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Lysine-linked viologen for substrate of hydrogenase on hydrogen evolution

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Abstract

Lysine-linked viologen was synthesized and was applied for hydrogen evolution with hydrogenase. The lysine-linked viologen has high affinity for hydrogenase than methyl viologen, resulting the high hydrogen evolution rate.

Keywords: Hydrogen evolution; Viologen; Hydrogenase; Lysine

1. Introduction

Hydrogenase from *Desulfovibrio vulgaris* (Miyazaki) is an enzyme that catalyzes the reaction, $2H^+ + 2e^- \rightleftarrows H_2$ [1]. Under reductive condition, the forward reaction, reduction of proton, is catalyzed preferentially. In this case, reduced electron carrier is shown to function as the substrate for hydrogenase. Reduced methyl viologen (radical cation, $MV^{+\bullet}$) has been used exclusively as a substrate in hydrogen evolution systems [2–6], because MV^{2+} has an enough redox potential (−440 mV) for electron transfer to iron–sulfur cluster of hydrogenase [1,7,8].

On the application of enzymatic within electron transfer reactions, electron-mediation between artificial non-enzymatic system and enzymatic system is one of the important factors to enhance the over all reactivity. To improve the hydrogen evolution system using hydrogenase, electron mediator, a substrate of hydrogenase, needs to donate an electron to hydrogenase effectively. One approach for effective electron-donation is to design a high affinity substrate which possesses some structural motif recognized by electron-accepting site of hydrogenase.

Hydrogenase complexes with cytochrome c_3, forming electrostatic enzyme–substrate, which is a basic protein containing 20 lysine residues in 108 amino acids [9]. Some of the lysine residues in cytochrome c_3 probably play important roles on the electrostatic interaction with hydrogenase and also on the electron transfer between them. Lysine residue is probably recognized by hydrogenase.

In this study, lysine-linked viologen ($LysV^{2+}$) was designed and synthesized in order to enhance the affinity for hydrogenase and the hydrogen evolution mechanism with $LysV^{+\bullet}$ is discussed.

2. Materials and methods

All the reagents were of analytical or of the highest grade available. The synthesis route of lysine-linked viologen is shown in Scheme 1. ^{1}H NMR spectra were measured by a Varian OXFORD NMR300. The chem-

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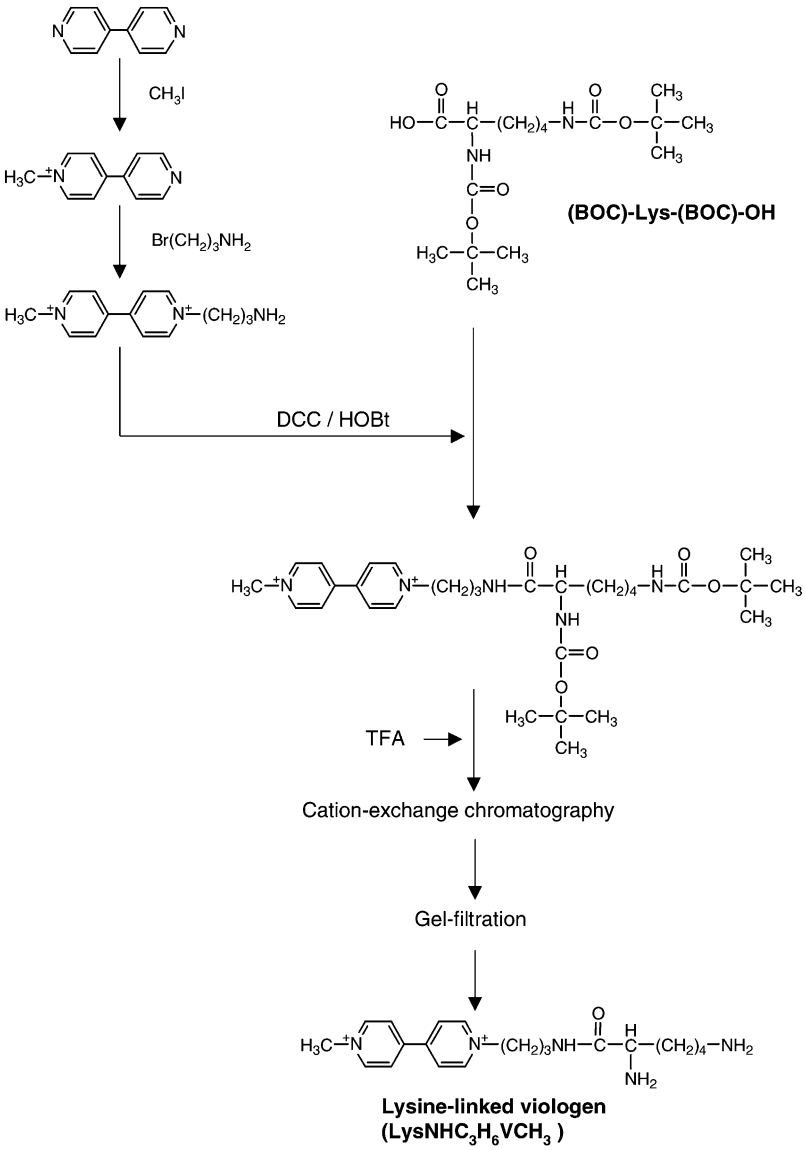

