CHEMISTRY LETTERS, pp. 693-697, 1972. Published by the Chemical Society of Japan

A NEW METHOD OF ASSAYING THE ENZYME ACTIVITY USING THE ENZYME FUEL CELL

Keisaku KIMURA, Hiroo INOKUCHI, and Tatsuhiko YAGI

Institute for Solid State Physics, the University of Tokyo, Roppongi, Tokyo 106
and Department of Chemistry, Shizuoka University, Shizuoka 420

A new assay method for the enzyme, hydrogenase, has been proposed in which the enzyme activity is expressed as a current density of a fuel cell composed of a hydrogen half-cell (anode) containing H_2 - H^+ - hydrogenase system and a saturated calomel electrode (cathode). The principle of the assay method is based on the fact that the short-circuit current density of the cell is proportional to the activity of the enzyme contained in the anode half-cell. The new method may be applied to any oxidoreductase, if properly modified.

One of the most important parameters in the study of an enzyme is its catalytic activity. To assay the activity of an enzyme, it is the common practice to measure the rate of the chemical reaction catalyzed by a given amount of the enzyme. The reaction rate (v) is defined either as the rate of decrease in the substrate concentration or as the rate of increase in the product concentration under the standard assay conditions. Thus,

$$v = \frac{s_0 - s_t}{t} \qquad \text{or} \qquad v = \frac{p_t - p_0}{t}$$

where s_0 and s_t, and p_0 and p_t, stand for the substrate and the product concentrations at the times 0 and t respectively. It is, thus, necessary to know the concentrations of the substrate or the product at two points at least, i. e., the times 0 and t. There is, in principle, no other method among the assay methods proposed so far, whether the reaction rate is determined spectrophotometrically, manometrically, electrochemically, or by other techniques, except where the amount of a pure enzyme which is proportional to the activity is determined spectrophotometrically or by weight.

In this paper, we will propose a new and different assay method for an enzyme. We have chosen hydrogenase [H_2: ferricytochrome c_3 oxidoreductase, Desulfovibrio vulgaris, Miyazaki] in developing the new assay method, but the method may be applied to any oxidoreductase, if properly modified.

PRINCIPLE OF THE METHOD

Hydrogenase catalyzes the following reaction:

$$\frac{1}{2}H_2 + Fe^{3+}\text{(in ferricytochrome } c_3) \rightleftharpoons H^+ + Fe^{2+}\text{(in ferrocytochrome } c_3)$$

In this reaction, cytochrome c_3 can be replaced by an artificial electron carrier such as methyl viologen. Thus,

$$\frac{1}{2}H_2 + MV^{2+} \rightleftharpoons H^+ + MV^+$$

where MV^{2+} and MV^+ stand for the oxidized and the reduced forms of methyl viologen respectively.

The hydrogenase reaction is observed as a current of an enzyme fuel cell composed of a saturated calomel electrode (cathode) and a hydrogen electrode (anode), in which hydrogenase is used as a catalyzer, and the viologen dye, as an electron carrier for the H_2 - H^+ equilibrium. It has been observed that the short-circuit current density of the cell is proportional to the activity of the enzyme contained in the hydrogen electrode of the cell. It is thus possible to assay the enzyme activity by the direct measurement of the current density of the cell at one time, instead of by measuring the change in reactant concentrations between the times 0 and t.

MATERIALS

Hydrogenase preparations with various degrees of purity were obtained from sulfate-reducing bacteria, Desulfovibrio vulgaris, Miyazaki.[1] The activity of the hydrogenase preparation used as the reference sample was measured by the H_2-evolution method.[1] The degree of the purity of the enzyme was expressed by the specific activity (activity units/absorbance at 280 nm of the preparation). The specific activity of the most highly purified preparations so far obtained was about 110. Methyl viologen was a product of the British Drug Houses.

