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Detection and interpretation of redox potential optima in the catalytic activity of enzymes

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Abstract

It is no surprise that the catalytic activity of electron-transport enzymes may be optimised at certain electrochemical potentials in ways that are analogous to observations of pH-rate optima. This property is observed clearly in experiments in which an enzyme is adsorbed on an electrode surface which can supply or receive electrons rapidly and in a highly controlled manner. In such a way, the rate of catalysis can be measured accurately as a function of the potential (driving force) that is applied. In this paper, we draw attention to a few examples in which this property has been observed in enzymes that are associated with membrane-bound respiratory chains, and we discuss its possible origins and implications for in vivo regulation.

Keywords: Oxidoreductase; Voltammetry; Respiration; Electron transport; Redox enzyme; Electrochemistry

1. Introduction

A long-established observation in enzyme kinetics is the way in which catalytic activity is optimised at a certain pH, typically giving rise to the familiar bell-shaped curves that were first explained nearly a century ago [1,2]. For redox enzymes, another controlling factor aside from pH is the electrochemical potential, which reflects the availability of reducing or oxidising equivalents, and which may be relevant both mechanistically and physiologically—for example with enzymes involved in respiratory chains; but it is generally much more difficult to measure catalytic activity under conditions of controlled redox potential than it is to explore pH effects with a buffered solution. Studies of enzyme kinetics in the 'potential domain' become relatively straightforward, however, with a technique called 'protein film voltammetry' (PFV) [3,4]. By linking the enzyme directly to an electrode in such a way that electron exchange is rapid, the catalytic current that is observed when a substrate is added relates easily to catalytic rate, and it becomes possible to probe, with high precision, the way in which this activity depends on potential. Useful results can be obtained even if the number of enzyme molecules adsorbed (and thus communicating with the electrode) is too low to observe stoichiometric signals from redox-active sites in the absence of substrate [5]. All that is required is that the enzyme activity is high; since this provides a large amplification of the current, which relates directly to rate in either reaction direction (oxidation or reduction).

Several enzymes have been found to exhibit interesting current-potential dependences in which an optimum rate occurs at a particular potential and the rate thereafter drops even though the thermodynamic driving force is increased.

Abbreviations: DMSO, dimethylsulfoxide; DmsABC, the membrane-bound DMSO reductase in *E. coli*; DQ, demethylmenaquinone; EPR, electron paramagnetic resonance; MQ, menaquinone; Mo-bisMGD, Mo-bismolybdopterin guanine dinucleotide; NarGHI, the membrane-bound nitrate reductase in *E. coli*; NarGH, the membrane-extrinsic subdomain of NarGHI; PFV, protein film voltammetry; PGE, pyrolytic graphite edge; SDH, succinate dehydrogenase; SQR, succinate quinone oxidoreductase; UQ, ubiquinone

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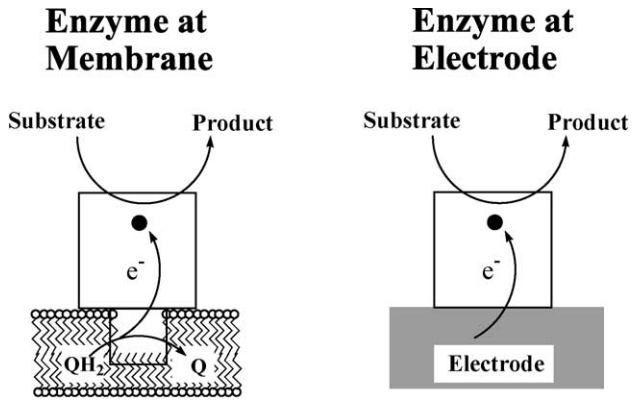


