Kinetics and thermodynamics of activation of quinoprotein glucose dehydrogenase apoenzyme *in vivo* and catalytic activity of the activated enzyme in *Escherichia coli* cells

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Apo-glucose dehydrogenase existing in *Escherichia coli* is converted to the holoenzyme with exogenous pyrroloquinoline quinone (PQQ) and Mg^{2+} . Catalytic behaviour of the *E. coli* cells with the holoenzyme is characterized by a Michaelis–Menten-type equation with a catalytic constant of the cell and apparent Michaelis constants for D-glucose and an artificial electron acceptor added to the *E. coli* suspension. The catalytic constant is expressed as the product of the number of molecules of the enzyme contained in an *E. coli* cell (z) and the catalytic constant of the enzyme (k_{cat}), which were determined to be 2.2×10^3 and $6.8 \pm 0.8 \times 10^3 \text{ s}^{-1}$ (phenazine methosulphate as an electron acceptor) respectively. Kinetics of the *in vivo* holoenzyme formation can be followed by an enzyme-electrochemical method developed by us. The rate constants for the reactions of apoenzyme with

PQQ ($k_{\text{r,PQQ}}$) and with Mg^{2+} ($k_{\text{r,Mg}}$) were determined to be $3.8 \pm 0.4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $4.1 \pm 0.9 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. Equilibrium constants for the binding of apoenzyme to PQQ and Mg^{2+} were determined as the dissociation constants $K_{\text{d,PQQ(Mg)}}$ and $K_{\text{d,Mg}}$ to be $1.0 \pm 0.1 \text{ nM}$ and $0.14 \pm 0.01 \text{ mM}$ respectively. The dissociation constants for Ca^{2+} were also determined. The holoenzyme, once formed in *E. coli*, returns gradually to the apoenzyme in the absence of PQQ and/or Mg^{2+} in solution. EDTA was effective to remove Mg^{2+} from the enzyme in the cells to deactivate the enzyme completely, while PQQ remained in the *E. coli* cells.

Key words: apo-glucose dehydrogenase, *in vivo* catalytic activity, *in vivo* reconstitution, pyrroloquinoline quinone.

INTRODUCTION

Escherichia coli has a membrane-bound dye-linked quinoprotein glucose dehydrogenase (mGDH). It is present in the form of an apoenzyme [1–3], since *E. coli* is unable to synthesize its prosthetic group pyrroloquinoline quinone (PQQ) [4]. Apo-mGDH purified from the membranes of *E. coli* has a molecular mass of 88 kDa [5] and its active site structure is modelled on that of methanol dehydrogenase [6,7]. It requires PQQ and Mg^{2+} for activation to form holo-mGDH [5]. The membrane fractions containing apo-mGDH are also activated in the presence of PQQ and Mg^{2+} [2,8], where divalent cations such as Ca^{2+} can be substituted for Mg^{2+} , although the activity is relatively low [2]. Holo-mGDH catalyses the oxidation of D-glucose (and other sugars [5,7]) to the lactone in the presence of such electron acceptors as ubiquinone-1, phenazine methosulphate (PMS), 2,6-dichlorophenol-indophenol (DCIP) and hexacyanoferrate (III) [2,5,8]. Ubiquinone 8 contained in *E. coli* phospholipids has been shown to be the compound accepting electrons directly from holo-mGDH in the D-glucose oxidase-respiratory chain reconstituted into proteoliposomes [9]. Kinetics of the catalytic reaction has been studied with the enzymes from *Acinetobacter calcoaceticus* [10,11], as well as the enzyme from *E. coli* [7], and the reaction mechanisms are discussed based on the structural model of the active site [6,7,12]. The reconstitution process of apo-mGDH to holo-mGDH with PQQ and Mg^{2+} has been studied extensively using the enzymes from both *E. coli* [2,5,7–9,12] and *A. calcoaceticus* [10,11], in which the binding constants of apo-mGDH to PQQ and Mg^{2+} have been determined [7,10,11,13]. However, little is known about the activation of apo-mGDH *in vivo* and the

catalytic reaction by the enzyme activated in the membranes of intact *E. coli*. There is indirect evidence for *in vivo* activation of apo-mGDH; the respiration rate [14] and glucose metabolism [1,15] of *E. coli* cells is accelerated by the presence of PQQ in the medium. It is suggested that apo-mGDH in the inner membrane of *E. coli* has a PQQ binding site at the periplasmic side of the membrane [3,16]. Therefore, we may expect that exogenous PQQ is able to readily reach the binding site through porins in the outer membranes.

