'Q-wires': Synthesis, electrochemical properties and their application in electro-enzymology

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CHAPTER 1

General Introduction

1.1 Redox enzymes and the respiratory chain

Redox (reduction/oxidation) enzymes play a fundamental role in the metabolism of all organisms. The reactions that are catalyzed by this class of enzymes are characterized by the transfer of electrons between substrates. In many redox enzymes, however, the active sites that are involved in the (half-)reactions are located relatively far apart, and – in order to facilitate sufficiently fast electron transfer through the insulating protein matrix between the active sites – (additional) cofactors are required [1]. These redox active prosthetic groups form an 'electron pathway' through the enzyme when located sufficiently close to each other; the edge-to-edge distance between the cofactors should not exceed 14 Å, the limiting distance for electron tunneling through a protein matrix [2]. The great diversity of cofactors encountered in redox enzymes allows for a broad reduction potential window that can be utilized for electron transfer reactions [3].

In prokaryotes, a number of redox enzymes associated with the cytoplasmic membrane participate in aerobic or anaerobic respiration. These enzymes are therefore referred to as respiratory enzymes, pertaining to a respiratory chain. Together, they form an electron transport pathway, starting with respiratory dehydrogenases, oxidizing relatively low-potential substrates, such as NADH or succinate, and transferring the liberated electrons to the 'quinone pool'. In the extensively studied model organism *Escherichia coli*, the quinone pool consists of ubiquinone, menaquinone and demethylmenaquinone: very lipophylic electron mediators that are located in the cytoplasmic membrane. They transport electrons to a second group of enzymes: terminal reductases, which transfer the electrons to a final, relatively high-potential electron acceptor, such as oxygen, DMSO, fumarate or nitrate. The purpose of the respiratory chain is to conserve the energy released by the inter- and intra-enzymatic electron transfer reactions. Either through proton pumping by a respiratory complex or by means of the redox-loop mechanism, energy is stored by generating an electrochemical proton gradient across the cytoplasmic membrane. This

energy can then be used for, for example, the synthesis of ATP or flagellar motion [4].

The composition of the respiratory chain can vary considerably, depending on the availability of electron donors and acceptors. Under aerobic conditions, for instance, the favored terminal quinol oxidase by *E. coli* is cytochrome *bo₃*, accepting electrons form ubiquinol and using oxygen as electron sink. In the absence of oxygen, alternative terminal reductases are expressed, such as fumarate reductase and DMSO reductase [4, 5, 6]. In this study, the latter three *E. coli* enzymes, together with *E. coli* succinate dehydrogenase, will be subjected to electrochemical experiments (see chapter 5). Figure 1 provides a schematic depiction of the aforementioned enzymes.
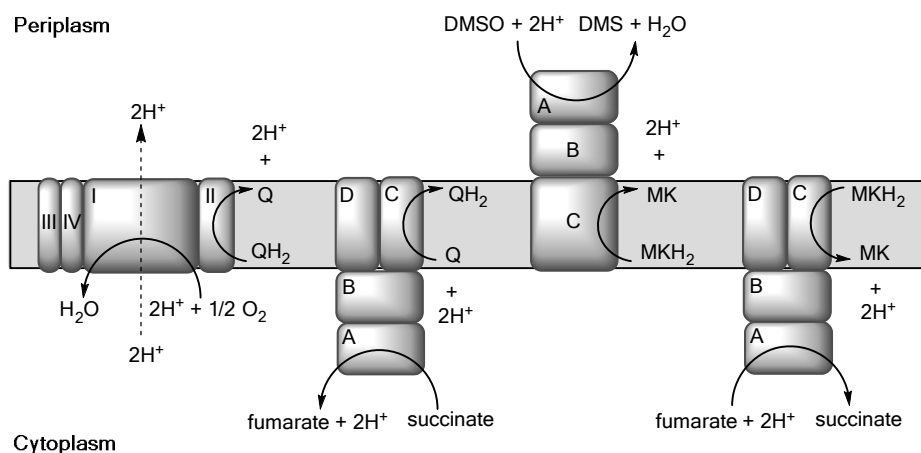

