J|A|C|S COMMUNICATIONS

Published on Web 04/20/2010

Metabolon Catalyzed Pyruvate/Air Biofuel Cell

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Energy conversion in eukaryotes occurs within a well organized packaged scheme in the form of an organelle called the mitochondria. The mitochondria contain two important metabolic pathways: the Krebs cycle and the electron transport chain. Protein−protein interactions of metabolic proteins within the mitochondria have long been studied.[1] Researchers suggest the existence of preferential intermolecular interactions between sequential enzymes of metabolic pathways often termed a metabolon.[2−8] This phenomenon has been demonstrated to enhance metabolic flux as a result of substrate channeling from enzyme to enzyme of the metabolic pathway.[9] This tunneling phenomenon has been demonstrated in particular in the Krebs cycle, which is the metabolic pathway responsible for the complete oxidation (i.e., metabolism) of pyruvate.[10−13] Many of the enzymes of this cycle have been shown to have noncovalent interactions that cause the localization of several enzymes to the inner membrane of mitochondria. This complex formation is induced by increased enzyme concentration, but it results in increased substrate concentration and channeling of enzyme products from one enzyme to the next enzyme in the cycle causing increased activity, thus imparting metabolic rate control.[1]

Although it has long been recognized that microbes can generate voltage and deliver current, in the early 1960s, researchers began studying the harvesting of energy from fuels and converting them to electrical energy via an enzymatic biofuel cell.[14] A biofuel cell is a fuel cell that converts the chemical energy stored in a fuel into electrical energy through the catalytic activity of living cells, organelles, or their enzymes. Biofuel cells have the advantage of not requiring precious metals and being able to utilize fuel from a wide variety of possible fuel sources (including alcohols, sugars, proteins, and fatty acids). However, the main problems that have hindered the advancement of biofuel cells are low power densities for microbial systems (due to transport limitations and low volumetric catalytic activity) and limited levels of oxidation with enzyme bioelectrocatalysis.[14,15] Efforts have been made to utilize enzyme cascades of whole metabolic pathways (i.e., Krebs cycle) for deep oxidation of biofuels; however, limitations to efficiency of these systems arise from the slow and undirected mass transport between sequential enzymes of the pathway.[15,16] One solution to this problem was the use of complete intact mitochondria for complete oxidation, because they contain all of the enzymes of the Krebs cycle and the enzymes are contained in metabolons for efficient substrate channeling. This led to the development of mitochondria catalyzed biofuel cells.[17] However, mitochondria contain phospholipid membranes, tRNA, DNA, and nonmetabolic proteins, so although the mitochondria improve oxidation efficiency, mitochondrial biofuel cells result in a dramatic decrease in volumetric catalytic activity compared to enzymatic biofuel cells.

Although enzymatic biofuel cell researchers have experimentally realized the importance of enzyme cascades in increasing the efficiency of biofuel cells,[16] they have not investigated the importance of three-dimensional protein−protein complexation and substrate channeling in the electrochemical performance of an enzymatic biofuel cell. *In this work, we demonstrate the enhancement in current and power density obtained from utilizing Krebs cycle metabolons as bioelectrocatalysts instead of uncomplexed enzymes.* The Krebs cycle proteins were covalently linked within the mitochondria to preserve the close proximity of sequential enzymes within the metabolon before isolation from the mitochondria and incorporation into a pyruvate/air enzymatic biofuel cell. A schematic representation of substrate channeling in the metabolon is outlined in Figure 1.

Saccharomyces cerevisea were grown in nonfermentable carbon source media to enhance mitochondrial yield and activity. The mitochondria were extracted through methods of differential centrifugation as described in the Supporting Information. Once extracted, the mitochondrial pellet was equally divided into three samples for incubation with cross-linking agents as per a procedure described in the Supporting Information. Two common bioconjugation reagents (glutaraldehyde and dimethyl suberimidate (DMS)) were used. These two reagents differ in both reactivity and spacer length between conjugated proteins; however, both are homobifunctional cross-linkers of primary amines. Three beakers containing 50 mL of 220 mannitol, 70 mM sucrose, and 50 mM HEPES at pH 7.4 were incubated at 30 °C. One beaker was left in this state as a control, while the other two contained 10 mM concentrations of the chosen cross-linking reagents.

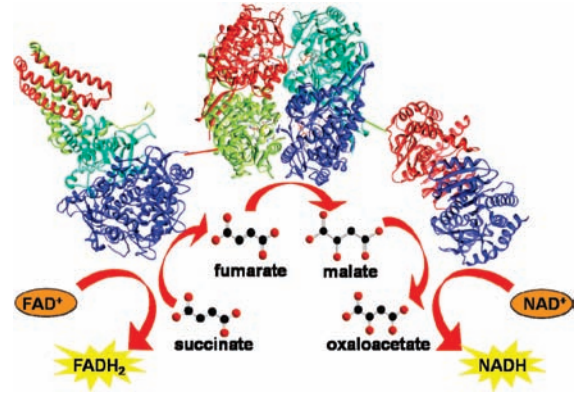

Figure 1. Schematic representation of substrate channeling achieved by cross-linked metabolon bioanode catalyst for a portion of the Kreb's Cycle.

