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Topographical characterization of the ubiquinone reduction site of glucose dehydrogenase in *Escherichia coli* using depth-dependent fluorescent inhibitors

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Abstract

Membrane-bound glucose dehydrogenase in *Escherichia coli* possesses a binding site for ubiquinone as well as glucose, metal ion and pyrroloquinoline quinone. To probe the depth of the ubiquinone binding site in the membrane environment, we synthesized two types of fluorenyl fatty acids which bear an inhibitor mimic moiety (i.e., specific inhibitor capsaicin) close to the fluorene located at different positions in the alkyl tail chain; one close to the polar carbonyl head group (α-(3,4-dimethoxyphenyl)acetyloxy-7-nonyl-2-fluoreneacetic acid, α-DFA), and the other in the middle of the chain (θ-(3,4-dimethoxyphenyl)acetyloxy-7-ethyl-2-fluorenenonanoic acid, θ-DFA). Mixed lipid vesicles consisting of phosphatidylcholine (PC) and α-DFA or θ-DFA were prepared by sonication method, and fluorescent quenching against a hydrophilic quencher, iodide anion, was examined. The vesicles containing α-DFA were more susceptible to quenching than those containing θ-DFA, indicating that the fluorene and consequently capsaicin mimic moiety are located at different depths in the lipid bilayer depending upon the position of attachment to the alkyl tail chain. The purified glucose dehydrogenase was reconstituted into PC vesicles which consisted of PC and α-DFA or θ-DFA with various molar ratios. For both types of reconstituted vesicles, the extent of inhibition of short-chain ubiquinone reduction activity increased with increases in the molar ratio of fluorenyl fatty acid to PC. The ubiquinone reduction activity was more significantly inhibited in the reconstituted vesicles containing α-DFA compared to those containing θ-DFA. Our findings strongly suggested that the ubiquinone reduction site in glucose dehydrogenase is located close to the membrane surface rather than in the hydrophobic membrane interior.

Keywords: Glucose dehydrogenase; Ubiquinone; Respiratory inhibitor; (*Escherichia coli*)

Abbreviations: DB, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; α-DFA, α-(3,4-dimethoxyphenyl)acetyloxy-7-nonyl-2-fluoreneacetic acid; θ-DFA, θ-(3,4-dimethoxyphenyl)acetyloxy-7-ethyl-2-fluorenenonanoic acid; FEDA, 1-(2-fluorenyl)ethyl 3,4-dimethoxyphenylacetate; GDH, glucose dehydrogenase; PB, 2,3-dimethoxy-5-methyl-6-*n*-pentyl-1,4-benzoquinone; PC, phosphatidylcholine; Q_n, ubiquinone-n

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1. Introduction

Glucose dehydrogenase (GDH), which resides in the inner membrane of *Escherichia coli*, functions in direct oxidation of D-glucose to D-gluconate by transferring electrons to quinol oxidase through ubiquinone (Q) in the respiratory chain [1,2]. Using reconstituted proteoliposomes with purified GDH, Yamada et al. [3] demonstrated that GDH has a Q reduction site close to the periplasmic side of the membrane and is incapable of generating a proton electrochemical gradient with electron transfer to Q. On the basis of the structure/electron-accepting efficiency relation of a systematic set of short-chain Q analogues, we demonstrated that the Q binding site of GDH very strictly recognizes dimethoxy groups in the 2- and 3-positions of the quinone ring, but loosely recognizes the 5-methyl group and 6-alkyl side chain [4]. We also identified some synthetic capsaicin analogues as potent inhibitors acting at the Q reduction site [4]. Although the topological structure of the *E. coli* GDH has been depicted [3,5], the structural features of the Q binding domain remain to be determined. To further characterize the Q binding site in GDH, information regarding the depth of the Q binding site in the membrane environment is important.

The molecular details of structure–function relationship in biomembranes must involve description of the topographical distribution of membrane components. One of the possible approaches, which is particularly suited to the study of natural as well as model membrane systems, is to employ spectroscopic probes which are located at different depths in the lipid bilayer and whose spectral characteristics change in response to the proximity of a given membrane component [6–9]. Lala and colleagues [10,11] carried out design synthesis of the fluorenyl fatty acids that bear a fluorenyl chromophore at different positions in the alkyl chain so that the lipid bilayer environment can be monitored at different depths. They reported that a hydrophobic tail attached to the fluorenyl group enables improved hydrophobic interactions and proper alignment in membranes. Since the depth of the fluorenyl group in the membrane can be regulated by modifying the hydrophobic tail structures, it is likely that this type of synthetic fatty acids can be used to locate various probe molecules at desired depths in lipid bilayer membranes. We hypothesized that if a specific biologically active chemical structure is attached to a hydrophobic tail as such a probe, topographical information regarding the interaction site of the membrane component could be obtained.