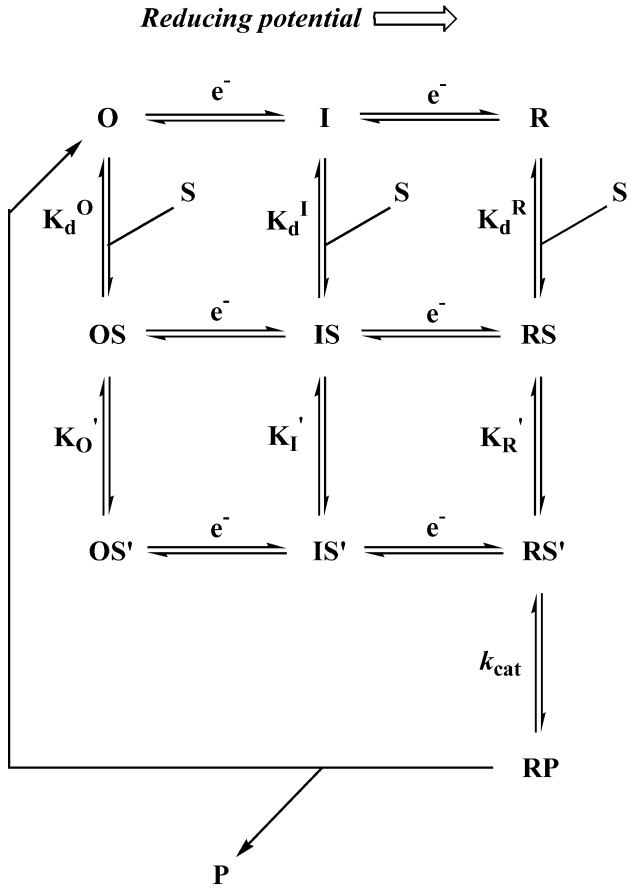
Fig. 1. Cartoon showing: (left) enzyme associated with a membrane and reacting with the quinone pool; (right) enzyme (membrane-extrinsic subdomain) adsorbed at an electrode and equilibrating with the electrochemical potential that is applied from a potentiostat.

This effect was first described for the soluble form of succinate quinone oxidoreductase (SQR) from beef heart mitochondria, and has since been observed in other enzymes, notably dimethylsulfoxide (DMSO) reductase and the SQR from *E. coli* and nitrate reductases from different sources [6–11]. These represent important respiratory enzymes, in which electrons from an aqueous substrate are exchanged with the membrane-bound quinone pool.

We may consider the membrane to provide a situation akin to that of interfacial electrochemistry with a thermodynamic driving force that is defined in part by the composition and oxidation level of the quinone pool, i.e. the ratio [Q]/[QH_2]. (We will not be concerned in this paper with the vectorial nature of membrane potentials.) *E. coli* and other facultative enteric bacteria synthesise three types of quinone: a benzoquinone, i.e. ubiquinone (UQ: E_{m7}=+100 mV), and two naphthoquinones—demethylmenaquinone (DQ: E_{m7}=+36 mV) and menaquinone (MQ: E_{m7}=−74 mV) [12]. The ratios of these components depend on the status of aerobicity, and the resulting quinone-based electrochemical potential can span a range of more than 200 mV [13]. As suggested in Fig. 1, there is an persuasive relationship between the action of an enzyme when bound at a membrane and at an electrode surface: consequently, exploiting the fact that isolated membrane-extrinsic subcomplexes can be adsorbed in an active form at certain electrodes, we are able to study their kinetics in the potential domain and relate the results to what might occur at a membrane. As we describe in this paper, use of the PFV technique reveals some subtle catalytic effects that prompt interesting mechanistic and physiological questions.

2. How activity-redox potential optima can arise

Potential optima can arise in different ways, but for simplicity, it is useful to consider a generic catalytic system such as that shown in Scheme 1, in which a substrate S is reduced. Here, the active site can exist in three different oxidation states, O, I and R (oxidised, intermediate and reduced), as exemplified by flavins and Mo-pterin cofactors. Each of these oxidation states has characteristic properties of substrate/product affinity (K_d) and equilibrium constants (K') of other processes that are mechanistically crucial. The catalytic pathway can take several different routes across the grid, influenced by the electrochemical potential which controls the rate and thermodynamics of electron supply for different states. As an example, a redox potential optimum might be expected if a process such as substrate binding is much more favorable for the O or I states than for the R state: at high potential, no reaction will occur because there is no overall thermodynamic bias; whereas application of a potential that is too low (and expected to drive the reaction more favourably) would also produce no reaction because the enzyme is forced into the relatively inactive R state. For an enzyme bound at an electrode, it is important that neither interfacial electron transfer nor transport of substrate is rate-limiting, otherwise the effect of the catalytic pathway is masked.



Scheme 1. Grid indicating the different reaction pathways that can be followed during the two-electron reduction of a substrate by an enzyme with an active site that can exist in three oxidation states O, I and R. The grid allows for O, I and R having different equilibrium constants for substrate binding (K_d) and other undefined transformations (K').

