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Classification of fumarate reductases and succinate dehydrogenases based upon their contrasting behaviour in the reduced benzylviologen/fumarate assay

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Reduction of fumarate by soluble beef heart succinate dehydrogenase has been shown previously by voltammetry to become increasingly retarded as the potential is lowered below a threshold potential of −80 mV at pH 7.5. The behaviour resembles that of a tunnel diode, an electronic device exhibiting the property of negative resistance. The enzyme thus acts to oppose fumarate reduction under conditions of high thermodynamic driving force. We now provide independent evidence for this phenomenon from spectrophotometric kinetic assays. With reduced benzylviologen as electron donor, we have studied the reduction of fumarate catalysed by various enzymes classified either as succinate dehydrogenases or fumarate reductases. For succinate dehydrogenases, the rate *increases* as the concentration of reduced dye (driving force) *decreases* during the reaction. In contrast, authentic fumarate reductases of anaerobic cells (and 'succinate dehydrogenase' from *Bacillus subtilis*) neither exhibit the electrochemical effect nor deviate from simple kinetic behaviour in the cuvette assay. The 'tunnel-diode' effect may thus represent an evolutionary adaptation to aerobic metabolism.

Succinate dehydrogenase; Fumarate reductase; Electron transport; Respiratory chain; Citric acid cycle; Electrochemistry

1. INTRODUCTION

Biological interconversion of fumarate and succinate is catalysed by fumarate reductase (FRD) and succinate dehydrogenase (SDH). These are closely related enzymes produced under anaerobic and aerobic growth conditions, respectively, to link the reduction of fumarate or oxidation of succinate to oxidation or reduction of the quinone pool (Q/QH_2) of respiratory membranes as shown by Eqn. 1.

$$\text{succinate} + Q \rightleftharpoons \text{fumarate} + QH_2 \quad (1)$$

Each of these membrane complexes consists of two hydrophilic subunits, containing the covalently bound FAD and the three iron-sulphur clusters of the enzyme, and one or two hydrophobic subunits that act as membrane anchors and provide the binding site(s) for Q and QH_2. The purified complexes can be resolved into the hydrophobic anchors and a soluble form of the enzyme that comprises the hydrophilic subunits and is still able to catalyse reduction of fumarate or oxidation of succinate by artificial redox partners (for a recent review, see [1]).

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Recently, it was shown that the soluble forms of SDH from beef heart and FRD from *E. coli* each give rise to catalytic voltammetry when adsorbed at a pyrolytic graphite 'edge-oriented' (PGE) electrode [2,3]. A major difference noted in these experiments was the shutdown of fumarate reduction at low potentials when SDH was the catalyst. This novel property is akin to the action of a tunnel diode, an electronic device for which the current-potential characteristic displays a region of negative resistance. We proposed that this type of behaviour could result from constraints in the ordering of events in the catalytic cycle that are more stringent for SDH. Specifically, for example, substrate binding to SDH would be required to precede the addition of electrons [2]. In this Letter, we outline further experiments that both support this preliminary result and demonstrate a means for distinguishing SDH and FRD on the basis of their contrasting behaviour in the classical assay of fumarate reduction by reduced benzyl viologen (BV^+).

2. EXPERIMENTAL

The spectrophotometric assay of fumarate reduction was conducted anaerobically in 50 mM Tris-HCl, pH 7.6 (38°C), containing 0.1 mM EDTA, 20 mM fumarate and 0.18 mM benzyl viologen. Glucose (20 mM) and glucose oxidase/catalase were added to ensure strictest anaerobicity. The cuvette pathlength was 1 cm. Substoichiometric amounts of dithionite, added anaerobically prior to addition of enzyme, produced BV^+ ($\varepsilon_{550} = 7.8 \times 10^3\ M^{-1} \cdot cm^{-1}$) in the concentration

