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Voltammetric measurement of Michaelis–Menten kinetics for a protein in a lipid film reacting with a protein in solution

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Abstract

Rotating disc voltammetry data for the reaction of myoglobin, a model "enzyme" in a thin lipid film, with ferredoxin was shown to fit the Michaelis–Menten kinetic model, but did not fit a simple linear EC' model. Advantages of this thin-film method are a lipid bilayer environment similar to that of membrane bound enzymes, the tiny amounts of proteins required, and simplicity of instrumentation compared to alternative methodology. The apparent K_M for ferredoxin reduction by reduced myoglobin was 112 μM, k_{cat} was 102 s^{-1}, and k_{cat}/K_M was 9.1×10^4 M^{-1} s^{-1} indicating relatively fast kinetics. Results suggest that this method should be amenable to kinetic studies of enzymes in lipid films with protein reaction partners in solution.

Keywords: Enzyme kinetics; Lipid films; Redox proteins; Voltammetry; Myoglobin; Ferredoxin

1. Introduction

There are many instances in natural systems in which a membrane-bound protein reacts with a solubilized protein. One example involves photosynthetic reaction center (RC) enzymes that play major roles in converting light energy into chemical energy [1,2]. RC's in plants, green algae and bacteria are embedded in biomembranes and after photoexcitation, terminal acceptors in the electron transport chain react with soluble proteins to reset redox states. For example, the terminal electron acceptor in the spinach photosystem I shuttles electrons to soluble ferredoxin [3,4].

A variety of techniques have been employed for kinetic studies of electron transfer reactions between proteins. Protein–protein reaction kinetics in solution have been studied by methods including stopped-flow spectrophotometry [5], pulse radiolysis [6], and laser flash photolysis [7]. Since these reactions are frequently fast, studies involving spectroscopic detection involve initiation of electron transfer by laser pulses for flash photolysis, radiation pulses for pulse radiolysis, or rapid mixing of reactants by stopped flow methods. The time course of the reactions are usually monitored spectrophotometrically, from which electron transfer kinetics are derived.

An alternative method for protein kinetic studies is offered by rotating disk voltammetry. Examples include electron transfer between physiological redox partners hydrogenases and cytochromes from several strains of sulfate-reducing bacteria as well as ferredoxins [8–10]. Rotating disk voltammetry has been elaborated for studying Michaelis–Menten reaction kinetics between adsorbed enzymes and soluble small molecule substrates, e.g., reduction of fumarate by fumarate reductase and oxidation of succinate by succinate dehydrogenase [11].

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We have immobilized various redox proteins in thin insoluble surfactant films that facilitate direct electron exchange with electrodes and are excellent vehicles for fundamental studies by thin-film voltammetry and spectroscopy [12]. Recent papers from our laboratory reported direct electron transfer in membrane bound RCs of purple bacterium *Rhodobacter Sphaeroides* [13] and spinach photosystem I [14] in thin dimyristoylphosphatidylcholine films on pyrolytic graphite electrodes. We also described catalytic voltammetry between terminal acceptors in both of these RCs in the lipid films with soluble proteins ferrous cytochrome *c* and ferredoxin in buffer solutions, respectively.

We were intrigued by the possibility of quantitative kinetics studies of reactions of enzymes in lipid films with redox proteins in solution by rotating disk voltammetry (RDV). As we were not aware of such studies previously, we first considered a simpler system than the complex RC proteins. We chose the reaction between myoglobin (Mb) in dimyristoylphosphatidylcholine (DMPC) films with spinach ferredoxin in solution. UV–Vis and FT-IR spectroscopy, ESR anisotropy and linear dichroism have shown that Mb is in its native conformation oriented within the lipid bilayers of these multi-bilayer films [15]. Mb gives quasi-reversible voltammetry in these films by a reasonably well-understood pathway [16]. Thus, Mb-DMPC provides a good model for a membrane bound, electroactive enzyme. In this communication, we show that RDV data for the reaction of reduced Mb in the lipid films with spinach ferredoxin in solution is well described by the Michaelis–Menten model, allowing estimation of kinetic parameters.