An obvious extension of this idea includes the effects of additional electron-transfer centers such as hemes or Fe–S clusters and the possibility that catalytic activity may depend upon their prevailing redox states. These influences can enhance (boost) the rate as well as depress it, so they provide further complexity that can be detected by voltammetry [14].

Far from being unusual, the appearance of a potential optimum for activity ought to be a common phenomenon in redox enzyme kinetics. But redox potential optima will usually evade detection because conventional steady-state kinetics typically utilize an excess quantity of standard electron donors or acceptors without any control or knowledge of what the effective electrochemical potential is. It is likely that in vivo, the prevailing steady-state oxidation levels of the active sites in quinone-oxidoreductases are determined largely by the composition of the quinone pool which has a fairly narrow potential window. By contrast, conventional solution assays utilise agents such as methyl viologen ($E_{m7} = -0.44$ V) or $Fe(CN)_3^{3-}$ ($E_{m7} = +0.4$ V) which each provide a large (and physiologically unlikely) driving force.

3. Results

3.1. Nitrate reductase

The membrane-bound dissimilatory nitrate reductase NarGHI of *E. coli* catalyses the oxidation of quinols by nitrate under anaerobic conditions. The active site is a Mo-bismolybdopterin guanine dinucleotide (Mo-bisMGD) cofactor located in subunit NarG [15]. Subunit NarH contains four Fe–S clusters, while NarI is a membrane anchor that coordinates two b-type hemes and contains the site of quinol binding. The detergent-solubilised holoenzyme can be adsorbed on a pyrolytic graphite 'edge' (PGE) electrode, at which it displays electrocatalytic activity in the form of a large reduction current that flows upon addition of nitrate to the solution.

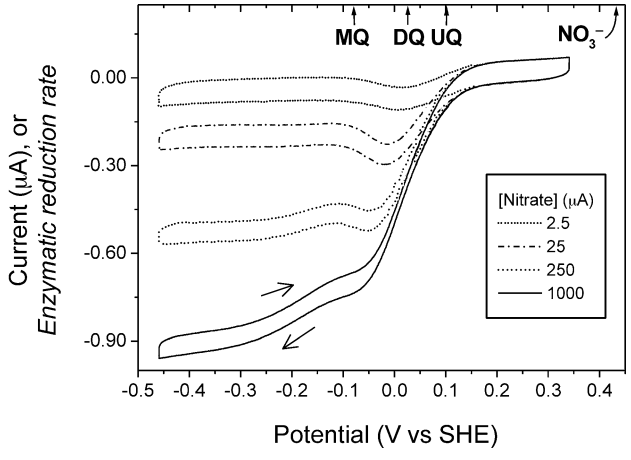


Fig. 2. Catalytic voltammograms of *E. coli* nitrate reductase adsorbed on a pyrolytic graphite 'edge' electrode, showing how the shape of the wave (and therefore the potential dependence of the catalytic activity) depends on nitrate concentration. Conditions: pH 7 and 30 °C. Arrows by the bottom trace indicate the direction of potential cycling, and apply to all the results in this paper. The reduction potentials of the various substrates appropriate for pH 7 are indicated.

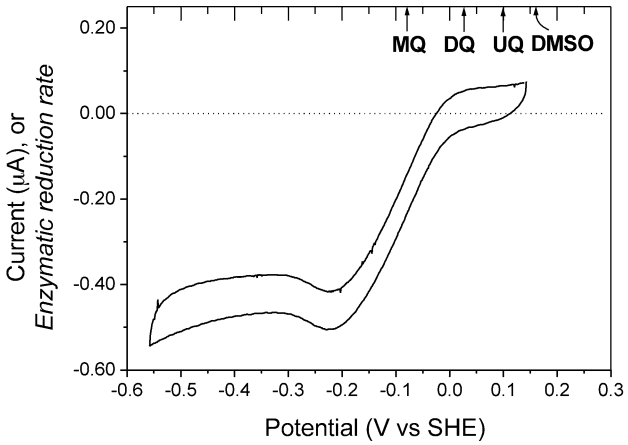


Fig. 3. Catalytic voltammogram of *E. coli* DMSO reductase adsorbed on a pyrolytic graphite 'edge' electrode, in the presence of 20 mM DMSO, pH 7, 25 °C. The reduction potentials of the various substrates appropriate for pH 7 are indicated.