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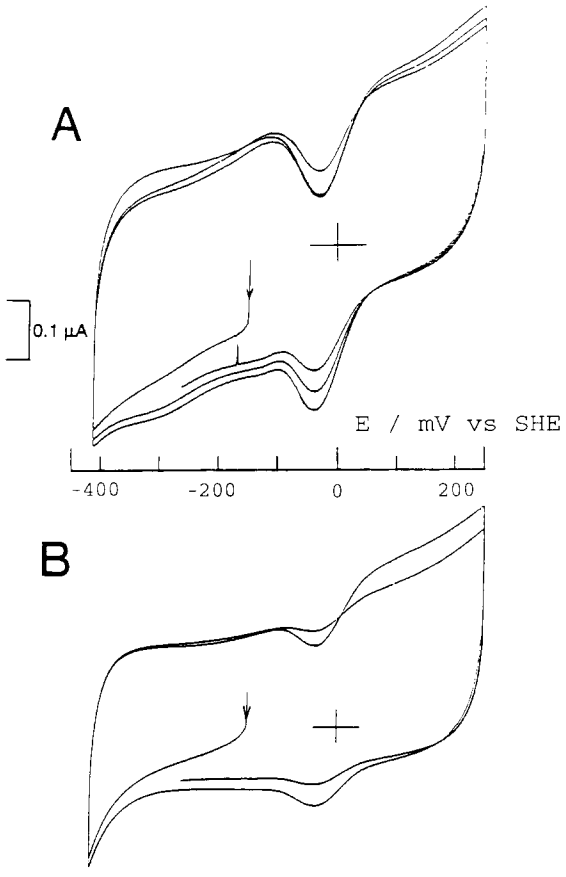

Fig. 1. Cyclic voltammograms showing reduction of fumarate catalysed by SDH adsorbed on electrodes. The enzyme was allowed to adsorb from a ca. 1 μM solution onto a pyrolytic graphite 'edge' (A) or gold (B) disk electrode. The voltammetric response was recorded in the presence of 0.65 mM fumarate and 0.65 mM succinate. Conditions: 0.1 M NaCl, pH 6.5 (MES), temperature 35.5°C, scan rate 10 mV/s. Although the voltammetric response at gold is much less stable, the reduction current maximum occurs at the same potential as for graphite.

range 0.1–0.15 mM. Where necessary, membrane samples were first clarified by using deoxycholate (final concentration 0.05%).

Enzyme preparations obtained by the procedures quoted or as gifts from colleagues included: submitochondrial particles (ETP) from beef heart [4], rat liver [5], human placenta [6] and *Ascaris suum* (from Dr. R. Komuniecki, University of Toledo, OH); aerobic cytoplasmic membranes of *E. coli* (Dr. R. Gennis, University of Illinois) and *B. subtilis* (Dr. L. Hederstedt, University of Lund, Sweden); purified FRD complex of *E. coli* (Dr. G. Cecchini, University of California, San Francisco); isolated complex (Complex II) [7] and soluble SDH [8] of beef heart; and the yeast cytoplasmic FRD isolated essentially according to the procedure of Muratsubaki and Katsume [9]. Protein was measured by the biuret method, after precipitation with trichloroacetic acid and, in the case of Complex II, an additional pre-wash with acetone/HCl [10].

Voltammetry at PGE or Au electrodes was carried out in an anaerobic glove box according to procedures described previously [2,3]

3. RESULTS AND DISCUSSION

Voltammograms in Fig. 1 show enzymatic interconversion of fumarate and succinate at pH 6.5 as catalysed by soluble beef heart SDH adsorbed on (A) a PGE electrode, and (B) a gold electrode. At this pH, oxidation of succinate does not proceed readily (c.f. [2]); however, succinate was included in order to stabilise the enzyme. In both cases the voltammogram is very unusual, being dominated by a pair of reduction peaks which effectively overlay in the directions of increasing negative or increasing positive potential. The reduction of fumarate is therefore slowed down by application of a higher driving force, a result that is contrary to that expected from simple considerations of electrochemical kinetics (for example, see [11]). Although the voltammetric response at the gold electrode is smaller and much less stable, the potential at which the peaks occur is otherwise identical in the two experiments. This shows that the effect is an inherent property of the protein and is not induced by the interaction with the electrode. (The electrode surfaces differ greatly in their electrical properties and thus in the manner in which they would be expected to interact with the enzyme.) It was further determined that the voltammetry of a fumarate solution initially devoid of succinate was unchanged when the PGE electrode was rotated at speeds up to 1,800 rpm. Thus the reaction does not depend upon mass transport of fumarate to the electrode, nor is it inhibited by build up of succinate, which in the rotating disk experiment is promptly removed from the vicinity of the enzyme.