Figure 1 Schematic representation of membrane-associated *E. coli* redox enzymes involved in respiration (adapted from e.g. [4]). Note that co-expression of these enzymes is highly unlikely. From left to right: cytochrome *bo₃* (subunit I-IV); succinate dehydrogenase (subunit A-D); DMSO reductase (subunit A-C); fumarate reductase (subunit A-D). The dotted, vertical arrow represents proton pumping; the curved arrows represent two-electron transfer. Q/QH_2: ubiquinone/ubiquinol (for molecular structure of Q, see figure 4); MK/MKH_2: menaquinone/menaquinol

Enzymological research is often focused on the study of substrate conversion. However, the other half of the catalytic cycle, in which the active site is regenerated by intramolecular electron transfer and where processes driven by this electron flow take place, remains poorly explored,

since these processes are difficult to address in solution. Only slow and indirect control of the redox processes in the enzymes can be achieved with freely diffusing electron-carrying mediators [7, 8]. This study aims to overcome these complications. During voltammetric measurements, a direct, well-defined and non-rate-limiting electron transfer pathway between the enzyme and an electrode will be established by means of molecular wires, which will be described in detail below. Anchoring redox enzymes to the electrode surface using these conductive wires abolishes the need for slowly diffusing mediators, and the direct and well-defined 'communication' between electrode and enzyme could ultimately allow for the unraveling of the mechanism of redox-coupled processes and proton-coupled electron transfer in the aforementioned large respiratory enzyme complexes [9-30].

1.2 Electrochemistry: cyclic voltammetry

Cyclic voltammetry is a widely used and versatile electroanalytical technique, performed using a three-electrode setup: the potential between a working electrode (e.g. a gold disk electrode) and a reference electrode (e.g. a saturated calomel electrode (SCE)) is controlled by means of a potentiostat, while measuring the resulting current between the working electrode and an auxiliary (or counter) electrode, the latter often simply consisting of a platinum wire, contacting the electrolyte in which both the working and reference electrode are immersed. In a typical experiment, a redox active compound of interest is added to the electrolyte or adsorbed onto the working electrode surface, after which the potential of the working electrode is swept linearly in time between two extreme 'switching' potentials in a cyclical fashion. An important parameter is the 'scan rate' (in V/s), which determines the rate with which the potential is swept between the switching potentials [31]. Figure 6 exemplifies a working electrode modified with a surface-confined redox active compound (i.e. a 'Q-wire', introduced below). By cycling the potential within an appropriate potential window, the compound is repeatedly reduced and oxidized, through a two-electron/two-proton redox reaction.

Following IUPAC conventions, when sweeping from a low to a high switching potential, a reduced compound is (re)oxidized at the electrode surface, resulting in a positive, anodic current. A negative, cathodic current is measured when the oxidized compound is (re)reduced during the reverse potential sweep. The desired voltammogram is obtained by plotting the measured current versus the applied potential. The resulting peaks, representing the measured anodic and cathodic currents, contain a wealth of information, as will be discussed in chapter 4 and 5 [31, 32].

In this study, a somewhat unconventionally sized three-electrode setup was used, permitting microscale electrochemistry: the electrodes are mounted in a 'Hagen cell' (figure 2 [33]), after which a very

Figure 2 Schematic representation of the 'Hagen cell' [33] used in this study. RE, WE, CE: reference (sat. calomel), work (Au disk) and counter electrode (Pt wire), respectively; (*a*) ~25 µl electrolyte; (*b*) glass cell containing controllable gas mixture; (*c*) injection port; (*d*) gas mixture inlet; (*e*) gas mixture outlet to O_2 sensor; (*f*) gas-tight CE holder (*g*) gas-tight WE holder