Scheme 1.

ical shifts were referenced to the solvent peak calibrated against tetramethylsilane.

2.1. Synthesis of lysine-linked viologen

2.1.1. 1-Methyl-4,4′-bipyridinium (VCH_3)

4,4′-Bipyridie (0.16 mol) and methyl iodide (0.15 mol) were dissolved in 300 ml of acetonitrile and stirred at room temperature for 24 h. Yellow precipitate was filtered and washed with acetonitrile and dried under vacuum overnight. ^{1}H NMR in DMSO-d_6: δ (ppm) 9.1–9.25 (d, 2H), 8.85–8.95 (multiplet, 2H), 8.62–8.7 (d, 2H), 8.05–8.1 (multiplet, 2H), 4.4–4.5 (s, 3H).

2.1.2. 1-(3-Aminopropyl)-1′-methyl-4,4′-bipyridinium ($NH_2C_3H_6VCH_3$)

The VCH_3 (10 mmol) was refluxed with excess 3-bromopropylamine hydrobromide in 300 ml acetoni-

trile for 24 h. Yellow precipitate was filtered, washed with acetonitrile and dried under vacuum overnight. The product was recrystallized from ethanol and water, and then dissolved in water and NH_4PF_6 solution was added to replace the counter-anion with PF_6^-. The PF_6^- salt was filtered, washed with water, and dried under vacuum overnight. ^{1}H NMR in DMSO-d_6: δ (ppm) 9.3–9.5 (multiplet, 4H), 8.7–8.9 (multiplet, 4H), 4.7–4.9 (t, 2H), 4.4–4.5 (s, 3H), 2.8–3.0 (t, 2H) 2.2–2.4 (quintet, 2H).

2.1.3. (Boc)-Lys-(Boc)-$NHC_3H_6VCH_3$

Coupling of (Boc)-Lys-(Boc)-OH to $NH_2C_3H_6$-VCH_3 used DCC (*N*,*N*′-dicyclohexylcarbodiimide)/HOBt (1-hydroxybenzotriazole) in dry DMF. (Boc)-Lys-(Boc)-OH (2 mmol) and $NH_2C_3H_6VCH_3$ (1.5 mmol) were stirred in the presence of DCC (1.2 equivalent to COOH group) and HOBt (equimolar to COOH group) in dry DMF at 30°C for 6 h. The solvent was removed under vacuum and the residue was dissolved in acetonitrile. After removing precipitate by filtration, the solvent was evaporated and the yellow oily residue was dried under vacuum overnight.