Mitochondrial suspensions were then injected into each beaker to begin cross-linking. Cross-linking was allowed to occur for 5 min after which reactions were quenched. This short duration of time was used to attempt to utilize the increased probability of collisions occurring between adjacent Krebs cycle enzymes and to limit less proximally close collisions. The resulting intact mito-

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10.1021/ja101326b

chondria were concentrated by centrifugation to a concentration of 100 mg/mL and were subsequently lysed by ultrasonic membrane disruption.

Figure S1 in the Supporting Information shows a representative 12% SDS-PAGE gel obtained for the resultant protein suspension. In comparing the two cross-linked samples to the native mitochondrial sample unexposed to cross-linking reagent, one can see a substantial decrease in mitochondrial protein bands while an increase in protein density in the gel above 100 kD. This qualitatively demonstrates not only the occurrence of cross-linking but also the increased reactivity of glutaraldehyde versus DMS for protein complexation.

These lysed mitochondrial samples were immobilized in tetrabutylammonium bromide modified Nafion on E-Tek Toray carbon paper bioanodes that were previously coated with poly(methylene green) to catalyze the oxidation of NAD(P)H and regeneration of $NAD(P)^+$, which are cofactors of several enzymes of the Krebs cycle. The physical test cell is fabricated as described in Arechederra et al.[18] The air-breathing cathode consisted of Nafion 212 fused together with a gas permeable ELAT electrode with 20% Pt on Vulcan XC-72. Test electrodes were then suspended in 100 mM sodium pyruvate solution containing 1 mM NAD^+, 6.0 M $NaNO_3$, and 1 mg/mL ADP at pH 5.75 and allowed to equilibrate for a period of 1 h and reach a stable open circuit potential. Polarization and power curves were obtained for each electrode fabricated. Representative data obtained are demonstrated in Figure 2.

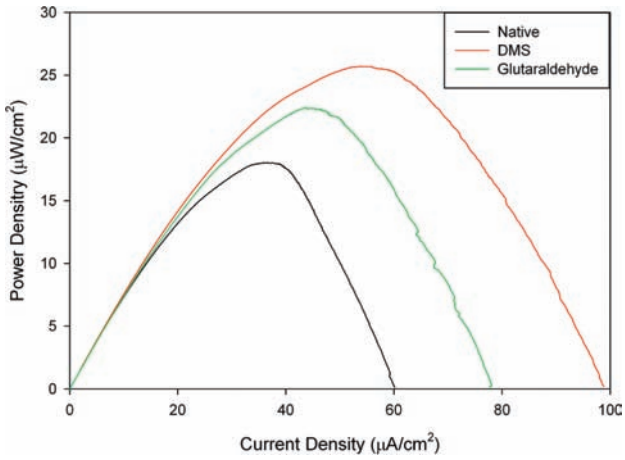

Figure 2. Representative power curves obtained for native and cross-linked mitochondrial electrodes in a pyruvate/air biofuel cell containing 100 mM pyruvate fuel at room temperature.

An increase in power and current density was observed for the cross-linked samples. This suggests that the increase in current density occurs due to the added efficiency of the oxidation of pyruvate by the covalent complex of the Krebs cycle enzymes in the metabolon. These results have also demonstrated the importance of the level of cross-linking achieved by the reagent used. Higher current density was achieved for samples treated with DMS in comparison to glutaraldehyde. This is expected, because glutaraldehyde is a harsher cross-linking agent and therefore may have more impact on enzyme activity. To differentiate effects of substrate channeling within the metabolon from effects of the cross-linker on enzyme activity, single enzyme activity assays for malate dehydrogenase were performed. As shown in Table S1, DMS cross-linking has no statistical effect on enzyme activity, but glutaraldehyde cross-linking decreases enzyme activity 5.5-fold. This shows the importance of balancing the need for a stable metabolon complex and the need to minimize cross-linking to maintain high catalytic activity.

Data obtained from four electrodes of each of the three preparations are compared in Table 1. Open circuit potential in the absence of pyruvate was insufficient for power curve measurements. Open circuit potentials for test electrodes in the presence of pyruvate were not statistically different for any sample and averaged 0.82 V. These cross-linked samples demonstrate a 49% and 38% increase in current density for the DMS and glutaraldehyde treated samples, respectively. The maximum power density was also increased by 32% and 33%, respectively.

Table 1. Average Maximum Current and Power Densities (±SD) of Bioanodes Prepared with Lysed Native Mitochondria, Dimethylsubimidate Cross-Linked Lysate, and Glutaraldehyde Cross-Linked Lysate

	Maximum Current Density (μA/cm^2)	Maximum Power Density (μW/cm^2)
Native	62.15 ± 8.07	18.35 ± 1.78
DMS	92.40 ± 10.98	24.14 ± 2.88
Cross-linked	($P = 0.004$)	($P = 0.014$)
Glutaraldehyde	85.56 ± 15.99	24.48 ± 4.71
Cross-linked	($P = 0.040$)	($P = 0.051$)

Results from this study show significant improvement in current and power density of pyruvate/air biofuel cells containing bioanodes prepared through the covalent linkage of matrix proteins of the mitochondria versus that of bioanodes prepared with mitochondria lysate. This shows the importance of mitochondrial matrix protein organization into concerted cycle complexes termed metabolons for efficient substrate channeling in bioanodes.

Acknowledgment. The authors would like to thank the Army Research Office, National Science Foundation, and the Air Force Research Laboratory for funding.

Supporting Information Available: Mitochondria preparation, cross-linking procedures, assay protocols, and fuel cell fabrication/operation protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA101326B