Assay of Enzymes with Insoluble or Unknown Substrates: The Membrane-Bound Quinone Reductases as an Example

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Abstract

The conventional assay method for the majority of enzymes envisages a reaction between substrates in aqueous solution. A measurable concentration of product is accumulated over time. This paradigm has served well for the characterization of many enzymes. Variations of the method, often using chromogenic or fluorogenic substrates, have been developed and are widely used for purposes such as clinical diagnosis and screening. There are some metabolically important enzymes for which the only published assay methods use artificial substrates. Some of these are oxidoreductases that use artificial mediators, and are listed in the EC list under EC 1.*x*.99. For computational reconstruction of the metabolism of a cell, however, it is necessary to use kinetic data from assays that reflect the physiological function in the cell, and the physiological substrates. For some oxidoreductases it is known, or considered likely that the acceptors are water-insoluble membrane-bound quinones such as ubiquinone or menaquinone, which present particular problems for measurement of kinetic parameters. Succinate dehydrogenase/fumarate reductase is considered as an example. The oxidoreductases from membranes must be rendered soluble by detergents, which alter their kinetic behaviour. Uncertainty about the way of measuring activity of such enzymes has led to confusion in textbooks and metabolic maps, such as the persistent myth that free FAD is the acceptor for succinate dehydrogenase and related enzymes. New strategies are discussed to measure electron-transfer flux, under conditions that reflect the physiological activity of

membrane-associated oxidoreductases. An example is direct electrochemistry of enzymes adsorbed onto carbon surface. In favourable cases this method is able to observe electron flux both within and through individual enzyme molecules. The kinetic parameters and substrate specificity of membrane-bound oxidoreductases may be obtained in this way.

Conventional Enzyme Assays

The STRENDA initiative aims for the standardization of enzyme assay protocols, with the prospect of simulating the metabolism of the cell by computational reconstruction of reaction pathways. It also underlines the need for the accurate classification of enzymes according to their physiological activities. The obvious first requirement for each enzyme is to know what its substrates are in the pathway. When determining the kinetic parameters, the natural substrates should be used, even if they are unstable, insoluble or expensive to produce. The assay method should reflect the conditions (pH, ionic strength, protein concentrations, etc.) in the cell for which the metabolism is being reconstructed. Ideally the flux through the enzyme should be measured in the presence of other metabolites, which might be allosteric activators or inhibitors, and of any proteins with which the enzyme interacts, all at the physiological concentration. At present, there are some enzymes for which the only published data fall well short of this ideal. Just as some enzymes are easier to assay than others, some enzymes are easier to classify than others. For some, the EC classification is incomplete, and their assay methods use artificial cosubstrates.

The classic enzyme assay, since the work of Michaelis and Menten, is one carried out by an enzyme in dilute solution, with a fixed, relatively high initial concentration of substrate(s) and in the absence of product. The reaction rate is determined by the change of concentration of product P or substrate S, for example by spectrophotometry:

$$v = d[P]/dt = -d[S]/dt \quad (1)$$

This approach is popular since it allows the accurate determination of catalytic parameters such as K_m and k_{cat}. As is well documented [1] however it deviates significantly from the situation in the cell where the enzyme substrate and product are in turnover but their concentrations do not change substantially.

The EC classification lends itself most easily to the description of enzyme-catalysed reactions involving up to two substrates, such as an X-transferase (EC class 2):

$$\text{X-donor} + \text{acceptor} = \text{donor} + \text{X-acceptor} \quad (2)$$

Oxidoreductases

For an oxidoreductase (EC class 1) the reaction can be written:

$$\text{reductant} + \text{oxidant} = \text{oxidized reductant} + \text{reduced oxidant} \quad (3)$$

the EC number of an enzyme of this type takes the form EC 1.*x.y.z*, where *x* represents the type of reducing substrate, and *y* the type of oxidant. The class EC 1 can be represented in terms of a matrix of *x* and *y*, as illustrated in Fig. 1, where the vertical (*z*) axis represents the number of enzymes of each type.