The present paper describes a quantitative investigation of the activation of apo-mGDH in *E. coli* with exogenous PQQ and Mg^{2+} , and the enzyme kinetics of the activated enzyme *in vivo*. The measurements have been carried out by means of an enzyme-electrochemical method developed by us [17–19]. The results are compared with those reported for GDH isolated from the bacterial membranes, and the characteristics of the *in vivo* activation and catalysis are discussed based on the comparison.

MATERIALS AND METHODS

Micro-organism and reagents

E. coli K-12 (IFO3301) was grown on a D-glucose-mineral medium to a late-logarithmic phase [8] and harvested by centrifugation. The cell paste was washed twice with distilled water and stored at -80°C until required. The stored cells were resuspended in a saline solution (0.85% NaCl) kept at 5°C and used for measurements within a few days. In some cases, the harvested cells were directly resuspended in a saline solution

Abbreviations used: PQQ, pyrroloquinoline quinone; mGDH, membrane-bound glucose dehydrogenase; Q_0 , 2,3-dimethoxy-5-methyl-1,4-benzoquinone; DCIP, dichlorophenol-indophenol; PMS, phenazine methosulphate.

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(0.85% NaCl) and used immediately for measurements. There was no appreciable difference in the experimental results obtained with the stored and freshly prepared cells. The cell population in the suspension was determined by using a haemocytometer; the suspension at $D_{610} = 1.0$ contained 3.4×10^8 cells/ml.

PQQ and 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0 , a ubiquinone homologue) were purchased from Sigma. All other chemicals used were of reagent grade and were used as received. The PQQ concentration was determined spectrophotometrically using ϵ (257 nm) = $19122 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [20].

Measurement of the catalytic activity of *E. coli* cell suspensions

E. coli cells were suspended in Mops buffer (pH 6.5), adjusted to an ionic strength of 1.0 with NaCl, and incubated in the presence of $1 \mu\text{M}$ PQQ and 5 mM Mg^{2+} for 15 min at room temperature. A dialysis-membrane-coated glassy carbon electrode, prepared as described previously [17], was immersed in the *E. coli* cell suspension, then an electron acceptor and D-glucose were added to the suspension in that order. The electrode measures the concentration of the electron acceptor as a reduction current; thus, the consumption of the electron acceptor by the *E. coli* cell-catalysed oxidation of D-glucose can be measured as the time-dependent decrease in the current [17].

In the titration experiment of apo-mGDH with PQQ, the buffer containing *E. coli* cells was incubated in the presence of lower concentrations of PQQ and 5 mM Mg^{2+} for more than 7 h to ensure equilibrium for the binding between PQQ and apo-mGDH in the cells. Then, the reduction current was measured in the suspension containing 10 mM D-glucose and 2 mM Q_0 .

The electrochemical measurements were carried out at a fixed potential with a three-electrode system using a BAS CV50W voltammetric analyser under anaerobic conditions at 25°C , in which a saturated Ag/AgCl electrode and a platinum disk were used as the reference and counter electrodes respectively [17]. The Mops buffer solution used in the measurements was adjusted to pH 6.5 with NaOH, and the ionic strength was adjusted to 0.1 with NaCl.

Preparation of an *E. coli*-modified electrode

A $5 \mu\text{l}$ aliquot of the *E. coli* cell suspension ($D_{610} = 28.6$) was dropped on to the surface of a carbon paste electrode (3.4 mm diameter) prepared as described previously [21]. The solvent was allowed to evaporate, then the surface was covered with a dialysis membrane. The electrode thus prepared, which is referred to as an *E. coli*-modified electrode in the following, was used for the measurements of holo-mGDH formation in the *E. coli* cells. When mGDH in the cells is activated, the *E. coli*-modified electrode produces a catalytic oxidation current in the solution containing D-glucose and an electron acceptor. The oxidation current is attributed to the mGDH-catalysed electrochemical oxidation of D-glucose with the electron acceptor mediating electron transfer between mGDH and the electrode [18,19]. Thus, the oxidation current is directly proportional to the rate of the mGDH-catalysed reaction proceeding in the immobilized *E. coli* cells on the electrode.

Measurement of holo-mGDH formation in *E. coli*

The measurement uses the *E. coli*-modified electrode immersed in a stirred buffer solution containing Mg^{2+} , Q_0 and D-glucose. When PQQ is added to the solution, the oxidation current starts to appear reflecting the holo-mGDH formation in the *E. coli*

cells. The magnitude of the oxidation current is proportional to the rate of the *E. coli*-catalysed oxidation of D-glucose, and thus, to the amount of holo-mGDH formed in the *E. coli* cells [18,19]. Accordingly, time-dependent increase in the current measures the rate of holo-mGDH formation in the cells. The current reaches a constant value when the reaction of holo-mGDH formation reaches equilibrium.

