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Letter

The role of specific lysine in cytochrome c_3 on the electron transfer with hydrogenase

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Abstract

Modification of specific lysine residue of cytochrome c_3 with 2,4,6-trinitorobenzenesulfonic acid sodium salt dehydrate (TNBS) was carried out. By comparing the hydrogen evolution rate with modified cytochrome c_3 and native cytochrome c_3 catalyzed by hydrogenase, the role of lysine residue of cytochrome c_3 was discussed. The modified lysine residue was responsible for the intermolecular electron transfer from cytochrome c_3 to hydrogenase.

Keywords: Cytochrome c_3; Lysine; Hydrogenase; *Desulfovibrio vulgaris*

Cytochrome c_3 [1,2] from *Desulfovibrio vulgaris* (Miyazaki) is a kind of redox proteins containing tetraheme [3,4] in the molecule. The multiple redox behaviors caused by tetraheme allow cytochrome c_3 act as electron donor and acceptor for hydrogenase, in vivo. In the reaction of hydrogenase and cytochrome c_3, the enzyme–substrate complex formation is caused by electrostatic interaction, because cytochrome c_3 and hydrogenase are basic and acidic protein, respectively [5,6]. As cytochrome c_3 involves 20 lysine residues in 108 amino acids [7], some of these lysine residues probably participate in the electrostatic interaction and in the electron transfer with hydrogenase.

As cytochrome c_3 has four heme in one molecule, a slight change of inner molecular structure may cause serious change of enzymatic character such as redox potentials, pathway of intramolecular electron transfer, resulting the activity as an electron mediator. To investigate the role of the lysine residue of enzyme–substrate complex, only one lysine residue existing on the cytochrome c_3 surface is modified without structural change. A selective chemical modification is an useful method, because structure of the protein does not change except modified functional group. Even if the amino acid on the molecular surface is selectively modified, the inner structure around heme such as the length between Fe and the amino acid residue is maintained.

In this study, one lysine residue in the cytochrome c_3 was modified with 2,4,6-trinitorobenzenesulfonic acid sodium salt dehydrate (TNBS) and cytochrome c_3-hydrogenase interaction within electron transfer events was discussed.

Cytochrome c_3 and hydrogenase from *Desulfovibrio vulgaris* (Miyazaki) were purified according to the literature procedure [8,9]. Cytochrome c_3 modified

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by TNBS (TNBS-cytochrome c_3) was prepared by mixing native cytochrome c_3 (24.8 nmol) and TNBS (37 nmol) in 10 mmol dm^{-3} EPPS buffer (pH 8.5) at room temperature. Modification of lysine was monitored by specific absorption of trinitrophenyl lysine (367 nm). The reaction mixture was applied to SP Sepharose Fast-Flow column (Pharmacia) with 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4) and eluted with NaCl concentration gradient. In this procedure, native cytochrome c_3 and TNBS-cytochrome c_3 was separated. Desalting of TNBS-cytochrome c_3 was carried out by ultrafiltration. Purification of TNBS-cytochrome c_3 was confirmed by native polyacrylamide gel electrophoresis. The results of ion exchange chromatography and polyacrylamide gel electrophoresis show that the cytochrome c_3 surface positively charge decreased by TNBS modification. Lysine residue of cytochrome c_3 surface is modified and inner structure probably is maintained. One lysine residue modification was identified by MALDI TOF-MS. Native cytochrome c_3 shows mass/charge = 14,160 ($M + H^+$) and TNBS treated cytochrome c_3 shows mass/charge = 14,378 ($M + H^+$).

Fig. 1 shows time dependence of hydrogen evolution with hydrogenase and dithionite reduced TNBS-cytochrome c_3. The reaction was started by injection of hydrogenase solution into 2 μmol dm^{-3} dithionite-reduced cytochrome c_3 solution (pH 7.4). The amount of evolved hydrogen was analyzed by gas chromatography. In the case of TNBS-cytochrome c_3, hydrogen evolution rate decreased remarkably compared with native cytochrome c_3. From the result, lysine of cytochrome c_3 probably effects on the interaction between hydrogenase and cytochrome c_3 or on the electron transfer from heme in cytochrome c_3 to hydrogenase. To clarify the difference of reactivity between native and TNBS-cytochrome c_3, the kinetic studies of hydrogen evolution were carried out.

