHN} (Hz)
Lanthanide Bound			
Oxidized P2Naq	4.63	6.18	4.83
Reduced P2Naq	5.46	6.23	5.34
Ligand Free			
Oxidized P2Naq	5.63	6.87	6.30
Reduced P2Naq	5.00	7.16	5.99
Random Coil Control			
Oxidized RCNaq	5.74	7.39	5.97
Reduced RCNaq	5.20	5.97	6.05
Helical Control			
Oxidized P2NaqExt	4.49	4.98	4.31
Reduced P2NaqExt	3.69	4.01	4.13

Spectroelectrochemistry of PolyNaq Peptides. To ascertain the electrochemical behavior of single and multiple Naqs in a helical peptide sequence, Naq was incorporated into soluble, alanine rich peptide sequences identical to the helical extension added to P2Naq to form P2NaqExt, Supplementary Table 1 – PolyNaqABC, PolyNaqAC and PolyNaqB. Even though the three peptides vary in the number of Naq residues, their electrochemistry is identical, Supplementary Figure 7, indicating that the Naq residues are not appreciably interacting directly with each other or through the peptide backbone. In addition, the observed potential for all Naqs, $E_{m,\,pH\,6.88}$ 30-32 mV, in these peptides is consistent with the value of predicted for our reported proline rich, soluble peptide, heptaNaq, $E_{m,\,pH\,6.88}$ 27 mV.[1]



Supplementary Figure 7. Spectroelectrochemical titrations of the PolyNaq peptides (sequences in Supplementary Table 1) in 20 mM phosphate buffer, 100 mM NaCl, pH 6.88. Titrations were performed as previously reported.[1]

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