Fig. 2 shows results obtained at pH 7.0, from which it is clear that the shape of the voltammogram depends in an unusual way upon the electrode potential and upon the nitrate concentration. At low nitrate levels (i.e. a few micromolar) the reduction current reaches a maximum at a potential of about 0 mV and decreases to a low value as the potential is lowered, even though the driving force for nitrate reduction is increasing. In other words, the catalytic activity is optimized at 0 mV. Upon reversing the scan direction, this response is retraced, even at quite high scan rates, showing that the process responsible for the change in activity is fast. At higher nitrate levels, the activity at more negative potentials increases, eventually exceeding that observed at higher potential and reaching a limiting value at approximately −300 mV in the presence of 1 mM nitrate. Identical wave shapes are observed for a sample of NarGH, which consists only of the membrane-extrinsic components, thus showing that the behaviour does not arise from properties/components of subunit NarI. (In conventional assays, NarGH cannot catalyse oxidation of quinols, but it does catalyse reduction of nitrate by benzyl viologen.)

These results share similarities to those reported recently by Anderson et al. [11], who have studied the nitrate reductase from *Paracoccus pantotrophus*. This enzyme is highly homologous with that obtained from *E. coli*, and the analogous NarGH complex adsorbs on a graphite electrode to display catalytic activity. They found that low levels of nitrate produced a peak-like response which disappeared as the nitrate concentration was raised, revealing a much higher sigmoidal activity. Importantly, they were able to observe similar behaviour using a gold electrode, thus making it highly unlikely that this behaviour arises from a potential-dependent change in the interaction with the

electrode. In conjunction with results from electron paramagnetic resonance (EPR) titrations, they suggested a model in which nitrate has a higher affinity for the Mo(V) state than for Mo(IV); therefore, as the potential becomes more negative, the weaker binding of substrate must be compensated for by using a higher nitrate concentration. In terms of Scheme 1, the substrate dissociation constant K_d^I is important in directing the reaction through the catalytic cycle. Alternatively, they suggested that the activity might be modulated depending on the oxidation level of one of the Fe–S clusters.

3.2. DMSO reductase

Like nitrate reductase, DMSO reductase (DmsABC) is expressed in *E. coli* under anaerobic conditions. DmsA contains the active site, Mo-bisMGD, while DmsB contains four Fe–S clusters, and DmsC is an intrinsic membrane 'anchor' [16]. From a detergent-solubilised solution, DmsABC adsorbs at a PGE electrode, and at pH 7 the resulting enzyme catalyses the reduction of DMSO, as shown in Fig. 3 [10]. The reverse reaction, oxidation of DMS, is not observed, even with a large excess of DMS, showing that the enzyme is highly biased to operate in the reduction direction. At pH 9, while DMSO reduction still occurs (Fig. 4A), a catalytic oxidation process can be observed when the more oxyphilic substrate trimethyl phosphine oxide is used (Fig. 4B). In both cases, the catalytic response is peak-shaped due to activity being optimized at a certain potential. The activities in either reaction direction yielded a catalytic potential 'window', to either side of which the activity is suppressed or even drops to zero. Potentiometric titrations monitoring the Mo(V) EPR signal suggested that this window correlated with the potential region giving the maximum level of Mo(V) [10]. However, unlike with nitrate reductase, there was little change in wave shape as the substrate concentration was varied; consequently, it is unlikely that the window arises due to preferential binding of substrate to the Mo(V) form, i.e. a particularly low K_d^I. Instead, it was proposed that other steps crucial to the catalytic cycle are facilitated when the Mo site is in this oxidation state [10]. In terms of Scheme 1, this would imply that process K_I' is an important gateway in the reaction.