APPARATUS

The cell used in this investigation is shown in Fig. 1. The container of the hydrogen electrode half-cell

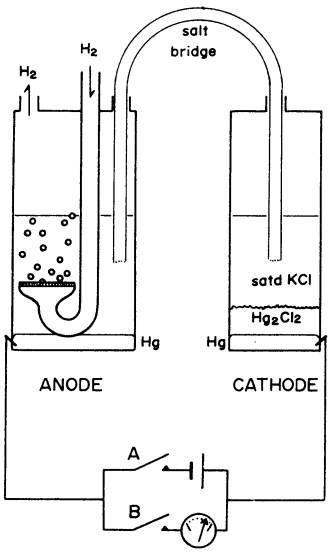


Fig. 1. The apparatus and the circuit.

was made of Pyrex glass, 40 mm in diameter and 105 mm high. Hydrogen gas which was purified by "Deoxo" (Engelhard Industries, Inc.) was supplied to the container through a glass filter (120 mesh) at a constant rate of 65 ml/min. The Hg electrode deposited at the bottom of the container was connected to the Hg deposit of the calomel electrode, a microammeter (PM-18C microvoltammeter, Toa Electronics, Ltd.) being inserted in-between. The reaction medium in the container was connected to the saturated calomel electrode by a salt bridge (4 mm in diameter and 300 mm long) composed of KCl-saturated 3 % agar gel. The distance between the Hg electrode and the salt bridge in the anode container was 25 mm.

The medium in the anode container was composed of 20 μmoles of methyl viologen and the enzyme preparation in 40 ml of 0.04 M phosphate buffer (pH 7.0). The amount of the hydrogenase preparation is illustrated in the legend to Fig. 2.

RESULTS

Measurement of the Short-Circuit Current of the Enzyme Fuel Cell

The colorless methyl viologen (oxidized form) in the enzyme solution in the anode container was reduced electrolytically by means of a UM-1 dry cell (with the A switch closed) with a constant flow of H_2-bubbling.

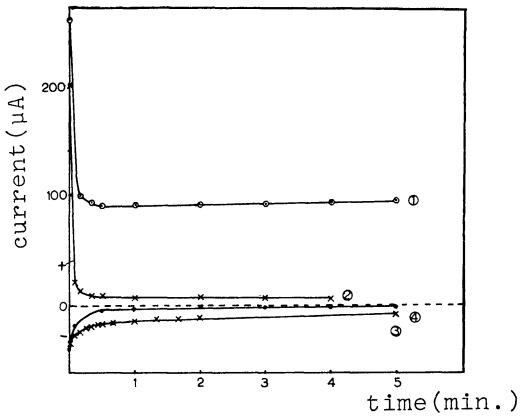


Fig. 2. Short-circuit currents of various media observed at 20°C. 1: complete system with hydrogenase (14 units) and methyl viologen (20 μmoles), 2: hydrogenase omitted, 3: methyl viologen omitted, and 4: the buffer solution only. In these experiments, methyl viologen was reduced enzymatically by H_2.

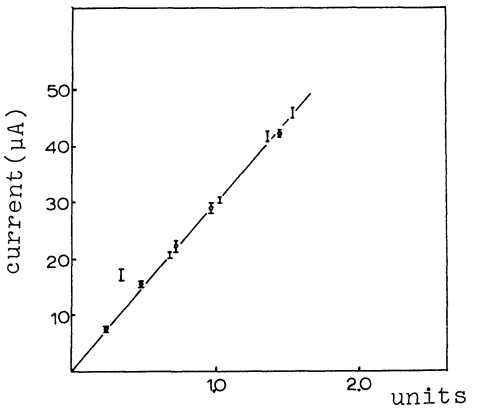


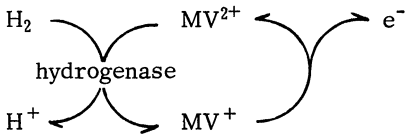
Fig. 3. Relation between I_S and the concentrations of hydrogenase preparations. I: for the hydrogenase preparation with specific activity 110, and ○: for the preparation with specific activity 2. All experiments were conducted in a thermostat (25.0 ± 0.2°C).

When methyl viologen was reduced to the reduced form with blue coloration, the dry cell was disconnected (with the A switch open); the B switch was thus closed, and the short-circuit current density was recorded. Then an additional portion of the enzyme preparation was added and the current density was recorded again. Similar processes were repeated with varying amounts of enzyme preparations of various degrees of purity. Figure 2 shows the time-course curves of the changes in the short-circuit current densities. As is shown in this figure, each current density approached a constant value, I_S. This figure also indicates that weak currents observed in cases 2, 3 and 4 remained for a few minutes. These residual currents must have been

caused by impurities in the anode electrolyte or by oxidation of H_2 catalyzed by the Hg electrode. However, the effect of impurities on the value of I_S for case 1 was negligible as illustrated in Fig. 2. Figure 3 shows the linear relation between I_S and the amount of the enzyme added to the medium in the hydrogen electrode half-cell. This linear relationship held in the range from 1 μA to 100 μA. When the current exceeded about 100 μA, however, the short-circuit current was restricted by the ohmic drop of the cell. I and ϕ in Fig. 3 show the fluctuation range of the dangling current caused by the H_2-bubbling. This fluctuation may be diminished by adopting a rotating electrode.