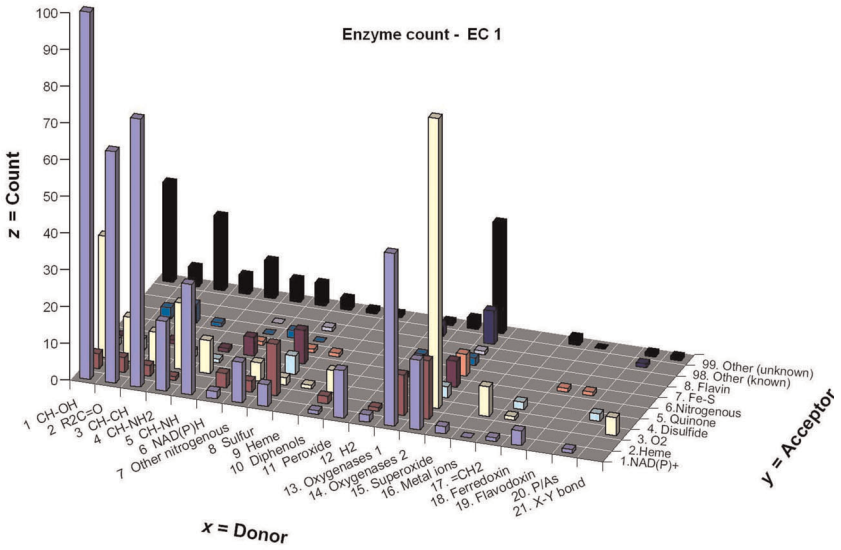

Figure 1. Classification of the oxidoreductases in Class 1 of the EC list. The vertical axis shows the number of enzymes of each sub-subclass, except that the count for EC 1.1.1.1 is truncated; there are currently 288 such enzymes.

Enzyme Assays Using Artificial Substrates

Enzyme assays are among the most widely used of biochemical measurements. They were developed for many other purposes, not just to characterize enzymes or study metabolism (Table 1). Colorimetric enzyme assays using chromogenic or fluorogenic substrates are routinely used. For example, in the pioneering studies of Jacob and Monod [2] on induction of the *lac* operon of *Escherichia coli*, hydrolysis of *o*-nitrophenyl-β-galactoside was used to measure the level of expression of β-galactoside, EC 3.2.1.45. The product nitrophenol has a bright yellow colour in alkaline solution. This well-studied detection system is still used to investigate gene regulation; for example the gene for β-galactosidase is coupled to a promoter of interest, and *E. coli* cells in which the promoter is activated can be observed by the colour reaction. The assay has been refined by the use of substrates such as X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) which generates an indigo precipitate, and 4-methyl

umbelliferyl β-D-galactoside, which releases a fluorescent product [3]. The colorimetric assay for β-galactosidase was found to be effective in the diagnosis and monitoring of Gaucher disease [4]; the enzyme deficiency being measured is in fact for a glucosylceramidase, EC 3.2.1.45.

Enzyme assays are widely used in clinical medicine for diagnosis and monitoring of disease [5]. They are used to measure unusually high or low levels of enzyme, or the presence of enzyme in an inappropriate location, as an indication of tissue damage. For such assays, all that is required is that the method be sensitive, robust, and specific to the enzyme of interest. Other compounds present in the sample should not interfere. It is not important that the assay should reflect the precise activity that the enzyme displays in the living cell. A few examples are listed in Table 1. Sometimes the physiological substrate of the enzyme being measured has not been established; for example alkaline phosphatase in blood is an indicator of bone disease. Cytochemical stains for respiratory enzymes are used in histology, and enzyme activity stains are used to visualize redox enzymes on non-denaturing electrophoresis gels. Vital stains using the reduction of a tetrazolium compound to a coloured formazan are basically tests for oxidoreductases [6]. The ability of an enzyme molecule to turn over many molecules of substrate represents a large amplification factor, which is exploited in enzyme-linked tests such as enzyme-linked immunosorbent assays (ELISA) [7].