small volume of electrolyte (~25 µl or less) is confined between reference and working electrode. Prior to voltammetric experimentation, the cell is flushed with an appropriate gas mixture (typically ~100% argon for anaerobic measurements). The small scale of the setup allows for minimization of the amount of required (precious) material (i.e. enzymes).
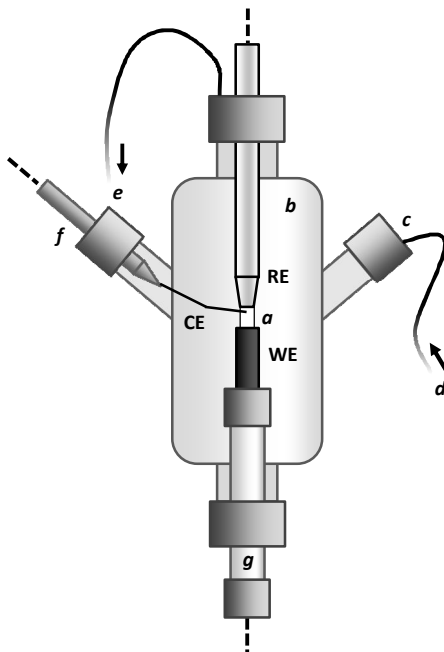
1.3 Electro-enzymology: immobilizing redox proteins

When redox enzymes are subjected to electro-enzymological experiments, an abundance of biochemically relevant information can be obtained; electron transfer within and between enzymes can be directly measured, as well as the catalytic current due to substrate conversion by the enzyme. In order for such experiments to be meaningful, well-defined and optimized interactions between electrode and redox enzymes are essential. Clearly, enzyme stability is of prime concern; direct contact with the (metallic) electrode surface often renders the enzyme inactive. Furthermore, slow diffusion of generally large proteins and transient interactions with the electrode surface complicate the interpretation of the measured data [7, 8].

A remedy addressing the aforementioned obstacles lies in the modification of the electrode surface. Gold electrode surfaces, for instance, can be modified using alkane thiols, which form self-assembled monolayers (SAMs) onto the surface by means of sulfur-gold bond formation [34]. Although the resulting SAM may prevent detrimental interactions with the electrode, the increased distance between enzyme and electrode may substantially limit electron transfer rates.

Further surface modifications may aid in the immobilization/adsorption of redox enzymes, eliminating the complications associated with slow protein diffusion. These modifications must ensure electrode-protein interactions that are both intimate and well-defined; in order for sufficiently fast electron transfer to occur, an appropriate cofactor or active site of the redox enzyme needs to be brought in close proximity to the electrode surface. Hence, proper orientation of the enzyme on the electrode surface is pivotal, while simultaneously preventing the electrode's potentially damaging effects on the enzyme. Although these requirements appear contradictory, several enzyme immobilization strategies are still capable of satisfying them [9-30].

One such strategy lies in the utilization of 'molecular wires' that facilitate fast electron transfer between the electrode surface and the enzyme. Figure 3 depicts two examples of such wires capable of binding azurin, a blue copper protein involved in electron shuttling between enzymes [35]. Both wires are highly conjugated, which – as will be discussed below – enhances electron tunneling, allowing for fast electron transfer over larger distances. The terminal methyl
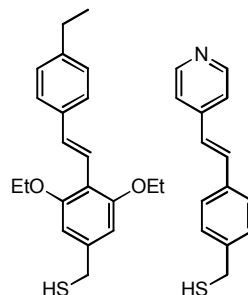

Figure 3 Two examples of molecular wires capable of binding azurin non-convalently [35, 36]

thiol permits the binding to gold electrode surfaces. The ethyl-terminated wire ensures non-covalent interactions of the ethyl moiety with a hydrophobic region near the copper (I/II) cofactor, resulting in (indirect) adsorption of azurin to the electrode [35], whereas the pyridine-terminated wire coordinates with the copper immediately [36]. Indeed, superior electron transfer rates were found for the former wire, in comparison with decanethiol, which is of similar length and also capable of binding azurin non-covalently [35]. In summary, the strategy outlined here ensures: a proper orientation of the enzyme with respect to the surface; the prevention of direct exposure to the bare electrode surface; and fast electron transfer over greater distances, making this strategy suitable for even deeply-buried cofactors/active sites. These requirements were also central in the design of the molecular wires presented in the following section.

