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## Electrochemical and Structural Coupling of the Naphthoquinone Amino Acid

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*p*-Benzoquinones and naphthoquinones are oxidation-reduction cofactors essential to proteins engaged in natural photosynthetic and respiratory energy conversion. Common to all natural *p*-quinones is their highly reversible two-electron, two-proton transition between the stable hydroquinone and quinone forms. Under physiological conditions, oxidation converts the aromatic hydrocarbon of the reduced form into an aliphatic, cyclic diketone quinone, which is accompanied by a considerable lowering of two pKa values (>12 units) and proton release.<sup>1, 2</sup> Despite the potential for catalytic control of these significant structural and electrochemical events in natural quinone oxidoreductases, there is little evidence of any consensus sequences, recurring structural motifs, high affinities, or specific differential interactions of significance between the reduced and oxidized forms of the quinone and the protein involved in regulating electrochemistry.<sup>3, 4</sup> In this respect the quinone contrasts sharply with many other biological cofactors, such as flavins and hemes, which are known to bind covalently or with high affinity in various protein sites where redox potentials are altered by hundreds of millivolts often with well-delimited specific and energetically significant protein interactions effecting the difference.<sup>5–7</sup>

In our goal to reproduce the quinone functions found in natural energy conversion proteins in non-natural designed proteins, the absence of specific natural quinone binding motifs as a guide for construction of a quinone site has led us to synthesize a naphthoquinone amino acid (Naq) for direct inclusion into polypeptides.<sup>8, ‡</sup> The steric bulk and geometry of Naq (Figure 1A) provides it with a nearly identical helical backbone dependent rotamer energy surface to tryptophan.<sup>9</sup> In this report, to quantify the influence of backbone conformation on the naphthoquinone side chain redox chemistry, we test the inclusion of Naq in a family of host-guest peptides that have proven valuable for understanding thermodynamic contributions to helix-coil transitions of guest amino acids included in this model system.

Derived from a lanthanide ion binding EF hand, and characterized by the Bierzynski and Makhatadze groups, the P2 family of guest-host peptides are composed of a lanthanide ion binding loop P1 (La<sup>3+</sup> K<sub>d</sub> of 9.4 μM) and a short alanine rich segment containing a surface exposed guest site.<sup>10–13</sup> Because binding of a lanthanide ion induces the formation of a helix in the alanine rich segment, the tendency to form a helix couples to lanthanide ion affinity offering a quantitative assessment of the helical propensity of the guest site amino acid.<sup>13</sup>

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<sup>†</sup>Electronic Supplementary Information (ESI) available: Synthetic methodology and spectroscopy, and NMR methodology and compiled data. See DOI: 10.1039/b000000x/

<sup>‡</sup>Aqueous E<sub>m</sub> values reported in<sup>8</sup> are in error due to mis-referencing of the E<sub>h</sub>, the correct values are 10 mV higher.

This allows direct comparison of the ability of the two stable redox states of Naq to form a helix, and thus provides a reliable measure of the effect of a backbone conformational change, from coil to helix, on Naq electrochemistry.

The incorporation of Naq into the guest site in the reported P2 peptide sequence (P2Naq; Figure 1B), a variant with an extended alanine rich helical region (P2NaqExt),<sup>14</sup> and a short peptide with a sequence nearly identical to the helical region of P2Naq (RCNaq), Table 1, was performed as described before<sup>8</sup> with modification of the deprotection conditions to account for increased sensitivity of the peptide sequences to oxidative modification (see Supplementary Information). The spectroscopy of reduced Naq, including circular dichroism and NMR, required the use of a relatively spectroscopically silent reducing agent that does not interfere with lanthanide ion binding. Using a colloidal platinum catalyst under a hydrogen atmosphere in the presence of a small amount of benzyl-viologen as a redox indicator proved most viable in maintaining Naq in a stable reduced, hydroquinone state.

As observed by circular dichroism, both oxidized and reduced P2Naq behaved as anticipated from previous reports on P2 host-guest peptides, showing a small amount of helicity in the absence of the lanthanide ion ligand as measured by ellipticity at 220 nm and becoming significantly more helical in the presence of saturating lanthanide (>2 mM), Figure 2. Similarly, in the presence saturating lanthanide, P2NaqExt demonstrated (Figure 3) a classic helical CD response in the amide region, while RCNaq failed to demonstrate any notable secondary structural signatures.