Table 1. Examples of enzyme assays used in clinical diagnosis and screening.

Enzyme assay	Test for	Enzyme	EC No
p-nitrophenyl phosphatase	liver function and bone disease	alkaline phosphatase	EC 3.1.3.1
Paraoxonase	antioxidant stress	aryldialkyl- phosphatase	EC 3.1.8.1
peroxidation of 3,3',5,5'-tetra-methylbenzidine	ELISA	peroxidase	EC 1.11.1.7
β-galactosidase or glucosidase	gangliosidosis	glucosylceramidase	EC 3.2.1.45

The use of redox-active dyes and other small molecules as electron acceptors and donors to enzymes derives from some of the earliest research in biochemistry, and was important in the discovery of important cellular processes such as the light reactions of photosynthesis [8] and respiration [9]. It has been known since early in the 20th century that extracts of living tissues can catalyse the oxidation and reduction of compounds such as 2,6-dichloroindophenol and $K_3Fe(CN)_6$ (reviewed by Keilin, [9]), long before the molecular properties of the protein complexes involved were established.

Some reducing compounds in the cell, such as NADH, react poorly with dyes but the process is facilitated by compounds such as phenazine methosulfate that will facilitate the transfer of electrons dyes such as tetrazolium compounds. Such compounds are known as *mediators*. Mediators can also facilitate electron transfer to platinum electrodes, and have been used in the determination of thermodynamic oxidation–reduction potentials of metabolites and proteins such as cytochromes [10, 11]. Mediators have their difficulties when used to study the kinetics of enzymes. The reaction of enzymes with redox mediators is unpredictable, and few systematic studies have been made since the early days [12]. The most effective mediators are usually one-electron carriers that can produce free radicals.

Mediators often react at sites that are not accessible to the physiological substrates; or they may not react at the active sites, for reasons of steric hindrance or electrostatic charge. Nevertheless, assays using the oxidation and reduction of mediator dyes have continued to be widely used.

Table 2. Midpoint potentials of some redox couples, at pH 7, in millivolts *vs* the hydrogen electrode.

Compound	E°, mV
$K_3Fe(CN)_6/K_4Fe(CN)_6$	+420
2,6-dichloroindophenol	+217
Phenazine	+80
Ubiquinone Q/QH_2	+60
Fumarate/succinate	+30
Methylene blue	-11
Menaquinone MK/MKH_2	-60
Indigodisulfonate	-125
$FAD/FADH_2$	-207
$NAD^+/NADH$	-320
Methyl viologen	-440

A considerable number of oxidoreductases were first studied by assay with mediators or artificial donors (Table 2). Succinate dehydrogenase was shown to act with methylene blue [13]. These assays helped to establish the specificity of the enzyme for their substrates and inhibitors, though they obviously could not be used to investigate the kinetics of reaction with the physiological cosubstrate. Some of them were documented in the first list of enzymes [14] which became the EC list. Rather than omit these enzymes from the classification, they were placed in the sub-subclass "99" [15]. They can be seen in the back row of the chart of EC 1 enzymes (coloured in black in Fig. 1). This sub-subclass included any enzyme which could not be listed anywhere else, including enzymes for which one substrate is uncertain; and enzymes for which the substrates are known, but for which no other subclass in the list is suitable. Eventually the "99" enzymes should all be deleted or relocated elsewhere in the list. A recent proposal in the list, not yet implemented, is to invoke the classification EC 1.*x*.98.*z* for enzymes where the acceptor is known but for which there is no suitable sub-subclass, and EC 1.97.*y*.*z* for enzymes where the donor is known but for which there is no subclass. Some of the sub-subclass "99" enzymes proved on further investigation to be degraded or incomplete parts of enzymes, and are being eliminated after further investigation. Others are from organisms that have been little studied. However some are from well-studied organisms, such as *E. coli* and they are particularly interesting as they point to gaps in our knowledge of metabolism, and possibly further complexities in the organization of the cell.