Fig. 2 shows the behaviour of (A) isolated FRD complex (*E. coli*) and (B) isolated SDH complex (Complex II from beef heart) as observed in the spectrophotometric assay in which a large excess of fumarate is reduced

Table I

Predominant behaviour of various succinate–fumarate oxidoreductases in the reduced benzylviologen/fumarate assay

Preparation	Reaction order with respect to [BV^+]	
	Positive	Negative
1 Membrane-bound SDH		
Beef heart		+
Rat liver		+
Human placenta		+
A.suum		+
B.subtilis	+	
E.coli		+
2 Isolated SDH complex		
Beef heart		+
3 Soluble SDH		
Beef heart		+
4 Isolated FRD complex		
E.coli	+	
5 Soluble FRD		
S.cerevisiae	+	

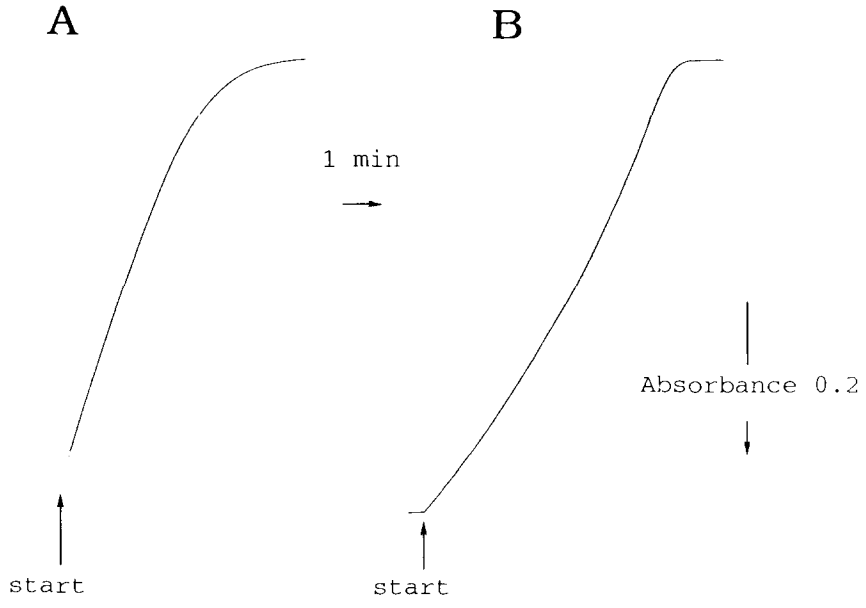

Fig. 2. Course of oxidation of benzylviologen radical by fumarate, as catalysed by isolated FRD or SDH complexes. Spectrophotometric assays using 3 ml of reaction mixture (see section 2) were started with (A) isolated FRD complex of *E. coli* (0.5 μg of protein) or (B) isolated SDH complex of beef heart (12.5 μg of protein). Initial concentrations of BV^+ radical were 85 μM (A) and 81 μM (B), respectively. Chart speed 2 cm/min.

by the blue benzylviologen radical. For catalysis by FRD, a rapid phase in which the reaction appears zero-order in BV^+ is followed by a first-order phase and ultimately exhaustion of BV^+. By contrast, the catalysis by SDH is dominated by a phase with an apparent *negative order*, i.e. the rate *increases* as radical is consumed. The reaction finally switches to positive order for a brief period just prior to depletion of radical. The increase in rate as benzylviologen radical is oxidised is analogous to the electrochemical result since the decrease in the concentration of BV^+ amounts to a decrease in the driving force of the reaction. The course of the reaction is not changed for preparations isolated in a partially deactivated state due to the presence of tightly-bound oxaloacetate (K_d < 1 μM) which dissociates once the enzyme is reduced in the assay mixture [1].

In the context of our previous suggestion, i.e. that substrate binding or release at SDH is much more favourable for the oxidised enzyme, the phenomenon may also be viewed from a kinetic standpoint [2]. Thus, the higher the rate that electrons are supplied to the enzyme, the shorter becomes the lifetime of the oxidised active form. In more conventional terms, an electron transport enzyme that exhibits 'tunnel diode-like' behaviour is in fact displaying substrate inhibition – the substrate in question being not molecular but the electron itself.

To explore this phenomenon more widely we have used the steady-state assay to compare other SDH and FRD preparations from different sources. Table I compares the results obtained, showing whether the predominant phase of the reaction has a positive or negative order with respect to benzylviologen radical. The data suggest that examples showing a positive order may be identified as fumarate reductases, while those displaying the negative order are succinate dehydrogenases, in as much as they appear to catalyse the reduction of fumarate only under conditions of moderate driving force. One exception to the rule is the SDH of the strict aerobe, *B. subtilis*, which behaves as an FRD in this assay. The mechanistic origins of this difference and the possible physiological implications are currently under investigation.

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