Proc. Indian Acad. Sci. (Chem. Sci.), Vol. 93, No. 7, September 1984, pp. 1221–1228.

Electron delocalisation during oxidation-reduction cycle of FAD: Design and fabrication of a coenzyme immobilized bioanode

RATNA S PHADKE, H M SONAWAT and GIRJESH GOVIL*

Biochemical Fuel Cell Project, Tata Institute of Fundamental Research, Bombay 400 005, India

Abstract. A biochemical fuel cell is an electrochemical power generation device that converts the chemical energy of a fuel (alcohol, glucose, hydrocarbons, etc) directly into electrical energy through enzyme-catalysed oxidation-reduction reactions. These systems possess several advantages over the conventional processes and are ideal for rural electrification in conjunction with biogas plants.

The major bottleneck in the design of such power sources is the electron transport from the substrate to the electrode. Biochemical systems which use coenzymes such as FAD or NAD seem to be the most promising in circumventing these difficulties. We have made systematic molecular orbital studies of the electron-flow diagrams of riboflavin during its oxidation-reduction cycle and it is possible to obtain very efficient electron transport from the coenzyme to the electrodes by immobilising flavin through semiconducting side chains, at certain selected positions, to electrodes such as graphite.

Based on these studies, we have immobilised FAD on graphite electrodes. The chemical steps involve creating active centres on graphite which are reacted with FAD to form covalent linkages between the electrode and the coenzyme. Cyclic voltamograms of the modified electrode show that FAD is active and undergoes the expected redox cycles. We hope that such electrodes will form suitable bioanodes for FAD-linked enzymatic reactions.

Keywords. Immobilized enzyme; FAD; molecular orbitals; oxidation-reduction cycle; coenzyme; electron transfer.

1. Introduction

A fuel cell (FC) is an electrochemical device in which part of the energy derived from a chemical reaction, maintained by the continuous supply of reactants, is converted directly to electrical energy (Austin 1967; Liebhasky and Cairn 1968; Govil 1980). Unlike chemical batteries, they can supply power intermittently and the electrodes are not consumed. Since they avoid the heat cycle, the efficiencies of FC are not limited by the second law of thermodynamics (Carnot cycle). The direct conversion of chemical into electrical energy is therefore inherently more efficient. Further, both the full load and part load efficiencies are better than those expected from conventional thermal power plants. Additional advantages of FC are environmental compatibilities, modular design, easy maintenance, versatility and possibility of on-site operation.

Fuel cells generally use hydrogen or hydrogen rich fuels such as methane, methanol, ethanol, etc. The energy producing reactions are the oxidation of hydrogen by oxygen:

$$SH_2 \rightarrow S + 2H^+ + 2e \qquad \text{(anode)}$$

$$2H^+ + \tfrac{1}{2}O_2 + 2e \rightarrow H_2O \qquad \text{(cathode)}$$

* To whom all correspondence should be addressed.

leading to the net reaction,

$$SH_2 + \tfrac{1}{2}O_2 \rightarrow S + H_2O.$$

Each cell based on the above reaction can produce a few hundred watts of d.c. power. Several such cells are stacked in a fuel cell power plant. A 4·5 MW demonstration unit recently set up in New York consists of 20 stacks, each containing 500 cells.

The actual design of the cell depends on the electrolyte medium. A phosphoric acid fuel cell (PAFC) operates in the temperature range 150–200°C and its technology is in a fairly advanced stage. Alkaline fuel cells which were used to provide power for the Apollo space programme operate in the temperature range 80–200°C and use pure H_2- and CO_2-free air for the chemical reaction. The molten carbonate fuel cells (MCFC) are tolerant to CO_2 but have serious technical problems due to high operating temperatures (600–700°C).

It may be noticed that inorganic FC use highly acidic or alkaline electrolytes and high temperatures. These conditions corrode the electrodes. In recent years, it has been proposed that the use of enzymes (or bacteria) may help to carry out electrochemical reactions under much milder conditions (Lewis 1966; Sisler 1971; Wingard *et al* 1971). An additional advantage of biochemical fuel cells (BFC) is that many natural sources or wastes can be used as fuels. However, enzymatic reactions have to be carried out under conditions which cause slow transport of the electrons from the active sites of the enzyme to the electrode. Consequently, fuel cells prepared with redox enzymes as catalysts deliver very low power. Attempts at immobilisation of enzymes on the electrode surface through semipermeable membranes or entrapment in some polymer gel have been made (Guilbault 1977; Gray *et al* 1977; Buck 1978). This has not resulted in the desired efficiencies of electron transport. However, efforts are being made in this direction.