As mentioned above, the immobilization/adsorption of redox enzymes onto an electrode surface circumvents the problems associated with slow difussion, enabling measurement of fast reactions. In 'protein film voltammetry' (PFV), such a (sub)monolayer 'film' – consisting of a stably adsorbed protein of interest on an electrode surface – is subjected to (cyclic) voltammetry experiments. Advantages of PFV include the possibility of fast screening under different (and extreme) circumstances: the modified electrode can be shortly exposed to different solutions of e.g. different (and extreme) pH. In addition, being directly controlled by the electrode

potential, the redox states of the entire enzyme sample can be synchronized and fine-tuned, thus allowing for unprecedented control over the redox states of the cofactors located within the immobilized enzymes. Finally, since only a (sub)monolayer of enzyme is required, very small amounts of protein are needed (1-10 pmol/cm^2). The surface concentration of the enzyme, however, is usually very high, enhancing the sensitivity of the measurements [37].

Besides their use in fundamental electrochemical research, enzyme immobilization techniques, as described in this section, may be of commercial interest as well. The formulation of well-defined, stable protein films on electrode surfaces should provide crucial advantages over current techniques in terms of stability, precision, accuracy and sensitivity, paving the way for new generations of biosensors and biofuel cells.

1.4 'Q-wires': quinone-terminated OPV molecular wires

The ultimate objective of this study was to immobilize respiratory membrane enzymes onto a rationally designed electrode surface, where the required surface modifications were to ensure fast, non-rate-limiting electron transfer. To achieve this, novel molecular wires were designed, capable of binding a quinol dehydrogenase or quinone reductase with one side, while binding a gold electrode surface with the other. Electrode surfaces modified with these 'Q-wires' (where 'Q' stands for 'quinone') are then expected to be capable of binding said enzymes by directly 'plugging' the substrate-mimicking terminus of the wire into the enzyme's quinone binding site. Immobilizing redox enzymes in this fashion results in direct and well-defined 'communication' between electrode and enzyme, which could ultimately aid in the unraveling of the mechanism of redox-coupled processes and proton-coupled electron transfer in these enzymes.

To appreciate the bio-mimetic design of the Q-wires, they are compared to the naturally occurring ubiquinone-8 in figure 4. Ubiquinone-8 is a substrate for many respiratory complexes.
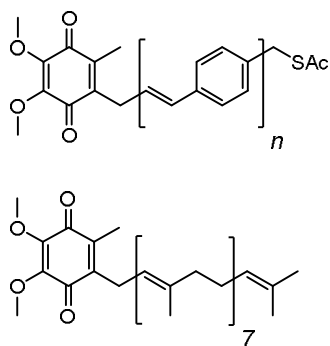
Figure 4 Comparison between naturally occurring ubiquinone-8 (below) and a ubiquinone-terminated 'Q-wire' (top, with acetylated (Ac) thiol), reflecting the biomimetic design of the Q-wires
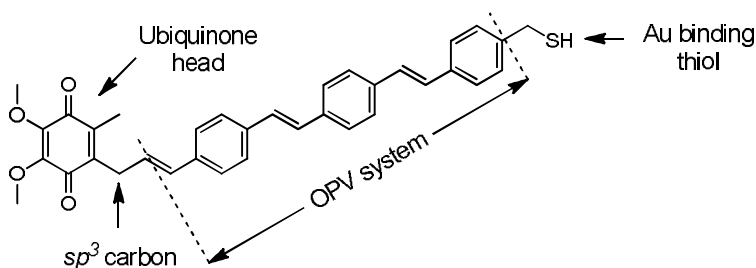

Figure 5 Composition of a ubiquinone-terminated 'Q-wire': a ubiquinone moiety is tethered to a highly conjugated OPV molecular wire, which terminates in a gold-electrode-binding (methyl)thiol. An sp^3 carbon disrupts the conjugation between the head group and the rest of the wire to preserve its electrochemical characteristics and to ensure the biocompatibility presented in figure 4