In the measurement of the current in the equilibrium state, the *E. coli*-modified electrode was first incubated in a buffer containing PQQ and Mg^{2+} for a given period of time, usually 15 h. Then, Q_0 and D-glucose were added to the same solution to measure the current. The electrochemical measurements were carried out in anaerobic solutions at pH 6.5 and 25°C with the potential fixed at 0.5 V, using the electrochemical apparatus described above.

RESULTS AND DISCUSSION

Catalytic activity of mGDH in *E. coli* cells

Figure 1 shows an example of the current–time (i – t) curve from which mGDH activity in *E. coli* cells is determined. The current at the electrode immersed in the *E. coli* cell suspension, which was pretreated with $1 \mu\text{M}$ PQQ and 5 mM Mg^{2+} for 15 min, increased with the addition of Q_0 and reached a steady state. The magnitude of the steady-state current (i) is proportional to the concentration of Q_0 (c_0) in the suspension, the proportionality constant (k) being given by $k = c_0/i$. The current begins to decrease by the addition of D-glucose to the suspension, which indicates that *E. coli* cells catalyse the oxidation of D-glucose with Q_0 as an electron acceptor. The rate of the *E. coli*-catalysed reaction (v) can be determined from the initial slope of the i – t curve ($\Delta i/\Delta t$) from $v = -(1/k)(\Delta i/\Delta t) = -\Delta c_0/\Delta t$. The rate was independent of the pre-incubation condition as long as the incubation was at concentrations higher than $1 \mu\text{M}$ PQQ and 5 mM Mg^{2+} for time periods longer than 15 min. This confirms that mGDH in the *E. coli* cells in the suspension was fully activated under the pre-incubation conditions. Such a decrease in current was not observed with *E. coli* suspensions not incubated with PQQ.

The value of v was proportional to the number of *E. coli* cells in the suspension in the range $D_{610} = 0.18$ – 5.4 (i.e. 6.1×10^5 to 1.8×10^9 cells/ml) and increased with the increasing concentrations of D-glucose and an electron acceptor to approach a saturation value. This is the behaviour observed with a number of bacterial species, and it has been successfully analysed by a conventional Michaelis–Menten-type equation [17]. Such analy-

Figure 1 Electrochemical measurement of the catalytic activity of *E. coli* cells for the oxidation of D-glucose with Q_0 as an electron acceptor

The current was recorded at -0.2 V in 1 ml of *E. coli* suspension ($D_{610} = 5.0$) pretreated with $1 \mu\text{M}$ PQQ and 5 mM Mg^{2+} for 15 min. Q_0 and D-glucose were then added successively to a final concentration of 2 mM and 10 mM respectively.

Table 1 Kinetic parameters for the catalytic oxidation of D-glucose by *E. coli* cells with activated mGDH

Experiments similar to that shown in Figure 1 were used to obtain the data analysed by a Michaelis–Menten-type equation by means of non-linear curve fitting [17]. A Clark-type oxygen electrode was used in the measurements using O₂ as an electron acceptor in air-saturated solutions.

Electron acceptor	K'_{Glc} (mM)	K'_M (mM)	$10^{-6} \times z k_{\text{cat}}$ (s ⁻¹)
PMS	0.90 ± 0.06	0.80 ± 0.07	15 ± 1.7
Q ₀	0.64 ± 0.10	1.1 ± 0.1	6.7 ± 1.1
DCIP	4.2 ± 0.5	0.80 ± 0.09	7.1 ± 1.2
Fe(CN) ₆ ³⁻	0.19 ± 0.02	3.5 ± 0.4	1.6 ± 0.3
O ₂	0.14 ± 0.01	< 0.05	0.45 ± 0.03

sis of the data obtained in the present experiments yielded apparent Michaelis constants for D-glucose (K'_{Glc}) and the electron acceptor (here Q₀; K'_M) and the catalytic constant of *E. coli* cell ($z k_{\text{cat}}$). It is noted that the 'apparent' Michaelis constants mean that the constants reflect the effect of permeability of the bacterial outer membrane and/or the equilibrium distribution [17]. Table 1 gives the K'_{Glc} , K'_M , and $z k_{\text{cat}}$ values for several kinds of electron acceptors including dioxygen. The values of K'_{Glc} as well as K'_M depend on the kind of electron acceptors. This indicates that the rate of the overall catalytic reaction is mainly controlled by the reaction step between mGDH in the cells and the electron acceptors [17]. Similar dependence of the Michaelis constants has been reported for the reaction of mGDH isolated from *A. calcoaceticus* with such electron acceptors as PMS/DCIP, DCIP and decylubiquinone (Q₂)/DCIP [11]. The Michaelis constant for D-glucose for the reaction of mGDH from *E. coli* has been determined in the presence of PMS and DCIP as 2.1 mM [7]. This can be compared with the values of K'_{Glc} = 0.9 mM for PMS and 4.2 mM for DCIP (Table 1). The relative magnitudes of $z k_{\text{cat}}$ for PMS, Q₀, DCIP and Fe(CN)₆³⁻ in Table 1 agree well with the relative enzyme activities reported for isolated mGDH with these compounds [5]. These results strongly support the idea that the electron acceptors penetrating into the *E. coli* cells accept electrons directly from the mGDH in the *E. coli* cells. The value of $z k_{\text{cat}}$ for O₂ in Table 1 ($4.5 \times 10^5 \text{ s}^{-1}$) is a quantitative expression of the acceleration of the respiration rate, as indirectly observed with *E. coli* in the presence of D-glucose and PQQ [14], in which ubiquinone 8 in the membranes should be the electron acceptor in the mGDH reaction linked to the respiratory chain, as has been reported for the respiratory chain reconstituted into proteoliposomes [9].

