Reversible proton coupled electron transfer in a peptide-incorporated naphthoquinone amino acid†

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Received (in Cambridge, UK) 11th September 2008, Accepted 5th November 2008
First published as an Advance Article on the web 25th November 2008
DOI: 10.1039/b815915g

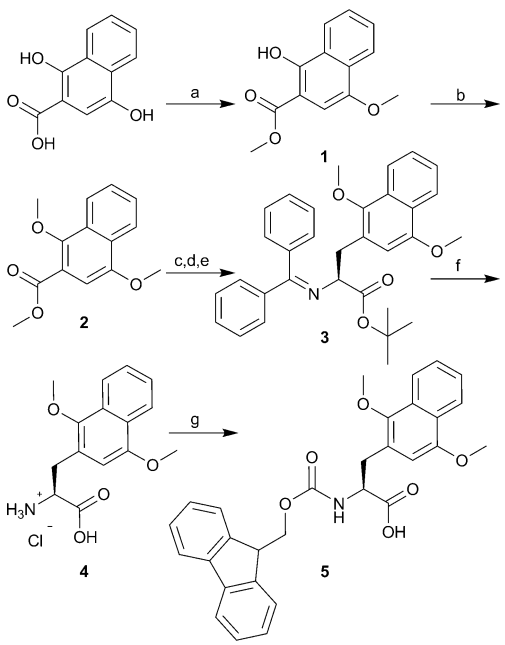
The synthesis of a naphthoquinone amino acid and its electrochemical characterization in a peptide is presented.

Quinones are critical cofactors in the generation of transmembrane electrochemical proton gradients that drive the production of ATP in photosynthesis and respiration.[1–5] Relatively weak binding, poorly resolved structural details, and indistinct spectroscopic characteristics of quinones in catalytic sites of membrane protein complexes have long challenged progress toward understanding their mechanistic roles in energy conversion. In contrast, work on the catalytic mechanisms of soluble quinoproteins has advanced in large part because their amino-acid derived quinonoid cofactors are covalently secured.[6–9] The development of simple, water-soluble, artificial proteins (maquettes) that bring the advantages of covalently secured bioenergetic quinone cofactors to the protein backbone provides both a platform to study membrane quinone catalysis and immediate access to novel biosynthetic devices. The key first step in producing such maquettes is the development of a quinone amino acid, reported here. We have chosen a naphthoquinone amino acid (Naq, ¶) based upon menadione, one of the quinone head groups common to energy conversion in the membranes of photosynthetic and anaerobic organisms.

Naq proves amenable to solid phase peptide synthesis (SPPS) and incorporation into synthetic proteins. It is an α-amino acid with a steric bulk similar to that of tryptophan, which promises incorporation into natural secondary structural elements of proteins with little of the perturbation encountered with cysteine ligation of quinones,[10–12] and hence facilitating catalytic site design. In addition, with substitution of the highly reactive Michael centers around the *para*-quinone ring, Naq is chemically robust, thereby allowing its study and use under a wide variety of conditions; this contrasts with non-substituted *ortho*- or *para*-quinone amino acids.[13,14]

The high yielding and readily scalable Naq synthesis centers around the asymmetric phase transfer based synthesis of amino acids developed by O'Donnell *et al.*[15] While methylation provides chemically rigorous protection of the hydroquinone hydroxyls throughout the synthesis and during peptide assembly, we were able to make use of a highly specific oxidative deprotection step, selective enough to avoid concomitant oxidative modification of tryptophan and tyrosine within the same peptide.

Synthesis begins with the protective acidic methylation of the natural product 1,4-dihydroxy-2-naphthoic acid to methyl 1-hydroxy-4-methoxy-2-naphthoate (**1**)[16] (Scheme 1). Further methylation of **1** under basic conditions affords methyl 1,4-dimethoxy-2-naphthoate (**2**)[17] in 84% yield after two steps. Arylbromide, derived from carboxylate **2**, combined with the third-generation cinchonidinium[18,19] asymmetric phase transfer catalyst (PTC) alkylated *tert*-butyl *N*-(diphenylmethylene)-glycinate to produce protected Naq (**3**) in 86% yield with 93% enantiomeric excess (ee). Standard acidic aqueous deprotection conditions on **3** result in a black tar.[20] While the tar is avoided in acidic methanol,‖ the yield is improved with deprotection of **3** in TFA with ethanedithiol (EDT) as a scavenger, which produces Naq(OMe/OMe) amino acid hydrochloride **4** nearly quantitatively. Protection of the amine



Scheme 1 Synthesis of Fmoc–Naq(OMe/OMe)–OH. (a) H_2SO_4, MeOH, reflux, 93.4%; (b) CH_3I, K_2CO_3, DMF, Δ, 90%; (c) $LiAlH_4$, THF, 0 °C, 99%; (d) PBr_3, CCl_4, 0 °C, 95.8%; (e) CsOH, *N*-(diphenylmethylene)glycine *tert*-butyl ester, cinchonidinium PTC, Tol, −20 °C, 86%, ee 93%; (f) EDT, TFA, 98.2%; (g) Fmoc–OSu, $NaHCO_3$, DMF, 85%.