As depicted in figure 5, the tether consists of a conjugated molecular wire, functionalized on one end with a thiol for immobilization onto gold, and on the other end with a ubiquinone (or menaquinone) moiety, which inserts into the substrate binding pocket of the enzyme. Should this binding prove stable, this strategy would yield a well-defined, vectorially immobilized and homogeneous protein monolayer, with several major advantages: a natural electron entry point and relay pathway is provided; the electrode functions as an artificial quinone/quinol pool, with the important advantage of full control over the redox state of the wires. Furthermore, the second, membrane-extrinsic substrate binding site faces solution and remains fully accessible. Finally, the conjugated wire allows for very fast interfacial

electron transfer [38, 39], enabling the measurement of potential-dependent enzyme kinetics over a broad time scale. In this way, the natural electron transfer pathway and coupled reactions can be studied by tuning the driving forces, both from the side of the quinone/quinol (electrode potential) and from the side of the soluble substrate (concentrations, inhibitors, pH, etc.).
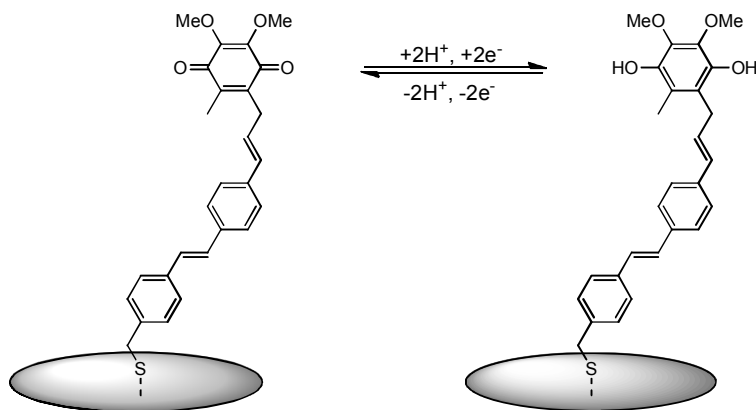

Figure 6 Schematic representation of a 'Q-wire' confined to a gold electrode surface, undergoing a two-electron/two-proton redox reaction. At low electrode potential, the head group exists in an oxidized state (left), while, at high electrode potential, the head group exists in a reduced state (right)

The introduction of a conjugated wire – here oligo(phenylenevinylene) (OPV) – is essential, because electron tunneling through a non-conducting alkanethiol becomes prohibitively slow with increasing wire length [35, 40-42]. However, direct conjugation of the quinone moiety to the OPV system influences the near-native quinone/quinol redox potential, as observed previously [43, 44]. Therefore, a saturated methylene bridge was introduced in order to uncouple the quinone from the conjugated wire. This methylene bridge also introduces a larger degree of rotational freedom for the quinone, which may aid in its interactions with enzymes. Binding may be further facilitated by the similarity of the first three carbon atoms (allyl) to the natural isoprenoid tail.