2.1.4. Lys-$NHC_3H_6VCH_3$ ($LysV^{2+}$)

To cleave the protected lysine resin (Boc), the yellow oily residue was stirred with trifluoroacetic acid (TFA) at 0°C for 1 h. Removing TFA at 10°C, white precipitate (containing Lys-$NHC_3H_6VCH_3{\cdot}2PF_6^-$) was washed with a large quantity of diethyl ether and dried under vacuum. The precipitate was dissolved in a minimum amount of acetonitrile and tetraethylammonium chloride salt was added to obtain Cl^- salt.

Lys-$NHC_3H_6VCH_3{\cdot}2Cl^-$ was applied to SP Sepharose Fast-Flow column (Pharmacia, 1 cm × 10 cm) with 30 mmol dm^{-3} CH_3COOH-CH_3COONa buffer (pH 4.9) and eluted with 90 ml linear gradient from 0.75 to 0.9 mol dm^{-3} NaCl. Desalting of the obtained Lys-$NHC_3H_6VCH_3$ solution was carried out by gel-filtration chromatography on Sephadex LH 20 (Pharmacia) with water. The solvent was removed under vacuum. ^{1}H NMR in D_2O: δ (ppm) 9.0–9.1 (d, 2H), 8.85–8.95 (d, 2H), 8.3–8.4 (multiplet, 4H), 4.6–4.7 (t, 2H), 4.3–4.4 (s, 3H), 3.65–3.75 (t, 1H), 3.1–3.4 (multiplet, 2H), 2.8–2.9 (d, 2H), 2.1–2.2 (quintet, 2H), 1.65–1.8 (quintet, 2H), 1.5–1.64 (quintet, 2H), 1.25–1.36 (quintet, 2H).

2.2. Purification of hydrogenase

Hydrogenase was purified from *Desulfovibrio vulgaris* (Miyazaki) according to [10]. Protein concentration was determined using the following molar absorption coefficient: $\varepsilon = 155{,}000\ (\text{mol dm}^{-3})^{-1}\ \text{cm}^{-1}$ at 280 nm.

2.3. Electrochemical measurement

Electron-reduction potentials were measured by cyclic voltammetry (Hokuto Denko Potentiostat/Galvanostat HA-501, Function Generator HB-111, Riken Densho X-Y recorder). The working electrode was a glassy carbon, and the counter electrode was a platinum wire. The electrode potentials were referred to the Ag|AgCl|sat. KCl electrode. All measurements were carried out in the solution containing 0.1 mol dm^{-3} KCl and 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4) under argon.

2.4. Measurement of hydrogen evolution rate

Hydrogen evolution was carried out in the solution (25 mmol dm^{-3} Tris–HCl buffer (pH 7.4)) containing hydrogenase and dithionite-reduced viologen in a 5 ml test tube sealed with Septa at 30°C. The reaction mixture contained dithionite-reduced viologen and. The reaction was started by injection 5 μl hydrogenase solution in the test tube. The amount of evolved hydrogen was analyzed by gas chromatograph (SHIMADZU GC-14B).

3. Results and discussion

3.1. Hydrogen evolution with lysine-linked viologen and hydrogenase

Time dependence of hydrogen evolution with (dithionite-reduced) $LysV^{+\bullet}$ by hydrogenase is shown in Fig. 1, showing $LysV^{+\bullet}$ can be a substrate of hydrogense. Hydrogen evolution with $MV^{+\bullet}$ is also shown in the same figure and the hydrogen evolution rate with $LysV^{+\bullet}$ was higher than $MV^{+\bullet}$.