The catalytic constant of an *E. coli* cell is expressed as the product of the number of mGDH molecules in a cell (z) and the catalytic constant of mGDH (k_{cat}) in the cell [22]. The value of z can be determined by the titration of an *E. coli* cell suspension with PQQ as illustrated in Figure 2. The titration curve satisfies a linear relationship almost to the equivalence point at the concentration of PQQ, [PQQ] = 29.5 nM. The value of z was calculated to be 2.2×10^3 from [PQQ] = 29.5 nM (i.e. $29.5 \times 10^{-12} \times N_A$ molecules/ml, N_A being the Avogadro constant) and the number of *E. coli* cells in the suspension $D_{610} = 23.9$ (i.e. 81.4×10^8 cells/ml). The experimental data is explained well by the solid curve in Figure 2, which is obtained by a theoretical equation with the z value and the dissociation constant of the binding of PQQ to apo-mGDH as described below. It should be noted that the value of z (2.2×10^3) is the average number of the enzyme molecules per cell in the *E. coli* suspension. The value of k_{cat} is calculated to be $3.0 \pm 0.5 \times 10^3 \text{ s}^{-1}$ for Q₀ and

Figure 2 Titration curve for the binding of PQQ to apo-mGDH in *E. coli* cells

The catalytic activities of 1 ml suspensions of *E. coli* cells ($D_{610} = 24.0$) that had been equilibrated with varying concentrations of PQQ and 5 mM Mg²⁺ were measured in the same manner as that shown in Figure 1. The rate of the catalytic reaction (v) is normalized by the maximum rate of the reaction (v_{max}), and the relative rate is plotted against the concentration of PQQ added to the suspension. Results are means $\pm$ S.D. ($n = 4$). The solid line is a theoretical curve as detailed in the text.

$6.8 \pm 0.8 \times 10^3 \text{ s}^{-1}$ for PMS, with the corresponding $z k_{\text{cat}}$ values in Table 1. This is the first report, as far as we know, on the determination of the number of enzyme molecules in a bacterial cell and the catalytic activity *in vivo*. Interestingly, the k_{cat} values were much larger than the catalytic constant for isolated mGDH (592 s^{-1}), which is calculated from the molecular mass of mGDH (88 kDa) and the V_{max} value determined with PMS/DCIP at pH 8.75 [7]. The catalytic constant calculated from the specific activity of isolated mGDH (285.7 units/mg, obtained with PMS/DCIP at pH 8.75 [5]) gave a smaller value (420 s^{-1}). This may be attributed to the relatively large Michaelis constants for the reaction with DCIP and PMS (0.80 mM for both dyes, Table 1), compared with the concentrations of DCIP and PMS (0.22 and 0.67 mM respectively) employed in the activity measurements [5]. The catalytic constants for mGDH from *A. calcoaceticus* and *Gluconobacter suboxydans* were calculated to be 958 s^{-1} (with the molecular mass of 80 kDa and $V_{\text{max}} = 720$ units/mg with PMS/DCIP at pH 8.5 [11]), and 559 s^{-1} (PMS/DCIP) and 301 s^{-1} (Q₁) (with the molecular mass of 87 kDa and $V_{\text{max}} = 720$ and 205 units/mg with PMS/DCIP at pH 8.75 and Q₁ at pH 6.5 respectively [23]). The large k_{cat} value in this study ($k_{\text{cat}} = 6.8 \pm 0.8 \times 10^3 \text{ s}^{-1}$ with PMS at pH 6.5) indicates that mGDH in the intact membranes has much higher catalytic activity than the isolated mGDHs. The k_{cat} value is as large as the catalytic constant ($6.2 \times 10^3 \text{ s}^{-1}$ with PMS/DCIP at pH 7.0) of soluble GDH from *A. calcoaceticus*, calculated from the molecular mass (50 kDa) and the specific activity with PMS (1 mM) and DCIP (50 μ M) at pH 7.0 [24].