2. Experimental

Horse skeletal muscle myoglobin from Sigma in 100 mM phosphate buffer pH 7.0 + 100 mM NaCl was passed through a YM 30 filter (Amicon, 30,000 MW cutoff) [16]. Dimyristoylphosphatidylcholine (DMPC, 99%) and spinach ferredoxin (Fd, MW 15 KDa) were from Sigma. The concentration of Fd was estimated by spectroscopy using molar absorptivity 9680 M^{-1} cm^{-1} at 420 nm [17].

Vesicle dispersions were made by sonicating DMPC suspensions in water until they became clear (~24 h). Mb-DMPC films were prepared by depositing 20 μl 1.5 mg mL^{-1} Mb in 2 mM DMPC vesicle dispersion onto a basal plane pyrolytic graphite (PG, $A = 0.16$ cm^{-2}) electrode and drying [14]. Voltammetry with a Pine Instruments rotating disk electrode was done as described previously [14]. Ohmic drop was compensated electronically to uncompensated resistance 5–7 Ω. All experiments were at 22 ± 2 °C. Atomic force images were taken in air with a Nanoscope IV microscope in tapping mode as described earlier [14].

2.1. Results and discussion

Cyclic voltammograms (CV) for Mb-DMPC films obtained several minutes after immersion in anaerobic buffer showed reproducible, reversible reduction and oxidation peak pairs with average midpoint potential of −0.31 V vs. SCE at pH 7.0. This is illustrated by the trace in Fig. 1 labeled "no Fd" (1000 rpm) and is consistent with thin layer Mb voltammetry reported in DMPC films earlier [16]. Integration of low scan rate CVs of the films gave an average surface concentration of electroactive Mb of 2.2×10^{-10} moles cm^{-2} with variability ±7%. A fit of corrected peak separation vs. scan rate data to the Butler–Volmer model gave an apparent surface electron transfer rate constant, k_s of 56 ± 2 s^{-1}, consistent with earlier value of 60 s^{-1} at pH 7 from square wave voltammetry [16].

To obtain atomic force microscopy (AFM) images, films of Mb-DMPC were prepared on PG disks with half of the disk surface masked with Teflon tape. Then the tape was stripped off before AFM analysis. Images in Fig. 2(a) and (b) show films after 30 s exposure to buffer and after the disk was rotated in buffer for 10 min. Steps connecting dark (no film) and light (film) areas indicate a film thickness ~1 μm for both films, suggesting that the films were stable during the rotating process.

Assuming that at high overpotential electron transfer is not limiting, rotating disc voltammetry (RDV) of thin enzyme films in the presence of substrate is described by the Koutecky–Levich approximation of the limiting current (I_L) [11]:

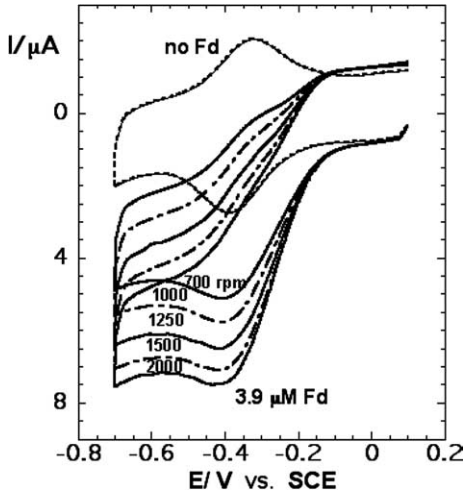

Fig. 1. Rotating disk voltammograms of myoglobin-DMPC films with and without 3.9 μM ferredoxin at various rotation rates in pH 7 phosphate buffer containing 100 mM NaCl. Voltammogram marked "no Fd" was at 1000 rpm.