The lanthanide binding affinity of the oxidized and reduced P2Naq peptides was measured using CD titrations, (Figure 2; see Supplementary Information for details) which revealed a significant difference between the affinities of the oxidized ( $K_d$   $3.2 \mu\text{M} \pm 0.1 \mu\text{M}$ )<sup>†</sup> and reduced ( $K_d$   $4.6 \mu\text{M} \pm 0.1 \mu\text{M}$ )<sup>§</sup> peptides (Table 1). These binding affinities fall within the range of those reported by Richardson *et al.* for natural amino acids in the P2 guest site (from P2Gly  $K_d$   $6.5 \mu\text{M}$  to P2Ala  $K_d$   $2.4 \mu\text{M}$ ).<sup>13</sup> To ascertain the thermodynamic effects of backbone conformation on the redox chemistry of Naq using these binding affinities, it is critical to note that the P2Naq peptides had significant amounts of helical character in the absence of lanthanide ion. As noted by Makhatazde *et al.*, by considering a two-state model, it is possible to isolate the energetic contribution to binding of the helix-forming region of the peptide from the lanthanide ion-binding loop (P1) by simply subtracting the intrinsic binding energy of the loop.<sup>12, 13</sup> In addition, the fraction of the peptide undergoing conformational change upon lanthanide binding needs to be taken into account:

$$\Delta G^\circ (P2Naq) = \frac{\Delta G (P2Naq)}{F_\alpha^+ - F_\alpha^-} \quad (1)$$

Where  $\Delta G(P2Naq)$  is the free energy associated with helix formation in P2Naq, and  $F_\alpha^+$  and  $F_\alpha^-$  are the helical fractions of the lanthanide bound and unbound form, respectively.

In previous reports it was shown that that  $F_\alpha^+$  is close to unity for the P2Ala peptide and the helical fractions as measured by the CD of lanthanide ion free and bound P2 host-guest peptide were accurate proxies for the more appropriate guest-site specific helical fraction.<sup>11, 12</sup> However, in our efforts with the P2Naq peptides, direct comparisons to P2Ala of the intensities of the CD spectra with and without ligand suggested that the helical fraction of the bound states were not close to unity. It was impossible to confirm that the helical fraction as measured by CD matched a computationally derived site-specific value

<sup>†</sup>Simultaneous fit of five identical titrations, reported error is from fit.

<sup>§</sup>Average of five titrations; reported error is propagated from the error of each measurement ( $\sigma = 0.04 \text{ mM}$ ).

due to the lack of tools like AGADIR<sup>15</sup> for predicting helical fraction of peptides containing artificial amino acids.<sup>13</sup>

To measure site-specific values of helicity in the P2Naq peptides, <sup>15</sup>N-backbone labels were incorporated at the naphthoquinone amino acid and its primary sequence adjacent alanines in P2Naq, P2NaqExt and RCNaq (see Supplementary Information for synthetic details). Nitrogen labeling was chosen because both backbone torsional angles calculated from scalar coupling measurements (via a <sup>15</sup>N-filtered HNHA experiment) and H<sup>α</sup> chemical shifts are readily obtainable. In addition, H<sup>α</sup> chemical shifts are highly correlated with secondary structure<sup>16, 17</sup> and have previously been used to accurately measure the thermodynamic properties of cation-π interactions in β-hairpin peptides.<sup>18, 19</sup> For each redox and ligand bound state of P2Naq, H<sup>α</sup> chemical shifts of Naq were used to calculate the fraction helicity with

$$F_{\alpha} = \frac{\delta_{\text{obs}} - \delta_{\text{coil}}}{\delta_{\text{helix}} - \delta_{\text{coil}}} \quad (2)$$

where  $\delta_{\text{obs}}$  is the measured chemical shift of the proton in P2Naq, and  $\delta_{\text{helix}}$  and  $\delta_{\text{coil}}$  are the H<sup>α</sup> proton chemical shifts of Naq in the P2NaqExt and RCNaq peptides, respectively, which serve as helical and random coil reference peptides. Importantly, the accuracy of this measurement is enhanced through the use of the nearly sequence identical control peptides for the reference states.