Determination of the rate constant of PQQ binding to apo-mGDH in *E. coli* cells

The *E. coli*-modified electrode allows the measurement of the time-dependent change in the catalytic activity of the cells immobilized on the electrode [18,19]. The rate of the catalytic reaction is measured as a current that is independent of time; thus the change in the current magnitude reflects the change in the catalytic activity. The current measured at the *E. coli*-modified electrode starts to appear after the addition of PQQ to the solution containing Mg²⁺, Q₀, and D-glucose and increases

Figure 3 Holo-mGDH formation in *E. coli* cells

Holo-mGDH formation in *E. coli* cells immobilized on an electrode was monitored by measurement of the current for the catalytic reaction of the immobilized cells and analysis of the kinetics of holo-mGDH formation. (A) The *E. coli*-modified electrode was incubated in buffer containing 5 mM Mg^{2+} for 15 min, then 0.3 mM Q_0 and 10 mM D-glucose were added to the buffer solution and the current was recorded at 0.5 V to measure the increase in current on the addition of PQQ at a final concentration of (a) 100, (b) 60 and (c) 30 nM. (B) The data used in these plots were taken from the current-time curves in Figure 3(A). The straight lines were drawn by least-square analysis of the data using eqn (2).

to approach the current at a steady state (Figure 3A). The slope of the current time curve becomes larger with increasing concentrations of PQQ (c_{PQQ}) added to the solution. The PQQ-dependent increase in the current is directly related to the rate of the holo-mGDH formation in the *E. coli* cells immobilized on the electrode [18,19], which can be written as reaction (1):

where $k_{\text{f,PQQ}}$ and $k_{\text{d,PQQ}}$ are the rate constants for the formation and dissociation of holo-mGDH respectively. The currents at the three PQQ concentrations attained the steady state of the same magnitude (Figure 3A), indicating that reaction (1) is almost completely shifted to the right hand side under these experimental conditions. Accordingly, the rate equation can be expressed as:

$$v = d\theta/dt = k_{\text{f,PQQ}}(1 - \theta)c_{\text{PQQ}} \quad (1)$$

where $\theta = [\text{holo-mGDH}]/\{[\text{holo-mGDH}] + [\text{apo-mGDH}]\}$ and $[\text{holo-mGDH}]$ and $[\text{apo-mGDH}]$ are the concentrations of holo-mGDH and apo-mGDH in the *E. coli* cells on the electrode. Since the current (i) is proportional to θ , as given by $i = \theta i_{\max}$, the dependence of i on time (t) is written as:

$$\ln[(i_{\max} - i)/i_{\max}] = -k_{\text{f,PQQ}}c_{\text{PQQ}}t \quad (2)$$

where $i_{\max}$ is the current at the steady state, which corresponds to

Table 2 Kinetic and thermodynamic parameters for the activation of apo-mGDH in *E. coli* cells

EDTA-treated *E. coli*-modified electrodes were used except for the results denoted by *, which were obtained using freshly prepared *E. coli*-modified electrodes not treated with EDTA. Results are means $\pm$ S.D. The thermodynamic parameters were estimated by adjusting them to give the best fit to the data by means of non-linear curve fitting as, for example, illustrated in Figure 4(B).

(a)	
c_{PQQ} (nM)	$10^{-4} \times k_{\text{f,PQQ}}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
30	3.3 ± 0.4 ($n = 3$)
	3.7 ± 0.5 ($n = 3$)*
60	3.1 ± 0.1 ($n = 3$)
	3.8 ± 0.1 ($n = 3$)*
100	4.8 ± 1.0 ($n = 3$)
	4.0 ± 0.1 ($n = 3$)*
(b)	
c_{Mg} (mM)	$k_{\text{f,Mg}}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
0.5	4.1 ± 1.0 ($n = 4$)
0.7	4.1 ± 0.8 ($n = 5$)
(c)	
K_{d}	
$K_{\text{d,PQQ(Mg)}}$ (nM)	1.2 ± 0.1
	0.82 ± 0.07 *
$K_{\text{d,PQQ(Ca)}}$ (nM)	12 ± 2
$K_{\text{d,Mg}}$ (mM)	0.14 ± 0.01
$K_{\text{d,Ca}}$ (μM)	42 ± 7.6

the full holo-mGDH formation. Applying eqn (2) to the current time curves in Figure 3(A), $k_{\text{f,PQQ}}$ can be determined from the slope of the $\ln[i_{\max}/(i_{\max} - i)]$ versus t plots as illustrated in Figure 3(B). Current-time curves were measured three times with different *E. coli*-modified electrodes at each PQQ concentration to obtain $k_{\text{f,PQQ}}$ values. The results are given in Table 2. The $k_{\text{f,PQQ}}$ values obtained with different c_{PQQ} agree with each other, indicating that eqn (1) is appropriate for the expression of the current-time curves for holo-mGDH formation.