As the redox potentials of $LysV^{2+}$ and MV^{2+} were −390 mV and −420 mV (versus SHE), respectively, $MV^{+\bullet}$ donates electron to hydrogenase easily

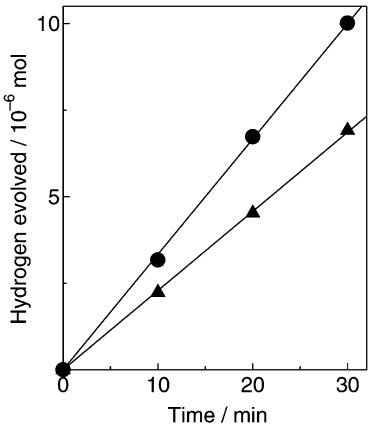

Fig. 1. Time dependence of hydrogen evolution from dithionite-reduced viologen: (●) $LysV^{+\bullet}$; (▲) $MV^{+\bullet}$. The solutions contain 21 nmol dm^{-3} hydrogenase, 38 μmol dm^{-3} viologen, and 30 mmol dm^{-3} dithionite in 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4).

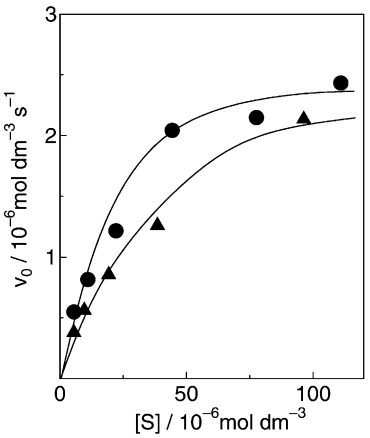

Fig. 2. Dependence of hydrogen evolution rate on concentration of dithionite-reduced viologen: (●) $LysV^{+\bullet}$; (▲) $MV^{+\bullet}$. The solutions contain 21 nmol dm^{-3} hydrogenase in 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4).

compared with $LysV^{+\bullet}$. However, the hydrogen evolution rate with $LysV^{+\bullet}$ was higher than $MV^{+\bullet}$. Thus, lysine group of $LysV^{+\bullet}$ probably forms the complex easily between hydrogenase and $LysV^{+\bullet}$, leading to the acceleration of the reaction rate. To compare the difference of the reactivity with $LysV^{+\bullet}$ and $MV^{+\bullet}$, kinetic studies of hydrogen evolution was carried out.

3.2. Kinetic parameters of $LysV^{+\bullet}$ and $MV^{+\bullet}$

Relation between hydrogen evolution rate and radical cation viologen concentration is shown in Fig. 2. In both case of $LysV^{+\bullet}$ and $MV^{+\bullet}$ hydrogen evolution rates increase with the concentration and reach constant values. The relation obeys Michaeris–Menten equation, and the obtained kinetic parameters are summarized in Table 1. K_m, V_{max}, and k_{cat}/K_m represent affinity for hydrogenase, maximum reaction rate, and total efficiency of the reaction, respectively.

Total efficiency of hydrogen evolution (k_{cat}/K_m) with $LysV^{+\bullet}$ was 1.5 times larger than $MV^{+\bullet}$. K_m value of reduced $LysV^{+\bullet}$ (22.6 μmol dm^{-3}) was smaller than that of $MV^{+\bullet}$ (27.2 μmol dm^{-3}), indicating that $LysV^{+\bullet}$ has higher affinity for hydrogenase than $MV^{+\bullet}$. The high affinity of $LysV^{+\bullet}$ shows the presence of high concentration of enzyme–substrate complex. In addition, maximum reaction rate (V_{max}) with $LysV^{+\bullet}$ was higher than $MV^{+\bullet}$. As V_{max} reflects the intermolecular electron transfer rate in the enzyme–substrate complex, $LysV^{+\bullet}$ may attach the complex site of hydrogenase where electron transfer occurs easily.

From these results, $LysV^{+\bullet}$ on the hydrogen-evolution reaction are play two important roles. One is the effective complex formation of hydrogenase–$LysV^{+\bullet}$. The other is the acceleration of electron transfer from $LysV^{+\bullet}$ to hydrogenase.

3.3. The role of lysine moiety on the reaction with hydrogenase

In order to investigate the role of lysine group of $LysV^{+\bullet}$, activation energy (E_a) was measured.