Measured H<sup>α</sup> chemical shifts and <sup>3</sup>J scalar coupling constants support the CD observations of the peptides. The P2NaqExt peptide can be considered helical in the environment around Naq with observed scalar coupling constants near to the helical 'ideal' of 4 Hz similar to a previously reported P2Ala peptide.<sup>10</sup> By comparison, the RCNaq peptide demonstrates significantly higher H<sup>α</sup> chemical shifts and scalar coupling constants of the three labeled amino acids corresponding to the expected behavior of a random coil peptide. The experimental P2Naq peptides lie between these two extremes and demonstrate shifts towards the behavior of P2NaqExt upon lanthanide addition (see Supplementary Information for tabulated data). As recognized from previous reports<sup>12, 13</sup> and our CD studies in the absence of lanthanide ion, the guest site of P2Naq is appreciably helical in both redox states (reduced:  $F_{\alpha}^{-}$  0.176; oxidized:  $F_{\alpha}^{+}$  0.220). While the backbone at Naq becomes significantly more helical with saturating lanthanide (reduced:  $F_{\alpha}^{-}$  0.295; oxidized:  $F_{\alpha}^{+}$  0.399), it does not become entirely helical (Table 1), precluding direct comparisons to previous work with P2 host-guest peptides. After correction, Eq 1, the isolated energies of helix formation with Naq in both the oxidized and reduced form are identical,  $\Delta G^{\circ}(P2Naq) -1.2$  kJ/mol (-6.0 meV).

The identical helix formation energies for Naq in both redox states reflects that there is no difference in midpoint potential between Naq on an unstructured loop and in the middle of a helix. This accounts for any potential changes in the redox midpoint due to specific interactions of the Naq side-chain with backbone and solvent in both redox states. It is clear that while specific interactions between Naq, backbone, and solvent may exist, backbone conformational changes do not impair the ability for Naq to find equally energetic electrochemical interactions within a new structural element. This fact simplifies the prediction of the electrochemical properties of Naq in a variety of protein backbone contexts, particularly those exposed to aqueous environments. Indeed, work on highly helical peptides containing one or more Naqs demonstrate electrochemical properties consistent with the expected values as measured in our previously reported heptaNaq peptide (See Supplementary Information).<sup>8</sup> It is also apparent that while Naq highly perturbs short and unstable helices, it is capable of being incorporated into a peptide that fully adopts a helical conformation, as P2NaqExt shows a classic helical CD response with a higher than

predicted ellipticity at 220<sup>20</sup> suggesting a single nucleation of the helix at the lanthanide binding loop that extends through Naq. In addition, in both redox states the <sup>15</sup>N-filtered NOESY spectrum of P2NaqExt proved capable of being used to sequentially assign the backbone amides of the Ala-Naq-Ala amino acid sequence as is typical for helical peptides. ◆

Quinones in Nature demonstrate an enormous range of two-electron, two-proton redox potentials, yet there do not appear to be specific hydrogen bonding interactions that drive this variance; instead, general hydration as well as proton uptake and release appear to be essential in determining the electrochemical properties of quinone cofactors. Similarly, we have now shown that the electrochemical properties of the naphthoquinone amino acid, are not perturbed by its backbone conformation; this opens new avenues for using Naq as a quantitative probe of the effects of local protein environment on quinone electrochemistry and as a general two-electron cofactor on the surface of proteins with predictable properties.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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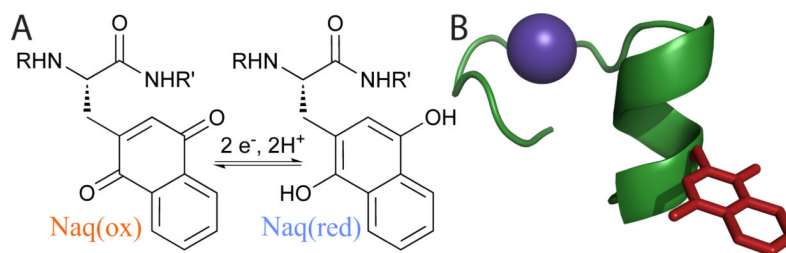
◆ A 24-mer with Naq at a buried helical site shares this property.