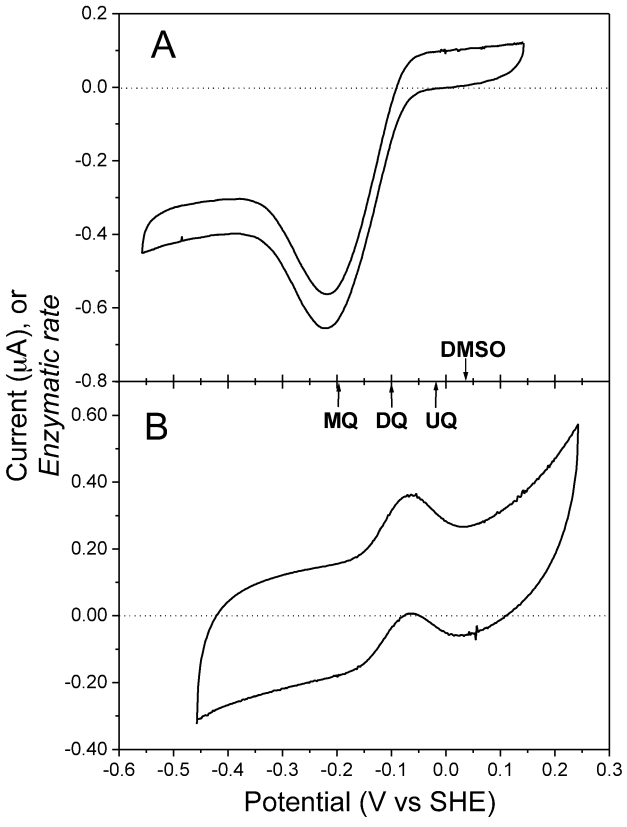


Fig. 4. Catalytic voltammograms of *E. coli* DMSO reductase adsorbed on a pyrolytic graphite 'edge' electrode and catalyzing (A) reduction of DMSO (20 mM) and (B) oxidation of trimethylphosphine oxide (10 mM). Conditions: pH 8.9, 25 °C. The reduction potentials of the various substrates appropriate for pH 8.9 are indicated.

3.3. Succinate:ubiquinone oxidoreductase

Succinate:ubiquinone oxidoreductase is found as a multisubunit membrane-bound complex (Complex II of mitochondria) comprised of two membrane-extrinsic subunits which contain the covalently bound active site FAD and three Fe–S clusters [17]. This 'catalytic' subdomain is bound to the membrane via membrane-intrinsic subunits that are essential for the reaction with ubiquinone and ubiquinol. The catalytic subdomain lacking the membrane anchors and called succinate dehydrogenase (SDH) can be obtained in soluble form. SDH catalyses succinate oxidation by water-soluble electron acceptors like $Fe(CN)_6^{3-}$ (but not quinones) and can be adsorbed on a PGE electrode. Addition of succinate to the solution results in a catalytic current that is due to the enzyme receiving electrons from succinate (which is converted to fumarate) and passing them to the electrode [6]. Results are shown in Fig. 5. The electrochemical response is sigmoidal and quite straightforward: i.e. as the potential is raised, succinate is oxidised more rapidly, until a limiting current plateau is achieved, the magnitude of which reflects the turnover number of the enzyme (Fig. 5A).

A very different result is obtained if fumarate is added, as shown in Fig. 5B. As expected, the reduction current increases as the potential is made more negative, but instead of a plateau, the current reaches a peak then drops as the potential is lowered further [6,8]. Upon scanning in the oxidative direction, this response is retraced, i.e. the enzyme activity switches back on. The peaks that are obtained lie at the same potential even at quite high scan rates, showing that the process responsible for this change in activity is fast. For equal concentrations of succinate and fumarate, it was easy to locate the equilibrium potential (E_{m7} for fumarate) from the point of zero net current, and inspection of the