Table 1
Kinetic parameters for hydrogen evolution with dithionite-reduced viologen and hydrogenase

Substrate	K_m (μmol dm^{-3})	V_{max} (μmol dm^{-3} s^{-1})	k_{cat}/K_m (10^6 dm^3 mol^{-1} s^{-1})
$LysV^{+\bullet}$	22.6	2.70	5.69
$MV^{+\bullet}$	27.2	2.22	3.89

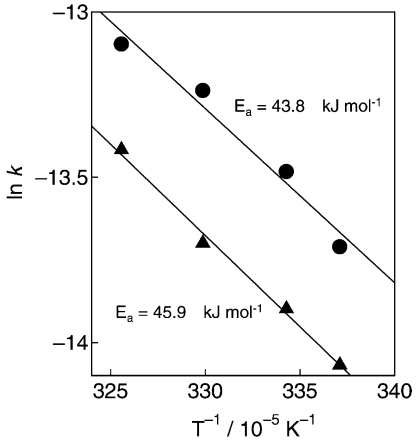

Fig. 3. Arrhenius plot of hydrogen evolution rate of hydrogenase with dithionite reduced viologen: (●) $LysV^{+\bullet}$: (▲) $MV^{+\bullet}$).

Fig. 3 shows Arrhenius plot of hydrogen evolution rate depending on reaction temperature. Each slope of $LysV^{+\bullet}$ and $MV^{+\bullet}$ in Fig. 3 corresponds to $-E_a/(RT)$. Activation energy with $LysV^{+\bullet}$ and $MV^{+\bullet}$ were estimated to be 43.8 and 45.9 kJ mol^{-1}, respectively. The activation energy of both reaction systems are almost the same, indicating that an intermediate on hydrogen-evolved reaction with $LysV^{+\bullet}$ is similar to that with $MV^{+\bullet}$.

As hydrogenase–$LysV^{+\bullet}$ and hydrogenase–$MV^{+\bullet}$ complexes are similar structures on hydrogen evolution, lysine ligand of $LysV^{+\bullet}$ strongly effects the enzyme–substrate complex formation. By the effect of lysine moiety, the equilibrium of the reaction, $E + S \rightleftarrows ES$ is sifted to complex formation, giving rise to high concentration of the complex compared with $MV^{+\bullet}$.

In this reaction, the concentration of hydrogenase–viologen radical cation complex is responsible for total efficiency of hydrogen evolution, so that k_{cat}/K_m with $LysV^{+\bullet}$ is 1.5 times larger than $MV^{+\bullet}$. $LysV^{+\bullet}$ has a high affinity for hydrogenase on *ES* complex, leading to effective hydrogen evolution.

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References

[1] S.P.J. Albracht, Biochim. Biophys. Acta 1188 (1994) 167.
[2] S.G. Mayhew, Eur. J. Biochem. 85 (1978) 535.
[3] A. Harriman, G. Porter, M.-C. Richoux, J. Chem. Soc., Faraday Trans. 77 (2) (1981) 833.
[4] A. Harriman, G. Porter, M.-C. Richoux, J. Chem. Soc., Faraday Trans. 77 (2) (1981) 1939.
[5] H. Tatsumi, K. Takagi, M. Fujita, K. Kano, T. Ikeda, Anal. Chem. 71 (1999) 1753.
[6] T. Hiraishi, T. Kamachi, I. Okura, J. Mol. Catal. A: Chem. 138 (1999) 107.
[7] H.R. Pershad, J.L.C. Duff, H.A. Heering, E.C. Duin, S.P.J. Albracht, F.A. Armstrong, Biochemistry 38 (1999) 8992.
[8] A. Volbeda, M.-H. Charon, C. Piras, E.C. Hatchikian, M. Frey, J.C. Fontecilla-Camps, Nature 373 (1995) 580.
[9] W. Shinkai, T. Hase, T. Yagi, H. Matsubara, J. Biochem. 87 (1980) 1747.
[10] T. Yagi, J. Biochem. 68 (1970) 649.