Figure 6 provides a schematic representation of a 'Q-wire' (i.e. U_2) undergoing a two-electron/two-proton reduction/oxidation reaction, which will be discussed in chapter 4. Figure 7 depicts the 'Q-wires' that were synthesized for this study, differing in length and quinone moiety (ubiquinone or menaquinone). The naming convention presented there will be used throughout this work.
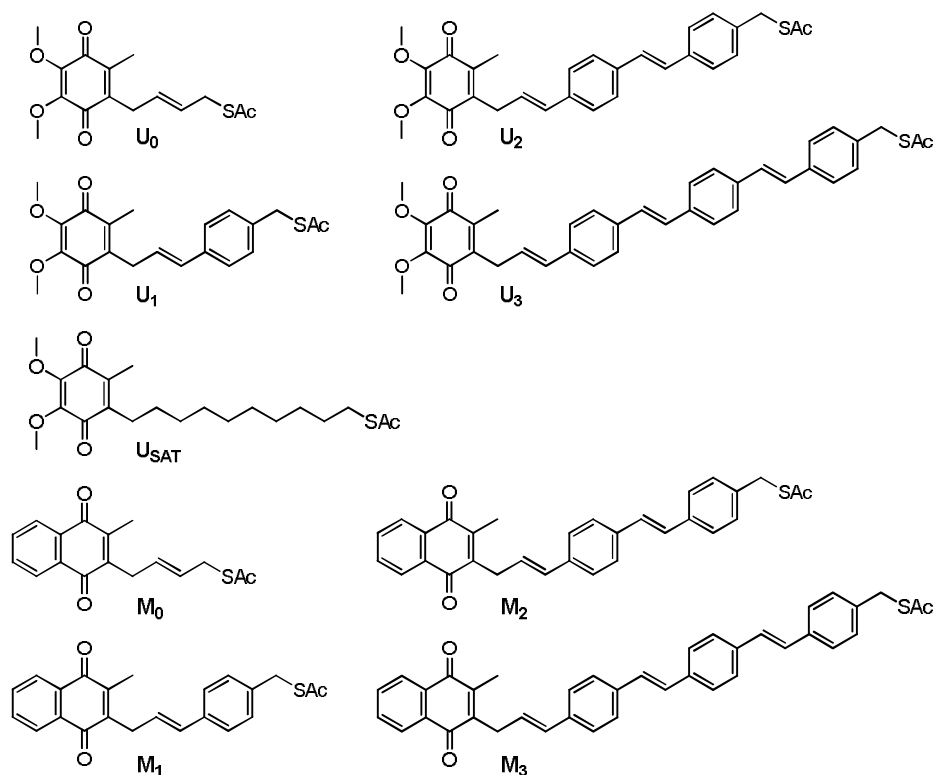

Figure 7 'Q-wires' synthesized for this study, differing in length and quinone moiety (ubiquinone (U) or menaquinone (M)). The naming convention (U_0-U_3, U_{SAT} and M_0-M_3) presented here will be used throughout this work

1.5 Thesis outline and scope

An objective of this research was to achieve direct, well-defined and non-rate-limiting electron transfer between respiratory enzymes and the electrode surface by means of 'Q-wires', which have been introduced

above. Ensuring direct and fast electron transfer, these molecular wires may ultimately be part of a series of electrode surface modifications leading to the complete, stable and well-defined immobilization of an enzyme of interest. Realization of such a stable protein film may aid in the elucidation of the enzyme's mechanism and may perhaps lead to (commercially viable) applications, such as biosensors or biofuel cells.

As will be described in chapters 2 and 3, a crucial step in the synthesis of the 'Q-wires' was the joining of two intermediate compounds by means of a Grubbs olefin metathesis. Usually being the final step, complications associated with said reaction forced a thorough reconsideration of the synthesis route. These efforts, which will be detailed in chapter 2, culminated in a final synthesis pathway, elaborated on in chapter 3. Although still based on a Grubbs metathesis reaction, the observation of certain consistent behavior of this reaction inspired the formulation of a new synthesis pathway, which ultimately allowed for the successful synthesis of the desired products.

As mentioned previously, the 'bridge' part of a Q-wire, which connects the quinone moiety to the electrode-binding thiol, consists of oligo(phenylenevinylene) (OPV), a highly conjugated moiety. In chapter 4 – as part of the electrochemical characterization of the Q-wires – it will be investigated whether the inclusion of an OPV section indeed enhances electron transfer rates, in comparison with fully saturated bridges. Additionally, the influence of bridge length on electron transfer kinetics will be assessed. Finally, the mechanism of the overall two-electron/two-proton reaction of the quinone head group will be investigated.

In chapter 5, the electro-enzymology of four *E. coli* respiratory enzymes – succinate dehydrogenase, fumarate reductase, DMSO reductase and cytochrome bo_3 ubiquinol oxidase – will be explored by means of cyclic voltammetry. Crucially, the Q-wires will be employed to provide electron transfer between electrode and enzyme. As will be discussed in this chapter, difficulties associated with reproducibility allowed only for qualitative analysis. Further optimizations are therefore still required to achieve quantitative electro-enzymology. Moreover, the stability of the

binding of the enzymes by the Q-wires remained unclear, perhaps suggesting a need for additional electrode surface modifications that result in full enzyme immobilization and allow for true PFV.

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