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† Electronic supplementary information (ESI) available: Synthetic methodologies, compound characterization and spectroelectrochemical experimental setup. See DOI: 10.1039/b815915g

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with Fmoc–OSu gives the SPPS-ready derivative Fmoc–Naq(OMe/OMe)–OH **5**.**

Fmoc–Naq(OMe/OMe)–OH (**5**) readily incorporates into peptides *via* SPPS but the standard TFA based cleavage cocktails fail to remove the methyl protecting groups of the quinone hydroxyls. We tried a variety of one-step on-resin deprotection/cleavage of peptides containing Naq using modifications of Fmoc-SPPS cleavage reagents. Addition of strong Lewis acids to these cocktails showed promise in simultaneously deprotecting Naq(OMe/OMe) and natural amino acids, but undesired side reactions associated with Naq were unavoidable. Similarly, although the reaction of Ac–Naq(OMe/OMe)–OMe with [bis(trifluoroacetoxy)iodo]-benzene (PIFA), one of the mild hypervalent iodine(III) oxidants used effectively in deprotecting a number of methyl-protected quinones,[21] quantitatively produced the electrochemically active quinone, we were unable to eliminate unwanted reactions of this reagent with other natural amino acids, notably tyrosine and tryptophan (data not shown).†† However, reaction in water at pH 1.0 with stoichiometric quantities of the oxidant cerium ammonium nitrate (CAN)[22] led to quantitative and selective oxidative deprotection of the Naq(OMe/OMe) side chain to yield the desired quinone.

Solution electrochemical characterization of Naq in a peptide was performed with a synthesized heptamer (heptaNaq, sequence PDPϘPDQ–NH_2). HeptaNaq was selected to be proline rich to decrease conformational flexibility while increasing water solubility and facilitating aqueous Naq electrochemistry. HeptaNaq(OMe/OMe) was readily synthesized *via* SPPS and the natural amino acids deprotected with 95 : 5 : 3 : 2 TFA–thioanisole–EDT–anisole. After HPLC purification and deprotection, heptaNaq was re-purified to produce oxidized, electrochemically active heptaNaq.

The reduced and oxidized electronic spectra of heptaNaq are nearly identical to those of 2-methylnaphthoquinone (Fig. 1). The oxidation–reduction midpoint potential (E_m) of heptaNaq as a function of pH was determined spectroelectrochemically by following the difference between the absorption of the reduced

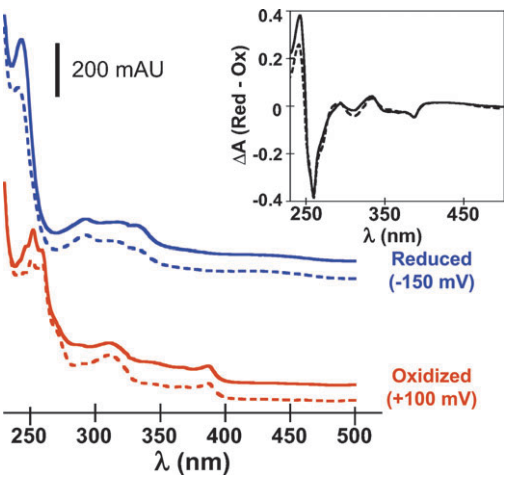

Fig. 1 Reduced and oxidized electronic spectra of heptaNaq (—) and 2-methylnaphthoquinone (---) in the presence of redox mediators. Inset: Difference spectra of the two species. See ESI for details.†

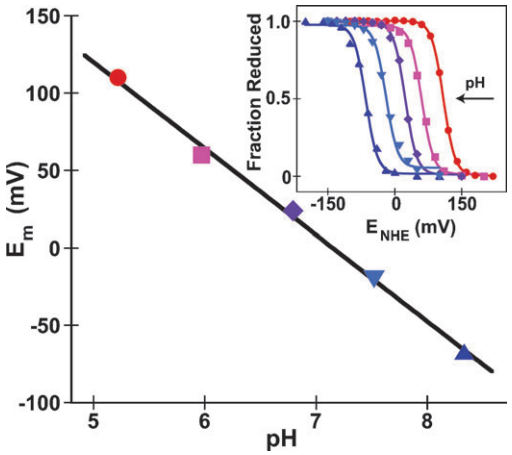

Fig. 2 Oxidation–reduction of heptaNaq. Line drawn is for a two-electron two-proton coupled redox center (−58 mV/pH at 20 °C). Inset: Spectroelectrochemical data fit to the two-electron Nernst equation from which the values of E_m were derived. See ESI for experimental conditions.†

naphthoquinone at 243 nm, and the isosbestic point at 252 nm as a function of redox potential (Fig. 2). Naq oxidation–reduction exhibits a two-electron reversible Nernstian behavior (n = 2.0) over the pH range 5.2–8.3 at 20 °C. The pH dependence of the E_m (−58 mV/pH unit) of heptaNaq matches the theoretical two-electron, two-proton coupled oxidation–reduction expected for quinones over this pH range.[24] The E_m of heptaNaq is −20 mV at pH 7.5, a value comparable to that of the Naq analogue 2-methylnaphthoquinone (E_m −47 mV at pH 7.5). The modest difference can be explained by subtle environmental effects caused by the peptide or more specific hydrogen bonding interactions of the Naq side chain polar groups with the backbone of the peptide.