Determination of the equilibrium constant for the reaction between apo-mGDH in *E. coli* cells and PQQ in solution

The value of $k_{\text{f,PQQ}}$ predicts that it will take a rather long time for reaction (1) to reach an equilibrium state at low c_{PQQ} . Consequently, the *E. coli*-modified electrode was incubated for 15 h in the solution containing both PQQ and Mg^{2+} before current measurements. Figure 4(A) shows examples of the currents measured with the *E. coli*-modified electrode, which attain steady states within 150 s after the addition of D-glucose. Pre-incubation for 17 h or more at $c_{\text{PQQ}} = 50$ pM (the lowest c_{PQQ} examined) yielded the same current magnitude as that at 50 pM in Figure 4(A) within experimental error. The steady-state currents (i_s) thus obtained were plotted against c_{PQQ} in Figure 4(B). Since i_s is proportional to $[\text{holo-mGDH}]_{\text{eq}}$, as given by $i_s = \theta_s i_{\max}$, where $\theta_s = [\text{holo-mGDH}]_{\text{eq}}/([\text{holo-mGDH}]_{\text{eq}} + [\text{apo-mGDH}]_{\text{eq}})$, the following equation is obtained:

$$i_s = i_{\max}c_{\text{PQQ}}/(K_{\text{d,PQQ(Mg)}} + c_{\text{PQQ}}) \quad (3)$$

Figure 4 Dependence of the steady-state current on the concentration of PQQ

(A) The *E. coli*-modified electrode was first incubated in a buffer containing 5 mM Mg^{2+} and (a) 1 μM , (b) 5 nM, (c) 0.5 nM and (d) 50 pM PQQ for 15 h. Then 0.3 mM Q_0 and 10 mM D-glucose were added successively to the same solution to measure the steady-state current. (B) Plot of i_s versus c_{PQQ} . A freshly prepared *E. coli*-modified electrode was used for each measurement. Results are means $\pm$ S.D. ($n = 3$). The solid curve was drawn using eqn (3) by means of non-linear curve fitting.

where $[\text{apo-mGDH}]_{\text{eq}}$ and $[\text{holo-mGDH}]_{\text{eq}}$ are the concentrations of apo-mGDH and holo-mGDH in *E. coli* cells respectively, in the equilibrium state, and $K_{\text{d,PQQ(Mg)}}$ is the dissociation constant defined by:

$$K_{\text{d,PQQ(Mg)}} = [\text{apo-mGDH}]_{\text{eq}} c_{\text{PQQ}} / [\text{holo-mGDH}]_{\text{eq}} \quad (4)$$

Non-linear-least-square analysis of the data in Figure 4(B) by eqn (3) yielded the $K_{\text{d,PQQ(Mg)}}$ value given in Table 2. The $K_{\text{d,PQQ(Mg)}}$ value (0.82 ± 0.07 nM at pH 6.5 for 15 h incubation) obtained in this study is the smallest among the dissociation constants reported so far: 40 nM at pH 6.5 (20 min incubation) for mGDH isolated from *A. calcoaceticus* [10], 270 nM at pH 7.0 (60 min incubation) for an anomalous form of mGDH isolated from an *E. coli* strain carrying the gene of *A. calcoaceticus* mGDH [11], and 90 nM at pH 7.0 (20 min incubation) for mGDH isolated from *E. coli* W3110 [13]. The small $K_{\text{d,PQQ(Mg)}}$ value confirms the high affinity for PQQ of the apo-mGDH in *E. coli* cells, as indicated in the titration curve (Figure 2). The solid curve in Figure 2 is obtained from the calculation by an ordinary equation for titration with the values of z (2.2×10^3) and $K_{\text{d,PQQ(Mg)}}$ (0.82 nM), which explains the experimental result very well. It has been reported that the dissociation constant for soluble GDH from *A. calcoaceticus* is estimated to be in the order of 10 pM, based on the titration data given in Figure 2 of [24]. This

is much smaller than the value of $K_{\text{d,PQQ(Mg)}}$, stating higher affinity for soluble GDH than mGDH.