2. Concept of coenzyme immobilised anodes for BFC

Most oxidation-reduction enzyme utilise a coenzyme in oxidation reactions. Here the coenzyme gets covalently linked to the enzyme and carries out the process of electron transport during biological oxidation-reduction. A typical example is glucose oxidase which uses flavin adenine dinucleotide (FAD) for carrying out oxidation reduction reactions. In fact, FAD and its analogues riboflavin and flavin mononucleotide (FMN) act as coenzymes in a variety of biological oxidation-reduction reactions (Bright and Porter 1975; Rowan and Wood 1963; Beinert 1960). Some of the important reactions in this series are: oxidation of glucose to gluconic acid and succinate to fumarate. The complete oxidation-reduction cycle of this coenzyme involves two protons and two electrons as schematically represented in figure 1. When a (reduced) substrate SH_2 is oxidised in presence of a suitable enzyme (E), it liberates two protons and two electrons. These can be taken up by the oxidised FAD (I) to give $FADH_2$ (III). The reactions involve a number of steps and can be viewed as a sequential addition of two protons (leading to II) followed by the capture of two electrons (III). Likewise, the regeneration of the oxidised form of FAD involves sequential release of two protons (IV) and two electrons (I). Protons can easily pass into the aqueous medium, which if properly buffered, will not make serious changes in the system, particularly in the enzyme activity. However the flow of electrons from the coenzyme to the environment is a relatively slow process

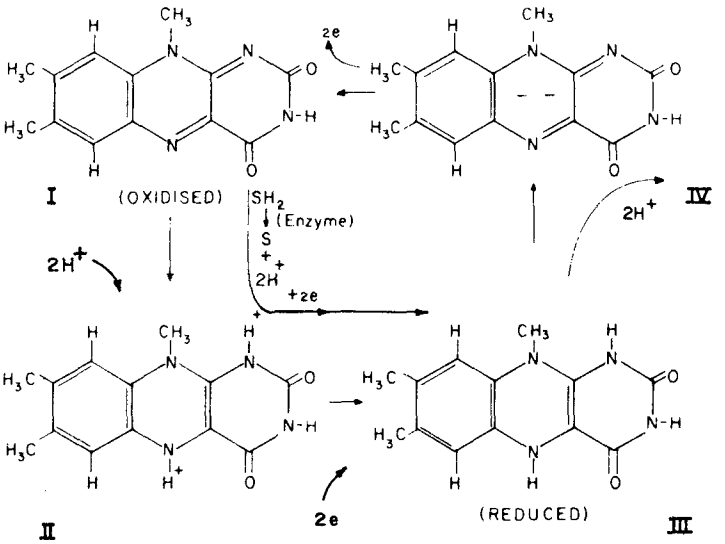

Figure 1. The oxidation-reduction cycle of FAD involves transfer of two protons and two electrons. Electrons are transferred to the electrode in stage IV → I while the oxidised form is enzymatically reduced to III by the substrate (S) which releases two protons and two electrons. The protons released in Steps III → IV are absorbed by the buffer and transported to the cathode compartment.

and requires an electron acceptor such as molecular oxygen.

$$H_2O + \tfrac{1}{2}O_2 + 2e \rightarrow 2OH^-.$$

The hydroxyl group may then combine with protons liberated in the previous steps to form water. In biological systems, the electron transfer from the substrate to molecular oxygen takes place through a number of steps involving several enzymes and coenzymes with the release of protons and coupled synthesis of adenosine triphosphate (ATP). ATP traps the free energy released in the oxidation of the substrate for use in various metabolic activities of the cell.

Our objective is to immobilise the coenzyme on an electrode in such a way that the electrons released in going from step IV to I can be directly transported to an external electrode. If this can be accomplished then we have a bioanode which in conjunction with an oxygen electrode can form the basis for the design of a biochemical fuel cell (figure 2). Such cells will involve a proton transfer in the internal circuit coupled with electron transport in the external circuits and can in principle have emf of about 1 V. If the rate of electron release and transport is fast then the chemical energy of the reaction

$$SH_2 + \tfrac{1}{2}O_2 \rightarrow S + H_2O \qquad (S = \text{Substrate})$$

can be converted directly into electrical energy. This can be achieved if, for example, FAD can be linked through conducting side chains to the electrode.