Theoretical Considerations

Figure 2 shows that the short-circuit current density recorded in the absence of hydrogenase or methyl viologen was negligible. This means that a cyclic redox system illustrated as in the following diagram must



have been constructed in the hydrogen half-cell. Since the viologen dye is oxidized at the surface of the Hg electrode and repeatedly reduced with H_2 in the medium by hydrogenase, the overall reaction is the mere oxidation of H_2 to protons, as is illustrated in the following equation:

$$H_2 \rightarrow 2\,H^+ + 2\,e^-$$

If this redox system is completely reversible, E_0' at pH 7.0 must be -0.414 V against the standard hydrogen electrode at 25°C, or -0.655 V against the saturated calomel electrode. The observed potential of this cell was -0.650 V.

When the electrical energy was withdrawn as an electric current, an actual enzymatic reaction had to take place. The current density must, thus, be a function of the catalytic activity of hydrogenase which equilibrates the H_2 - H^+ redox couple, the rate of the diffusion of H_2 gas to the medium, the rate of the diffusion of the reduced and the oxidized viologen dyes to and from the Hg electrode, the surface area of the Hg electrode, or other parameters. The linear relation between I_S and the amount of the enzyme illustrated in Fig. 3 clearly indicates that the catalytic activity of the enzyme limits the overall reaction rate under the experimental conditions described in this paper, and thus is proportional to the observed current density of the cell.

Table I. The enzyme activity evaluated from the standard calibration curve

Activity of hydrogenase[a] (in units, at 30°C) added to the medium.	0.8	1.2	1.6	2.0
Activity of hydrogenase (in units, at 25°C) evaluated from the calibration curve shown in Fig. 3.	0.94	1.30	1.64	1.90

a) The specific activity of the enzyme preparation was about 50.

Assay Method for Hydrogenase

When once the standard calibration curve for hydrogenase as exemplified in Fig. 3 has been given, the enzymatic activity of any hydrogenase preparation can be read and recorded as a current density, I_S, after the short-circuit current stabilizes. Some examples are given in Table I.

DISCUSSION

Biochemical fuel cells using microorganisms or enzymes have been widely investigated.[2-4] In these studies, energy extraction from metabolic reactions catalyzed by microorganisms or enzymes has been the main subject from the view point of technology. It has been observed in these investigations that microorganisms and enzymes are effective convertors of the chemical energy to the electrical energy. One example of an enzyme fuel cell using hydrogenase was reported by Mizuguchi et al.[5] However, no one has yet attempted to use the enzyme fuel cell as a method of assaying the activity of the enzyme. In this study, hydrogenase was chosen as the enzyme in order to develop a new technique for measuring the enzyme activity. We found that the short-circuit current density of the cell was proportional to the activity of the enzyme contained in the cell, and that the activity of any hydrogenase preparation could be read as a current density from a standard calibration curve.

In order to conduct this measurement, the current density of the cell has to be measured a number of times before the current stabilizes. Thus, it would appear that this new method produces no new advantages over the normal assay methods of an enzyme; where the reactant concentration is normally measured several times in order to determine the slope. However, this is not the case. We are observing the current density, and not the rate of change in the current density of the cell.

Since any redox couple can be a component of a half-cell, like the H_2 - H^+ couple described in this paper, any oxidoreductase activity can be measured by a similar technique if it is properly modified. As the enzyme activity is expressed as a current density of the cell, the recording of the activity of the enzyme in a series of samples is possible. This means that the method can be automatized to allow successive determinations of a series of samples or continuous monitoring of enzyme activity of a single preparation. The simple and rapid method proposed in this paper will have wide application in the field of biochemistry and medical technology as well as in other fields of kinetic studies where the activity of a catalyzer is to be determined.

We wish to thank Dr. M. Fujihira for his stimulating discussion.

REFERENCES

1) T. Yagi, J. Biochem., 68, 649 (1970).

2) M. F. Wolff, Electronics, 35, 30 (1962).

3) E. L. Colichman, Proc. IEEE, 812 (1963).

4) W. van Hees, J. Electrochem. Soc., 112, 258 (1965).

5) J. Mizuguchi, S. Suzuki, K. Kashiwaya and M. Tokura, Kogyo Kagaku Zasshi, 67, 410 (1964).

(Received May 26, 1972)