The flavins, FAD and FMN, are redox-active in their own right, and can act as mediators. However apart from a few enzymes (dioxygenases in subclass EC 1.14) that appear to use the free flavin as donor, FMN and FAD form part of an enzyme and are considered as

prosthetic groups. In some enzyme assays FMN or FAD are required to supplement a flavin that is a dissociable prosthetic group in the enzyme. Despite this, some textbooks and metabolic pathways indicate that $FADH_2$ is the product of succinate dehydrogenase. The reaction may sometimes be found written in the form:

$$\text{succinate} + \text{FAD} = \text{fumarate} + \text{FADH}_2 \quad (4)$$

which treats FAD as a dissociable substrate. This is usually mentioned in the context of the citrate cycle, in which the soluble products of pyruvate oxidation are described as CO_2, 4 NADH and 1 $FADH_2$. Although this analogy to NAD is superficially attractive, it represents confusion between a prosthetic group (which is part of the enzyme) and a cosubstrate (a cofactor that is a substrate of the reaction catalysed). We now know that the enzyme in mitochondria that oxidizes succinate is a membrane-bound enzyme, succinate dehydrogenase (ubiquinone), EC 1.3.5.1, also known as Complex II of the respiratory chain [16]. This is a four-subunit enzyme, which contains FAD, iron–sulfur clusters and heme (Fig. 2a).

Thus Equation 4 should be written:

$$\text{succinate} + \text{Q} = \text{fumarate} + \text{QH}_2 \quad (5)$$

The origins of the idea of FAD as an acceptor may date from the 1950 s, when Massey and Singer [17] showed that FAD could act as a mediator with soluble "succinic dehydrogenase"; this was a preparation of the two membrane-extrinsic subunits, still in the list as EC 1.3.99.1, succinate dehydrogenase. These authors did not suggest that $FADH_2$ was the acceptor, which is unlikely for several reasons.

- $FADH_2$ could not dissociate from the enzyme, as it is covalently bound to a cysteine residue in the protein.
- Free $FADH_2$, unlike NADH, is readily oxidized by O_2, producing toxic oxygen radicals.
- The equilibrium of the reaction of Equation 4 would lie in the direction of reduction of fumarate to succinate since the midpoint potential of the fumarate/succinate couple (30 mV at pH 7; Table 2) is more positive than that of FAD/$FADH_2$ (-210 mV).
- FAD is a carrier (as are the iron–sulfur clusters and heme) in the flux of reducing equivalents from succinate to ubiquinone (Fig. 2a).
- Equation 5 is formally a transfer of two hydrogen atoms from succinate to ubiquinone. In fact, the process involves electron transfers. Flavins can be reduced in one-electron steps with the formation of an intermediate semiquinone; hence they act as a transformer between hydrogen- and electron-transfer reactions. Quinones such as ubiquinone are also best considered as electron carriers, the reaction going through the formation of a semiquinone radical; to preserve

charge neutrality, transfer of each electron is usually accompanied by a proton [18].

Classification of Membrane-Bound Enzymes and the Reactions Catalysed

When annotating a metabolic pathway, the aim is to identify the enzyme by the reaction catalysed in a particular step. The identification of an enzyme in the EC list is not quite the same; it depends on the observed substrate preferences of enzymes that have actually been isolated [18, 19]. This distinction is reflected in the use of the term "reaction class" (RC) in the KEGG annotation of genomes [20]. The same EC number may be attached, quite correctly, to different reactions in different organisms or cells. This happens because the enzyme is of broad specificity, but it only encounters a particular substrate in that organism. For example, an alcohol dehydrogenase that oxidizes one alcohol in a particular organism may be indistinguishable from one that oxidizes another alcohol in another organism [21]. An enzyme only needs to be specific enough for the purposes of catalytic efficiency, and to avoid unwanted reactions with other metabolites found in the cell. There will be no evolutionary pressure to avoid reactions with molecules that the enzyme never encounters. The enzyme is only induced in the presence of that particular substrate in that organism; the specificity lies not in the enzyme, but in the regulatory systems that induce its biosynthesis.