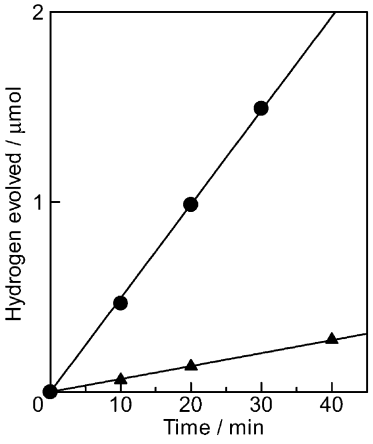

Fig. 1. Time dependence of hydrogen evolution with dithionite-reduced cytochrome c_3: (●) native cytochrome c_3; (▲) TNBS-cytochrome c_3. The solutions contain 32 nmol dm^{-3} hydrogenase, 2 μmol dm^{-3} cytochrome c_3, and 2.5 mmol dm^{-3} dithionite in 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4).

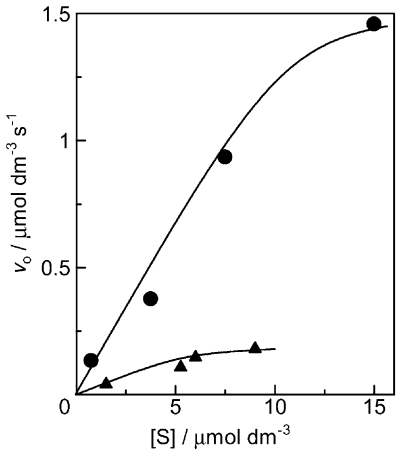

Fig. 2. Dependence of hydrogen evolution rate on concentration of dithionite-reduced cytochrome c_3: (●) native cytochrome c_3; (▲) TNBS-cytochrome c_3. The solutions contain 32 nmol dm^{-3} hydrogenase and dithionite-reduced cytochrome c_3 in 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4).

Relation between hydrogen evolution rate and reduced cytochrome c_3 concentration is shown in Fig. 2. In both cases of TNBS-cytochrome c_3 and native cytochrome c_3, 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. The total efficiency (k_{cat}/K_m) with TNBS-cytochrome c_3 was very low and the affinity for hydrogenase is very low from K_m value of TNBS-cytochrome c_3. Maximum reaction rate (V_{max}) of TNBS-cytochrome c_3 is smaller than native cytochrome c_3, indicating that electron transfer to hydrogenase is suppressed by

Table 1
Kinetic parameter for hydrogen evolution with dithionite-reduced cytochrome c_3 and hydrogenase

Substrate	K (μmol dm^{-3})	V_{max} (μmol dm^{-3} s^{-1})	k_{cat}/K_m (10^6 mol dm^3 s^{-1})	E_a (kJ mol^{-1})
Native cytochrome c_3	8.17	1.58	6.05	32.1
TNBS-cytochrome c_3	18.4	0.536	0.913	50.5

TNBS. These results suggest that the low efficiency is caused by the difficulty in intermolecular electron transfer of hydrogenase–TNBS-cytochrome c_3 complex. Hydrogenase–TNBS-cytochrome c_3 complex and hydrogenase–native cytochrome c_3 complex probably are different in structure and in ability of intermolecular electron transfer.

In order to investigate the precise role of modified specific lysine, activation energy was measured. Activation energy (E_a/kJ mol^{-1}) of two reactions with native and TNBS-cytochrome c_3 are listed in Table 1. Activation energies were calculated from Arrhenius-plot of temperature dependence hydrogen evolution rate. In the case of TNBS-cytochrome c_3, activation energy was remarkably large. This result shows activated species of native cytochrome c_3 is strongly different from TNBS-cytochrome c_3. Hydrogenase–TNBS-cytochrome c_3 complex has low ability of electron transfer compared with hydrogenase-native cytochrome c_3, requiring high thermal energy for electron transfer within hydrogen evolution. The result suggests that the electron accepting site of hydrogenase far away from electron donating site of cytochrome c_3 by TNBS modification. The modified lysine residue has important roles on the intermolecular electron transfer of hydrogenase–cytochrome c_3 complex.

In hydrogen evolution, electron probably transfers from one of the heme in cytochrome c_3 to 4Fe–4S cluster of hydrogenase. The specific lysine may be located near the electron-donating heme on the reaction. The modified site of cytochrome c_3 has not been determined, but many interesting results were obtained from this work. So, determination of the position of modified lysine residue will be carried out and intermolecular electron transfer between cytochrome c_3 and hydrogenase will be clarified.

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