Observation of proton-limited redox potentials was accomplished using cyclic voltammetry in the aprotic solvent dimethylformamide. Under slow scan rates, heptaNaq oxidation–reduction peaks display clear scan-rate dependency likely to originate from restricted protonation by protons associated with the peptide itself. Indeed, at 50 mV s^{-1} a "protonation peak" is observed indicative of the availability of protons (Fig. 3). Moreover, although in this scan rate range, the half-wave potential ($E_{1/2}$ −328 mV *versus* NHE) is significantly depressed relative to the aqueous midpoint, a complex ece(c) electrochemical mechanism is evident which is absent from studies of 2-methylnaphthoquinone under identical conditions (data not shown). However, with increasing scan rates both anodic and cathodic peaks split into two one-electron steps and approach scan rate independence consistent with kinetic bypass of protonation. Thus, at fast scan rates in the range of 2 V s^{-1} the two proton uncoupled half-wave potentials are −362 mV and −1088 mV (*vs.* NHE), again modestly positive of the values of 2-methylnaphthoquinone in anhydrous, aprotic DMF (−408 mV and −1142 mV *versus* NHE).[23] The difference in electrochemical potentials between heptaNaq and 2-methylnaphthoquinone can as before be explained by redox state dependent interactions of Naq with the peptide or by the possible presence of minute amounts of residual water in the experimental setup.[25]

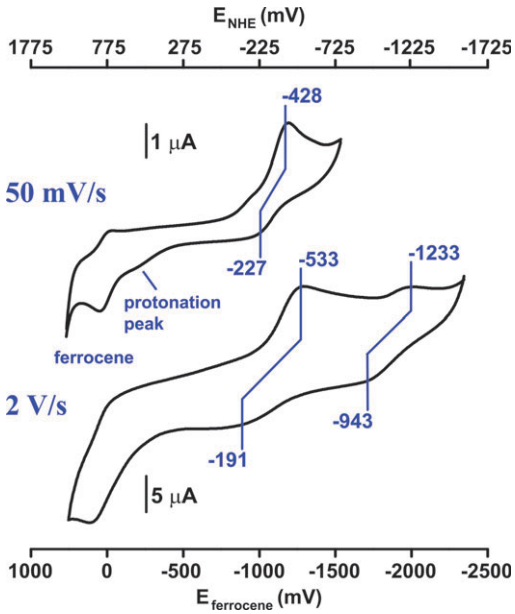

Fig. 3 Cyclic voltammetry of heptaNaq in dimethylformamide. Slow (50 mV s^{-1}, top) and fast (2 V s^{-1}, bottom) scan rates reveal proton dependent and independent redox-couples. Peak potentials are presented relative to NHE using the ferrocene couple (766 mV *versus* NHE)[23] as an internal standard. See ESI for experimental details.†

The ease with which Naq can be synthesized and incorporated into robust protein scaffolds offers new ways to examine the interplay between the protein structure and quinone redox and protonation states without the immense complexity attendant with natural quinone proteins. Electrochemically, the two-electron two-proton couple of Naq is central to the known range of biological redox cofactors and near the midpoint of the quinones critical to respiration and photosynthesis. Thus Naq is well positioned to foster new concepts and constructions of multicofactor artificial proteins incorporating Naq and designed to explore mechanistic aspects of natural oxidoreductases, or to modulate quinone properties for novel specific catalytic purposes. Potentials observed in the aprotic electrochemical studies of heptaNaq represent an upper limit on the expected values for these couples within a protein matrix, supporting the utility of Naq in very low potential electron transfer chains within a protein core. In addition, the proton-decoupled electron transfer potentials are favourable for the implementation of constructs using Naq as a very low potential reductive intermediate for the generation of stable strongly reducing fuels such as hydrogen.

The authors thank Tammer A. Farid, Anne K. Jones and Christopher C. Moser for useful discussions. This work was supported by DOE Grant DF-FG02-05ER46223; USPHS Grant GM41048 (P.L.D.); NSF-MRSEC Grant DMR05-20020.

Notes and references

¶ The Greek character quoppa (ϙ) was chosen as the one-letter code for Naq.
‖ We suspect that in aqueous acid the methoxy groups are substituted with hydroxyls which results in oxygen dependent quinone polymerization.
** Interestingly, the 9-methylenefluorene by-product is polymerized if the crude reaction mixture sits.
†† We believe that the hypervalent iodine species react selectively with tryptophan and tyrosine due to the lower steric bulk of the covalent intermediate formed with these amino acids.

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