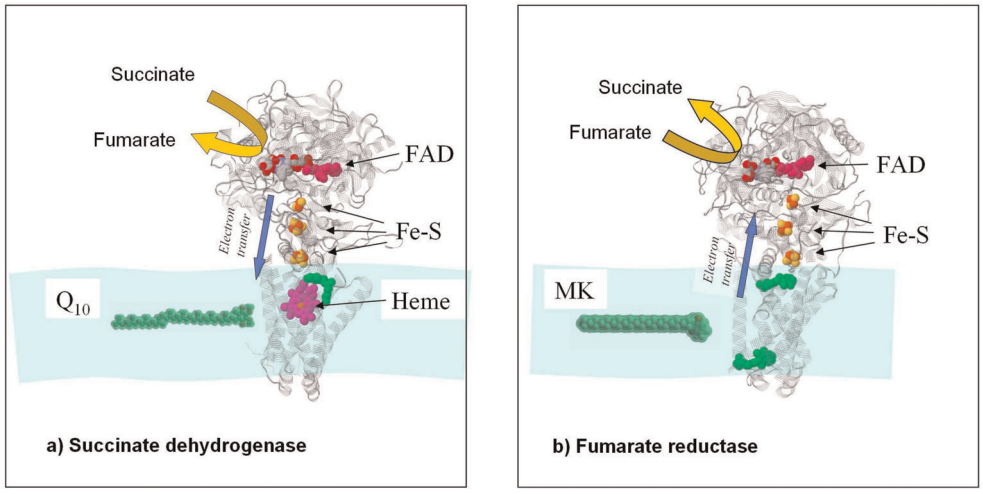

Figure 2. Proposed organization of electron carriers in a) succinate dehydrogenase in the membrane of aerobic *E. coli* cells and b) fumarate reductase in the membrane of anaerobic *E. coli* cells. The membrane is indicated in pale green. The structures were determined from Protein Databank files and 1LOV, respectively, drawn with RasMol version 2.7.2.1. The position of ubiquinone (Q) and menaquinone (MK) are indicated in green, drawn with Chem3D (Cambridgesoft). Two binding sites for the MK headgroup in fumarate reductase are indicated in b).

A simplifying principle of the EC classification, which affects their use in annotation of metabolic pathways, is that the direction of reaction is not considered in allocating a subclass or sub-subclass. Enzymes that catalyse the same reaction in opposite directions will have the same EC class, unless it can be demonstrated that they have different substrate specificity. For example in aerobic conditions the enzyme that oxidizes succinate to fumarate, as in mitochondria, is Complex II, succinate: ubiquinone reductase [16]. Under anaerobic conditions expression of this form of the enzyme is suppressed, and a similar enzyme, fumarate reductase, is expressed, which uses fumarate as an oxidant. Both enzymes are listed as EC 1.3.5.1, and they have a similar molecular architecture (Fig. 2). These enzymes might be classed separately if it could be demonstrated that they are specific for a particular quinone. In fact the quinones present under the two different growth conditions are different, ubiquinone under aerobic conditions, and menaquinone, which has a lower midpoint reduction potential (Table 2) under anaerobic conditions. However so far there have been few cases where it has been possible to demonstrate specificity of membrane-bound oxidoreductases for particular naturally-occurring substrates. This may be due to the difficulty of measuring kinetic parameters of such reactions. There are biophysical methods to do this, for example in photosynthetic reaction centres, where it was shown that the length and structure side-chain of the quinones has a significant influence on the rate of reaction [22].