3. Theoretical considerations

Our approach is to understand the electron and proton transport mechanisms in the oxidation-reduction reactions of FAD by the molecular orbital approach, followed by

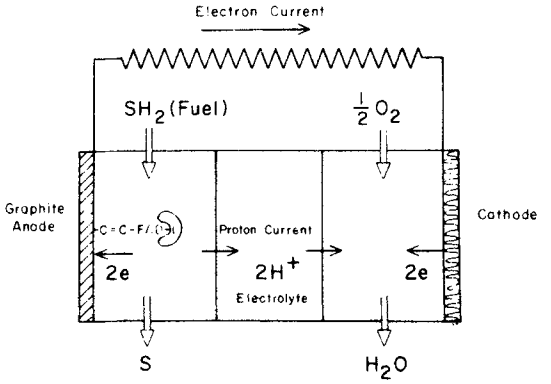

Figure 2. The conceptual drawing of a FAD based biochemical fuel cell. The FAD-immobilised graphite electrode is used as a cathode. Protons are transferred from the anodic to the cathodic compartment giving rise to an internal proton current as opposed to electron current in the external circuit. (S = substrate).

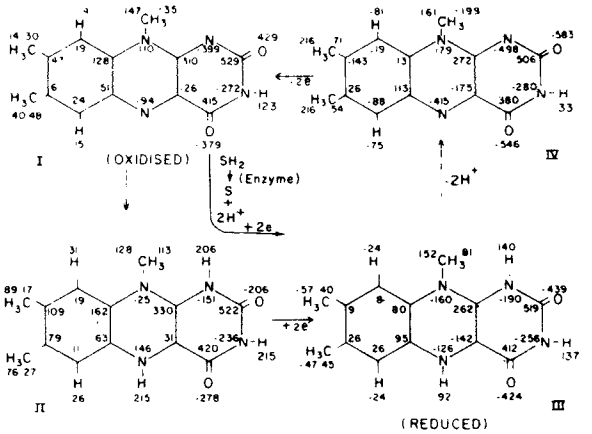

Figure 3. Results of INDO calculations of the charge densities in various redox states of FAD. The charge densities at various sites are in units of 10^{-3} atomic units (S = substrate).

the utilisation of these results for covalently attaching the FAD molecule to a graphite electrode through a short π electron system. To achieve the first aim, we have used the semiempirical quantum chemical technique of INDO (Pople *et al* 1967) which has been earlier shown to provide a satisfactory description of charge distribution in organic molecules (Pople and Beveridge 1970). The geometry for the flavin ring in these calculations has been chosen from x-ray data (Tanaka *et al* 1967). Standard geometries for C=C, C–C and C–H bonds were adopted wherever the x-ray data was not available. The ribose ring has been replaced by a methyl group, to economise on computer time. Since the oxidation-reduction cycle is carried out in the flavin ring, this does not make much difference to the conclusions. The results of the atomic charge distribution in the various oxidation states of FAD are shown in figure 3.

The reduced form of FAD (III) is a neutral molecule. However, the molecule shows a highly delocalised charge distribution with certain sites forming local dipoles. In accordance with general expectations these sites are the two carbonyl groups in the isoalloxazine ring and the four nitrogens in the two heterocyclic rings. Of these, one of the four nitrogens is substituted by ribose and two are involved in proton transfer reactions. Of the other possible sites for immobilisation of FAD, the four carbons in the benzenoid ring have a small electronic charge.

The removal of two protons from III leads to an anionic form of FAD (IV) with two negative charges. This leads to a higher negative charge at almost all the positions in the molecule. The ionic character of the two N-H bonds involved in the proton transfer (III → IV) make them sufficiently acidic to enable the release of the two protons. Present calculations indicate that in the third N-H group the proton is more tightly bound making its release more difficult.

In the process IV → I, the maximum change in electron density occurs at the two oxygen atoms (about 0·16 a.u.)—this is the stage in which the electrons are released and have to be passed on to external electrodes. The two carbonyl sites therefore appear to be potential sites for hooking up the semiconducting side chains.

During the reduction cycle of the coenzyme, it may be noticed that structure I has two negatively charged nitrogen atoms. These sites will have a high affinity to the two protons which are released in the coupled oxidation of SH_2 to S. Thus it can be concluded that the proton intake will precede the electron intake. Going from I to II, makes the molecule electrophilic almost all over its external surface. Thus II can easily absorb the two electrons released in the enzyme catalysed oxidation of SH_2.

Thus it appears that it should be possible to form an efficient coenzyme to electrode transport bridge by immobilizing FAD on a graphite electrode through alternate hydrocarbon chains at one of the two carbonyl groups. It may be argued that the prediction of atomic charges in INDO formalism is approximate. However, the type of questions we are asking are qualitative and can be fairly satisfactorily answered by the semi-empirical approach used here.