Effects of Mg^{2+} and Ca^{2+} on the activation of apo-mGDH in *E. coli* cells

In order to remove endogenous Mg^{2+} and Ca^{2+} , the *E. coli*-modified electrode was treated with 5 mM EDTA for 20 min, and the same series of experiments as above were carried out using the EDTA-treated *E. coli*-modified electrode to determine the values of $k_{\text{r,PQQ}}$ and $K_{\text{d,PQQ(Mg)}}$. The results are given in Table 2, which shows that the effect of EDTA-treatment is small on the *in vivo* holo-mGDH formation reaction. In a similar manner, the rate constant ($k_{\text{r,Mg}}$) and the dissociation constant ($K_{\text{d,Mg}}$) for the Mg^{2+} binding to apo-mGDH were determined. The EDTA-treated electrode was incubated in the buffer containing 1 μM PQQ for 15 min, then 0.3 mM Q_0 and 10 mM D-glucose and a given concentration of MgSO_4 were added to the buffer. The $k_{\text{r,Mg}}$ value was obtained from the time-dependent increase in the current observed after the addition of MgSO_4 to the EDTA-treated electrode (curves similar to those in Figure 3A were obtained). The $K_{\text{d,Mg}}$ value was obtained from the dependence on the MgSO_4 concentration of the steady-state current at the EDTA-treated *E. coli* electrode that had been incubated in the buffer containing 1 μM PQQ and a given concentration of MgSO_4 for 90 min (curves like those in Fig. 4A were obtained). The $k_{\text{r,Mg}}$ and $K_{\text{d,Mg}}$ values are given in Table 2. Ca^{2+} was also effective at producing a steady-state current of almost the same magnitude as that in the presence of Mg^{2+} , although the time course for the activation of apo-mGDH with Ca^{2+} was not as simple as that for the activation with Mg^{2+} , taking a longer period of time to reach the equilibrium state. Consequently, only the dissociation constants for Ca^{2+} ($K_{\text{d,Ca}}$) and for PQQ in the presence of Ca^{2+} ($K_{\text{d,PQQ(Ca)}}$) were determined.

The $K_{\text{d,Mg}}$ value ($1.4 \pm 0.1 \times 10^{-4}$ M) is between the values reported for isolated mGDH (22 μM [13] and 1.4 mM [7]). It is noted that the $K_{\text{d,Mg}}$ value is in the same order of magnitude as those for Mg^{2+} complexes with organic acids, such as oxalate and citrate [25], and is larger than the conditional dissociation constant of the Mg^{2+} /EDTA complex at pH 6.5 (1.2×10^{-5} M [26]). The $K_{\text{d,Mg}}$ value seems to be of an appropriate order of magnitude for Mg^{2+} to form a moderately stable metal complex with the amino acid residues (Asp-354, Asn-355 and Thr-424) and PQQ (C-5 carbonyl oxygen, N-6 pyridine nitrogen and C-7 carboxy group), as proposed for the model of the active site [6,27] and removal of Mg^{2+} from the active site by treatment with EDTA. Calcium ions have been reported to be effective in activating isolated mGDH [2,10]. Catalytic activity of the enzyme relative to that of the enzyme activated by Mg^{2+} is 87% for mGDH isolated from *A. calcoaceticus* ACIII [10] and 40% for the membrane fractions of *E. coli* K-12 cells [2]. Our results for mGDH in *E. coli* K-12 cells showed that the catalytic activity of mGDH with Ca^{2+} was very similar to that of mGDH with Mg^{2+} . GDH in the intact membranes of *E. coli* cells might have a flexible conformational state to allow for the incorporation of Ca^{2+} (of a larger size than Mg^{2+}) into a proper position in the protein to fix PQQ in the active site, although the incorporation is not as easy as the incorporation of Mg^{2+} , as mentioned above. The $K_{\text{d,Ca}}$ value ($42 \pm 7.6 \times 10^{-4}$ M) is approx. three times smaller than the $K_{\text{d,Mg}}$ value ($1.4 \pm 0.1 \times 10^{-4}$ M; Table 2). This is in line with the fact that the dissociation constants for Ca^{2+} with organic acids are slightly smaller than the corresponding constants for Mg^{2+} [25]. The $K_{\text{d,Ca}}$ value is larger than the dissociation constant for the Ca^{2+} /EDTA complex (0.12 μM at pH 6.5 [26]), assuring complete removal of Ca^{2+} from the enzyme by the EDTA

Figure 5 Effect of EDTA on the deactivation of *E. coli* cells and reactivation of the deactivated cells

(A) The current was measured in the solution containing 0.3 mM Q_0 and 10 mM D-glucose with the *E. coli*-modified electrode that had been fully activated by incubation for 15 min in the solution containing 5 mM $MgSO_4$ and 1 μ M PQQ. EDTA was added to the solution to a concentration of 5 mM. (B) The electrode treated with EDTA was washed with distilled water and immersed in a buffer at pH 6.5 containing 0.3 mM Q_0 and 10 mM D-glucose to measure the current; $MgSO_4$ and PQQ were added, in this order, to obtain concentrations of 5 mM and 1 μ M respectively. (C) The experiment was the same as for (B) except that PQQ was added first.