Within a membrane such as the mitochondrial inner membrane, a “pool“ of quinones such as ubiquinone (Q) or menaquinone (MK) diffuses in this phase, and interacts with specific quinone binding regions of the membrane protein complexes [23]. These quinones have long prenyl chains, and are virtually insoluble in water. They are located in the hydrophobic region between the bilayer leaflets of cell membranes [24]. The quinone/quinol headgroups are somewhat hydrophilic, and tend to orient toward the aqueous layers on either side of the membrane (Fig. 2). The quinones interact with substrates in the aqueous phases by electron transport through the membrane protein complexes [25].

For membrane-bound enzymes that react with water-insoluble quinones, the paradigm for the enzyme assay described above (Equation 1) cannot be readily applied. The amount of quinone is confined to the small volume of the lipid bilayer, so the “initial rate“ of an enzyme reaction will produce a very small amount of product. Because the quinones are virtually insoluble in water, their oxidation and reduction cannot readily be followed by conventional solution methods such as spectrophotometry. In order to study them in solution, detergents are added, so that both the enzyme and substrate are present in the form of detergent micelles. Now, if the oxidation–reduction of the quinone is measured, the kinetics of diffusion in and between micelles is a complicating factor.

Smaller quinone molecules such as menadione or Q_1 are more water-soluble, and may be used instead of the native substrates. However any quinone with a shorter chain length than 5 prenyl units will not partition correctly in the membrane, and so its interaction with the quinone-binding sites may be different [26]. Small quinone molecules such as menadione or Q_1 can act as general mediators, accessing redox centres outside of the membrane bilayer, and transferring electrons inappropriately. They can also react with oxygen to produce

reactive oxygen species. A compromise is to use synthetic substrates such as ubiquinone or menaquinone with a decyl side-chain; these artificial mediators have reasonable solubility in water, and partition into the membrane in a similar way to the natural cofactors [27].

Alternative methods are needed to investigate the kinetic properties of enzyme assays with membrane-bound substrates. One way to measure the rates of enzymes with membrane-bound substrates is to couple the reaction to another enzyme, of which the product can accumulate and be more easily measured. An example would be the succinate:cytochrome c reductase activity of the mitochondrial membrane, where the reduction exogenous cytochrome c can be monitored spectrophotometrically. This still assumes however that the binding and dissociation of cytochrome c into the membrane is not rate-limiting.

Protein Film Voltammetry

A considerable number of oxidoreductases, containing redox centres such as heme, flavin, and/or iron–sulfur clusters, have been found to adhere, under suitable conditions, to a carbon electrode in such a way that they transfer electrons [28]. Membrane-associated oxidoreductases appear to work particularly well (Fig. 3). Direct electrochemistry of a film of these proteins provides information about kinetic parameters that is difficult to obtain by other means. When the substrate is present, the electric current is equivalent to the rate of substrate oxidation, v. The voltage dependence of the current i is equivalent to the dependence on concentration of an electron donor.

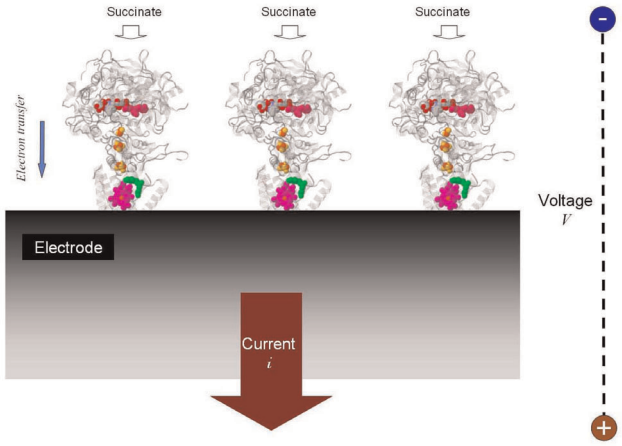

Figure 3. Diagram of succinate dehydrogenase on the surface of a carbon electrode. A monolayer of active molecules is adsorbed, so that succinate from solution can bind to the surface. The electrode is maintained at a voltage V, which can be swept, and the current i of electrons flowing through the enzyme molecules is measured. In the absence of succinate, the current observed is due to electrons flowing into the flavin, iron–sulfur clusters and heme.