4. Experimental

FAD used in the experiment was obtained from the Sigma Chemical Co., USA. All other reagents used were of analytical grade. The electrodes were either lead battery graphite electrodes or refills of lead pencils. All the reactions were carried out on the surface of the electrodes, thereby eliminating the additional steps of isolation and purification of the products.

Before use, the electrodes were refluxed with acetone for about an hour and vacuum-dried. Carboxylic acid groups were created on the surface by treating the electrodes first with concentrated HNO_3 for 15 minutes at room temperature followed by concentrated H_2SO_4 for one hour at 170°C. The electrodes were then thoroughly washed with distilled water and vacuum-dried. The carboxylic acid groups were reduced to alcoholic groups by treatment with sodium and ethanol. The electrodes were placed in a round bottomed flask fitted with a large-bore reflux condenser along with 60 ml of absolute ethanol. Then sodium (3·2 g) was added through the condensor rapidly enough to keep up a vigorous reaction with occasional shaking. After the initial reaction had subsided 8 ml more of absolute ethanol were added and the mixture was

heated on a steam bath until all the sodium had reacted. Twenty ml distilled water were added and the mixture refluxed for one hour. After cooling 24 ml of water were added and then the electrodes taken out, washed thoroughly with water and vacuum-dried.

The alcoholic group was brominated by KBr/H_2SO_4 treatment. KBr (30 g) was dissolved in 50 ml water and 25 ml of concentrated H_2SO_4 were slowly added with constant stirring and cooling, the mixture was then filtered. The electrodes were immersed in the filtrate, 15 ml of concentrated sulphuric acid added and the mixture refluxed for 3–4 hr. The electrodes were then taken out and washed first with distilled water and then with chloroform.

Phosphoniumylide derivatives were prepared by refluxing the electrodes for 4 hr in 20 ml chloroform to which was added 1 g of triphenyl phosphine, cooling to 0°C and washing with cold methanol. The electrodes were then placed in a solution of FAD (0·5 mg/ml methanol) and sodium added (0·012 g/ml) with stirring. They were left overnight at room temperature, then taken out, washed and vacuum-dried. The overall scheme of the modification is sketched in figure 4.

5. Cyclic voltametric studies

Cyclic voltametric studies were carried out to check if FAD had been immobilised on graphite in the above reactions. In all the cyclic voltametric studies, 1 M KCl was used as supporting electrolyte, saturated calomel electrode as the reference electrode and platinum mesh as counter electrode. A multipurpose electrochemical instrument fabricated at the Institute was used (Bhagat 1971). For comparison cyclic voltamograms with FAD in 1 M KCl (1 mg/ml) were also done.

The cyclic voltamograms of electrodes modified with FAD (figure 5a) indicate the presence of two redox couples representing the reduction of the two nitrogens at positions 1 and 5 and their subsequent oxidation on the return sweep. No difference in the peak potentials was observed when different rates were used for sweeping the voltage. None of these peaks was observed in the cyclic voltamograms of the unmodified graphite electrode (figure 5b). That the species attached to the electrode was FAD was proved by the cyclic voltamogram of FAD in the supporting electrolyte (figure 5c) which showed similar redox couples. However, at low sweep rates no peaks were observed.

The appearance of peaks both on the forward and return sweeps indicates the reduction and oxidation of FAD attached to the electrode. This also implies the chemical stability of FAD in the attached form as compared to its light-induced instability in

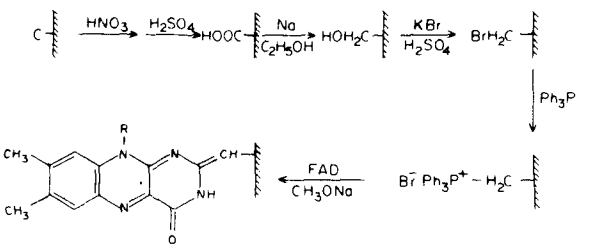

Figure 4. Reaction scheme for immobilising FAD on the graphite electrode.