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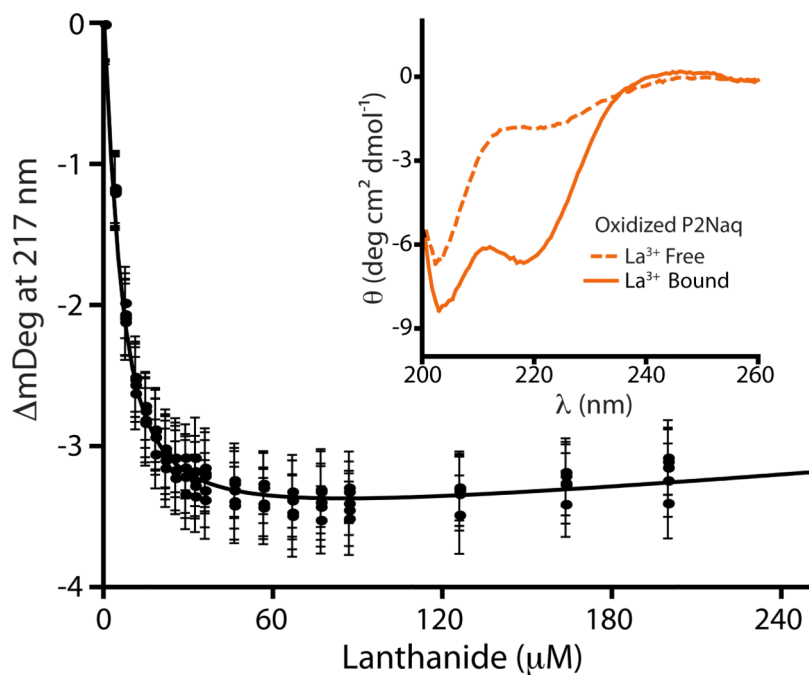
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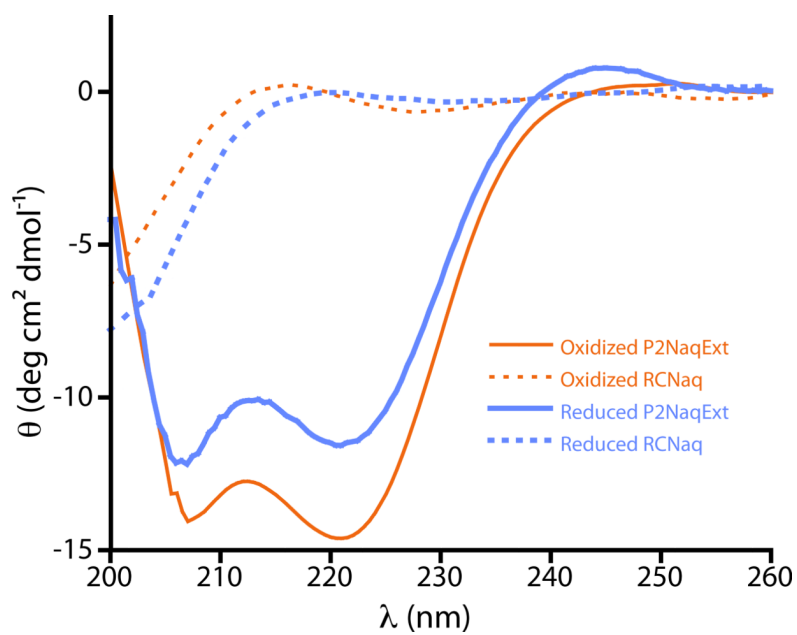
**Figure 1.**

A. The naphthoquinone amino acid (Naq) demonstrates two electron, two proton electrochemistry in aqueous solutions.<sup>8</sup> B. Naq modelled into the guest site of the lanthanide bound P2Ala peptide.<sup>⊗</sup>

<sup>⊗</sup>Figure created with PyMOL ([www.pymol.org](http://www.pymol.org)) using the NMR structure of lanthanide bound P2Ala, PDB accession code 1NKF.<sup>10</sup>



**Figure 2.** CD response of oxidized P2Naq in the presence and absence of lanthanide ion (inset). Five identical titrations of oxidized P2Naq with lanthanide ion are fit to the single site, tight binding equation with an observed  $K_d$  of  $3.2 \mu\text{M} \pm 0.1 \mu\text{M}$ . (See Supplementary Data for experimental details as well as equivalent data for reduced P2Naq).



**Figure 3.** Amide region CD response of the reduced (blue) and oxidized (orange) P2NaqExt (solid lines) and RCNaq (dotted lines).



**Table 1**

## Peptide Sequences and Experimental Parameters

Peptide	Sequence*	$K_a$ ( $\mu\text{M}$ )	$F_{\alpha}^+ / F_{\alpha}^-$
P2Naq(ox)	Ac-DKDGDGYISAAEAQAQ-NH <sub>2</sub>	$3.2 \pm 0.4$	0.399 / 0.220
P2Naq(red)	“ ”	$4.6 \pm 0.1$	0.295 / 0.176
RCNaq	Ac-AEAQAQ-NH <sub>2</sub>		
P2NaqExt	Ac-DKDGDGYISAAEAQAQAAAAEAAAEEAAAE-NH <sub>2</sub>		

\*In these sequences Q is the one letter character for Naq.