The adsorption of an oxidoreductase onto a membrane, or onto a carbon electrode, transforms the kinetics from homogeneous catalysis to heterogeneous (at a two-dimensional surface). The rate of reaction depends not only on the concentration of substrate, but also on the rate of diffusion of substrate molecules to the surface. This can be studied by use of a rotating disk electrode; the rate of diffusion is proportional to the square root of the rate of rotation, ω [29]. When extrapolated to limiting value of ω, the current *i* is then proportional to the rate of catalysis by the enzyme. The Koutecky–Levich equation, which describes the quantitative relationship between *i* and ω, for an enzyme at a rotating electrode, takes a form analogous to the Michaelis–Menten equation, and provides values that are equivalent to K_m and V_{max} for substrate oxidation [29]. The method is very sensitive, needing only a monolayer of enzyme molecules over a surface of a few square millimeters.

Protein film voltammetry, in which the current *i* is measured as a function of the applied voltage *V* is swept, makes it possible to examine other features of the enzyme-catalysed reaction. As the applied voltage is swept, the current rises in a „catalytic wave“, usually at the midpoint potential of the substrate, for example the fumarate/succinate potential for succinate dehydrogenase. Cyclic voltammetry, in which the field is repeatedly swept up and down, shows that the reaction was nearly perfectly reversible [29, 30]. However some unusual kinetic properties of the enzymes emerged. Succinate dehydrogenase showed a „diode-like behaviour“ at higher driving potentials, the current decreased, a situation analogous to high substrate inhibition by the reducing agent [31]. However by judicious choice of the conditions of measurement it was possible to measure enzyme-catalysed rates much higher than those observed with artificial electron acceptors. The method can be used to measure the specificity of enzymes with different substrates and inhibitors, and study the effect of parameters such as pH.

In order to determine k_{cat} by this method, it is necessary to calculate the number of protein molecules on the surface that are giving rise to the catalytic current. This may be obtained by voltammetry of the enzyme in the absence of substrate, when catalytic waves can be measured from the redox centres in the protein itself. In the case of succinate dehydrogenase these are identified as flavin, iron–sulfur clusters and heme, for which the oxidation–reduction potentials can be measured.

Cyclic voltammograms of adsorbed enzyme layers containing membrane lipids offer a solution to the problem of determining the specificity of oxidoreductases for membrane-soluble quinone cosubstrates. Electrochemistry has been applied to thin films of ubiquinone [32]. A recent development is the construction of “tethered“ membranes on gold electrodes [33]. These bilayer membranes are connected, both physically and electrically, to the electrode, by a cholesterol tether which allows electron transfer. They can be loaded with protein complexes and quinones, and in favourable cases appear to behave kinetically like the native proteins.

Conclusions

The “99” enzymes represent an area of uncertainty in the description of enzymes. Ultimately they should be removed or transferred to other parts of the enzyme list. Meanwhile they indicate a fertile area for future studies. If the function of an enzyme is not clear, it may indicate interesting new biochemical processes.

For the purposes of metabolic reconstruction, the hydrophobic interiors of membrane bilayers represent separate, mobile compartments in the cell. Membrane-bound quinones such as ubiquinone-10 communicate through the membrane-bound protein complexes. Assays that assume a simple two-substrate, two-product reaction in dilute solution do not apply in such cases. New methods are needed for studying their activities and kinetics.

Membrane-bound oxidoreductases, which are not amenable to conventional solution enzyme assays, may be studied from their reactions at a carbon electrode surface. This makes it possible to examine their reactivity with different substrates, and the thermodynamic and kinetic properties of the redox centres within the enzymes.

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Abbreviations

MK, Menaquinone
Q, Ubiquinone
STRENDA, Standards for Reporting Enzymology Data

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