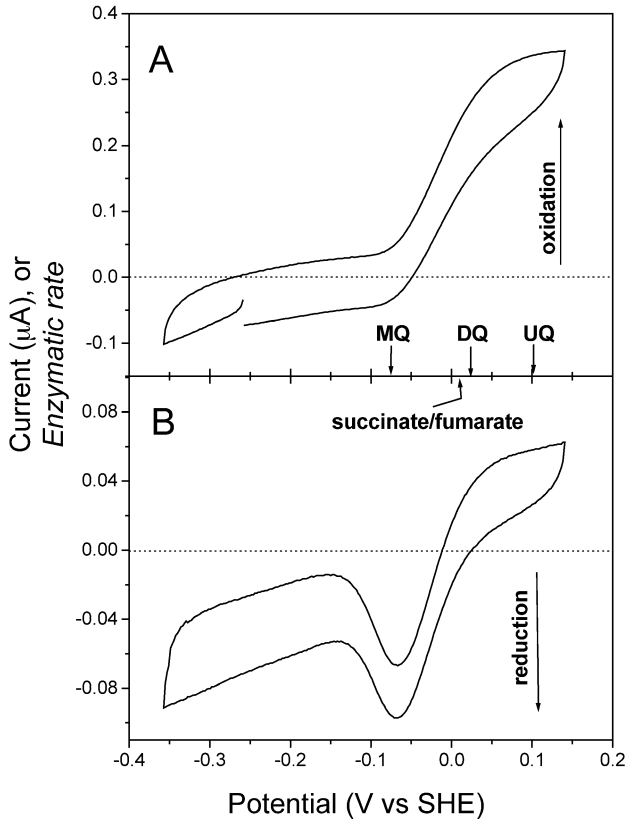


Fig. 5. Catalytic voltammograms of beef heart mitochondrial succinate dehydrogenase (membrane-extrinsic subdomain of SQR) adsorbed on a pyrolytic graphite 'edge' electrode. (A) Succinate 10 mM, pH 7.5, 38 °C. (B) Succinate 0.1 mM, fumarate 0.1 mM, pH 7, 38 °C. The reduction potentials of the various substrates appropriate for pH 7 are indicated.

current response in this region revealed that the enzyme is actually biased to function better in the direction of fumarate reduction than succinate oxidation. But as the potential is lowered, providing a higher driving force for fumarate reduction, this activity is suppressed. There was little change in wave shape as the concentrations of fumarate or succinate were varied, and a weak response with the same features and potential values was obtained when a gold electrode was used instead of PGE. It was noted that the current response resembles that of a device known as a tunnel diode, which displays negative resistance over a certain potential range [6]. Similar results were obtained using the analogous SDH from *E. coli*, but not the fumarate reductase [9,14]. The SQR from *E. coli* shows high similarity with the beef heart enzyme, particularly in terms of residues that are close to the active site [9].

Using a conventional solution assay, it was observed that the rate of oxidation of benzyl viologen radical increased as benzyl viologen was consumed (i.e. as the solution potential increased) [7]. On the basis of simulations, in which the best fit was obtained using a two-electron reaction for the switch in activity, the results were interpreted in terms of the enzyme being less active when the FAD was maintained in the reduced state. In terms of Scheme 1, this means that the process K_R' is less effective than the other pathways in the direction of fumarate reduction.

4. Do redox potential optima have any physiological relevance?

The question that stems from these results is whether these redox potential optima might have any physiological relevance. To be influential, the activities of any of these enzymes would have to be limiting factors in their respective electron-transport chains. In all three examples, modulation of activity by the electrode potential is essentially instantaneous and capable of providing a rapid feedback response. We may draw the analogy that like the electrode, the membrane provides a variable potential in the form of the ratio of quinone to hydroquinol (Q/QH_2). In *E. coli*, this potential will depend further on what quinones are present since, depending on aerobicity, at least three different quinones are synthesised: thus ubiquinone is the dominant redox carrier during aerobic growth, whereas menaquinone and demethylmenaquinone dominate under anaerobic conditions in which nitrate, DMSO or fumarate are used as electron acceptors [13]. To assist our discussion, the formal potentials of substrates relevant to each enzyme reaction are indicated in Figs. 2–5, and we have included those of the aqueous substrates.

For nitrate reductase, the interesting features of the voltammogram span the potential range defined by variable quantities of oxidised and reduced ubiquinone, demethylmenaquinone and menaquinone (Fig. 2). The low K_M activity that is optimized around 0 mV might still allow reduction of nitrate by the higher potential ubiquinol, while the high k_{cat}–high K_M activity at lower potential would be suited for menaquinol oxidation. With DMSO reductase, the results suggest that activity would be depressed if the level of reduced menaquinol were to reach a very high level, i.e. if $[MQ]/[MQH_2]$ became very low, although we note that the drop in activity is not very marked at pH 7 (compare Figs. 3 and 4A) . For SQR, one possible outcome would be that under anoxic conditions, any thermodynamic pressure to pass electrons back to reduce fumarate and thus reverse the direction of the citric acid cycle would be countered—the enzyme thus acting as a 'ratchet' [18]. Given the more oxidising nature of ubiquinone, it is unlikely that this pressure could be exerted effectively in mitochondria by a low $[UQ]/[UQH_2]$ ratio. However, the effect might be more influential in *E. coli*, in which there is a cocktail of quinones, and the SQR might experience redox potentials far below that produced in mitochondria [12].

At present, these suggestions remain hypothetical, but the observations are clear and reproducible. At an electrode, enzyme activity can be modulated in rapid response to changes in the electrode potential, and it is useful to consider how this may occur and what the implications are for linking activity to the status of the quinone pool in

respiratory membranes. This might give rise to various kinds of advantage in vivo, including feedback fine control of respiratory rates.

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