treatment. Interestingly, the $K_{d,PQQ(Ca)}$ value (12 ± 2 nM) is approx. ten times larger than the $K_{d,PQQ(Mg)}$ value (1.2 ± 0.1 nM; Table 2). This suggests that Mg^{2+} allows PQQ to take a more appropriate position in the active site. A ten times difference in the dissociation constant corresponds to an energy difference of 5.7 kJ/mol at 25 °C, which is the amount easily attained by a small change in the weak interactions of PQQ with the amino acid residues and the metal ion in the protein. Considering that the catalytic activity of mGDH with Ca^{2+} is very similar to that of mGDH with Mg^{2+} , as mentioned above, we may say that the catalytic activity of mGDH is insensitive to the difference in the dissociation constants, although the difference may reflect a small change in the structure of the active site.

Deactivation and reactivation of mGDH in *E. coli* cells

EDTA was effective at removing Mg^{2+} from the active site of mGDH in *E. coli* cells, as has been reported for *E. coli* membrane fractions previously [2,8]. Figure 5(A) demonstrates that the current obtained with the *E. coli*-modified electrode carrying activated mGDH decreases rapidly to almost zero magnitude with the addition of EDTA. This proves that the catalytic activity of the *E. coli* cells is completely lost by the EDTA treatment. When the EDTA-treated electrode is immersed in a buffer at pH 6.5 and $MgSO_4$ is added to the buffer, a rapid increase in the current is observed attaining almost 70% of the maximum current at this stage; the maximum current is obtained by further addition of PQQ (Figure 5B). The result suggests that a considerable portion of PQQ remains in the *E. coli* cells, at least within the experimental time period after EDTA treatment. When PQQ and $MgSO_4$ were added to the solution in this order (Figure 5C), the current at the EDTA-treated electrode started to increase only after the addition of $MgSO_4$, which confirms that the EDTA treatment removes Mg^{2+} from the *E. coli* cells to deactivate mGDH completely.

Deactivation of mGDH once activated in *E. coli* cells also occurred without EDTA treatment, as observed from the gradual decrease in the current for the mGDH-catalysed reaction. When the solution contained both PQQ and Mg^{2+} the current remained practically unchanged after 17 h of incubation (Table 3). On the other hand, the current was decreased to approx. 80% in a solution lacking either PQQ or Mg^{2+} , and to approx. 50%

Table 3 Deactivation of holo-mGDH in *E. coli* cells in solutions of several different compositions

Relative activity is expressed as the ratio of the current measured at the *E. coli*-modified electrode with activated mGDH before and after the incubation for 17 h in the stirred solutions. Results are means $\pm$ S.D.

Solution (pH 6.5)	Relative activity (%)
1 μ M PQQ + 5 mM Mg^{2+}	92 ± 4.7 ($n = 4$)
1 μ M PQQ	80 ± 14.5 ($n = 4$)
5 mM Mg^{2+}	81 ± 6.2 ($n = 4$)
None	53 ± 10.3 ($n = 4$)
3 mM Glycine	21

in a buffer solution containing neither PQQ nor Mg^{2+} . The deactivation rate, $k_{b,PQQ}$, in reaction (1) can be estimated to be $3.8 \times 10^{-5} s^{-1}$ from the $k_{r,PQQ}$ and $K_{d,PQQ(Mg)}$ values in Table 2 using the relation $k_{b,PQQ} = k_{r,PQQ} K_{d,PQQ(Mg)}$. It predicts that 50% of the holo-mGDH in *E. coli* cells is converted into apo-mGDH after 5 h incubation in a PQQ-free solution and that only 10% of mGDH remains active after 17 h. The result that the observed decreases are smaller than the prediction is conceivable in view of the effect of the backward reaction (activation reaction). It will soon become appreciable by the liberation of free PQQ with the progress of the dissociation, owing to the very small $K_{d,PQQ(Mg)}$ value and the small volume of the test solution (2 ml); PQQ liberated after 5 h is estimated to be 0.44 nM. The decrease in the current in the solution containing either PQQ or Mg^{2+} is smaller than that in the buffer solution. This is consistent with the active-site model, in which Mg^{2+} and PQQ are kept attached together at the active site [6,27]; the presence of either Mg^{2+} or PQQ in the solution will retard both Mg^{2+} and PQQ from the dissociation because of their mutual interaction in the active site. Further decrease in the current was observed when the solution contained glycine, a compound known to react with PQQ to form oxazole [28–31]. The oxazole formation will scavenge PQQ liberated from holo-mGDH and thus prevent the backward reaction.

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