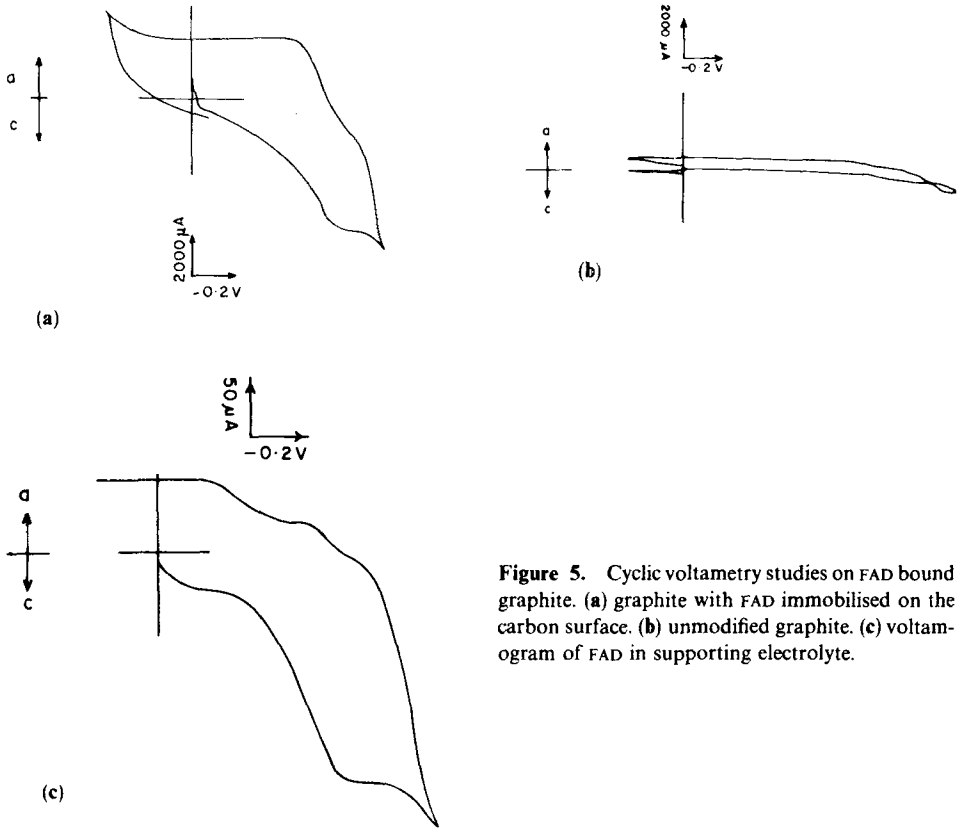

Figure 5. Cyclic voltametry studies on FAD bound graphite. (**a**) graphite with FAD immobilised on the carbon surface. (**b**) unmodified graphite. (**c**) voltamogram of FAD in supporting electrolyte.

solution. The oxidation of $FADH_2$ is important in that its conversion to FAD will result in the transfer of two electrons to the electrode which will eventually appear as a current in the external circuit. This being a non-enzymic reaction, its absence due to any reason whatsoever, will amount to no current in the external circuit.

However, the nature of attachment of FAD to the electrode is yet to be established by surface studies like x-ray photoelectron spectroscopy. Also non-porous electrodes have to be tried and the overall procedure of the modification to be improved upon. The cyclic voltametric graphs suggest that the attachment of glucose oxidase apoenzyme to FAD, fixed on the electrode, will be successful. This also has to be checked experimentally.

Acknowledgement

The authors are grateful to Dr K S V Santhanam for his help in the cyclic voltametry experiments. They are also grateful to Dr Anil Saran for his interest in the work and to Dr C K Mitra for some preliminary calculations.

References

Austin L G 1967 Fuel Cells, NASA report, Washington
Beinert H 1960 in *The enzymes* (ed.) P D Boyer (New York: Academic Press) Vol. 2 p. 339
Bhagat V R and Santhanam K S V 1971 *J. Sci. Ind. Res.* **30** 235
Bright H J and Porter D J T 1975 in *The enzymes* (ed.) P D Boyer (New York: Academic Press) 3rd ed. Vol. 12 p. 421
Buck R P 1978 *Anal. Chem.* **50** 178
Govil G 1980 *Phys. News* **11** 134
Gray D N, Keyes M H and Watson B 1977 *Anal. Chem.* **49** 1067
Guilbault G C 1977 in *Comprehensive analytical chemistry* (ed.) G Svehla (Amsterdam: Pergamon Press) Vol. 8
Lewis K 1966 *Bacteriol. Rev.* **30** 101
Liebhasky H A and Cairn E J 1968 *Fuel cells and fuel batteries* (New York: John Wiley)
Pople J A and Beveridge D L 1970 *Approximate molecular orbital theory* (New York: McGraw-Hill)
Pople J A, Beveridge D L and Dobosh P A 1967 *J. Chem. Phys.* **47** 2026
Rowan T and Wood H C S 1963 *Proc. Chem. Soc.* 21
Sisler F D 1971 *Prog. Ind. Microbiol.* **9** 1
Tanaka N, Ashida T, Sasada Y and Kakudo M 1967 *Bull. Chem. Soc. Jpn* **40** 1739
Wingard L B, Liu C C and Nagda N L 1971 *Biotechnol. Bioeng.* **13** 629