In this study, we synthesized fluorenyl fatty acids bearing a structure mimicking capsaicin, a specific inhibitor of GDH, in different positions in the alkyl tail chain (Fig. 1); one close to the polar carboxyl head group (α-DFA), and the other in the middle of the tail (θ-DFA). To probe the depth of the Q binding site of GDH in the membrane environment, we reconstituted purified GDH into phosphatidylcholine (PC) vesicles which contain α-DFA or θ-DFA with various molar ratios relative to PC. We found that Q reduction activity of GDH was more significantly inhibited in the reconstituted vesicles containing α-DFA compared to those containing θ-DFA. Our study strongly suggested that the Q reduction site in GDH is located close to the membrane surface rather than in hydrophobic membrane interior.

2. Materials and methods

2.1. Materials

2,3-Dimethoxy-5-methyl-6-*n*-pentyl-1,4-benzoquinone (PB), 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone (DB), ubiquinone-1 (Q_1) and ubiquinone-2 (Q_2), synthetic capsaicin (C26) and piericidin A were the same as described previously [4,12]. Phosphatidylcholine (egg yolk) of highest grade (>99%) was purchased from Wako Pure Chemical Industries (Osaka). Other chemicals were commercial products of analytical grade.

2.2. Synthesis

2.2.1. 1-(2-Fluorenyl)ethyl 3,4-dimethoxyphenylacetate (FEDA)

$NaBH_4$ (1.2 g, 29 mmol) dissolved in 0.2 M NaOH was added dropwise to a solution of commercially available 2-acetylfluorene (5.1 g, 24 mmol) in methanol. The reaction mixture was stirred for 1 h at room temperature. After removal of methanol in vacuo, the mixture was neutralized with dilute HCl and

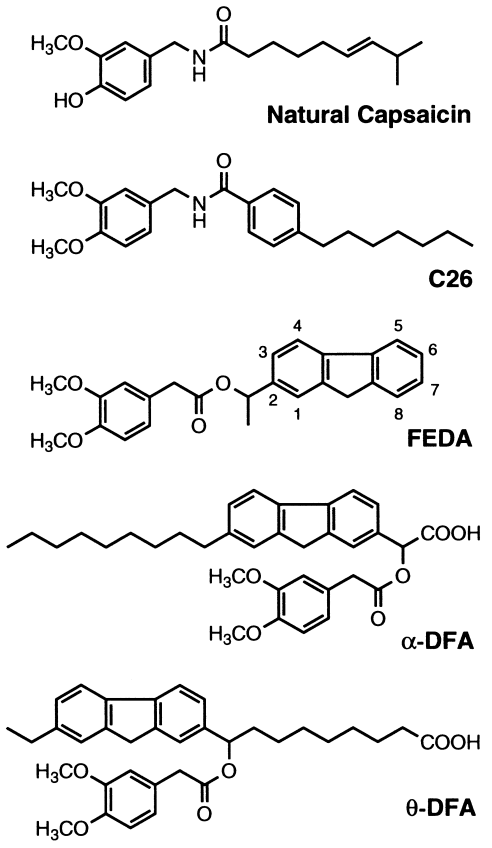

Fig. 1. Structures of natural capsaicin and fluorescent capsaicin mimic probes synthesized in this study.

extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc, 7:3) to give 2-fluorenethanol in 83% yield. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ 1.42 (1H, br, OH), 1.49 (2H, d, $J=6.4$ Hz, CH_3), 3.84 (2H, s, fluorene C9-H), 4.88 (1H, q, $J=6.5$ Hz, C*H*CH_3), 7.1–7.8 (7H, fluorene H).

3,4-Dimethoxyphenylacetyl chloride (0.75 g, 3.8 mmol) dissolved in CH_2Cl_2 was added dropwise to a stirred solution of a mixture of 2-fluorenethanol (0.81 g, 3.8 mmol) and triethylamine (1.7 g, 16 mmol) in CH_2Cl_2 at 0°C. The reaction mixture was left at room temperature for 3 h, then diluted with H_2O and extracted with EtOAc. The organic layer was washed with dilute aqueous HCl, saturated aqueous $NaHCO_3$, and finally with brine, then dried over Na_2SO_4. The reaction mixture was purified by silica gel column chromatography (EtOAc/toluene, 1:19) to give FEDA in 35% yield. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.57 (3H, d, $J=6.6$ Hz, CH_3), 3.59 (2H, s, CH_2Ph), 3.80 (2H, s, fluorene C9-H), 3.86 (6H, s, $2OCH_3$), 5.97 (1H, q, $J=6.6$ Hz, C*H*CH_3), 6.80 (3H, br, phenyl H), 7.3–7.8 (7H, fluorene H). Anal. calcd for $C_{25}H_{24}O_4$: C, 77.30; H, 6.23. Found: C, 77.49; H, 6.24.

2.2.2. α-(3,4-Dimethoxyphenyl)acetyloxy-7-nonyl-2-fluoreneacetic acid (α-DFA)

α-DFA was synthesized as shown in Fig. 2. To a stirred solution of fluorene (27.1 g, 163 mmol) in CS_2 was added anhydrous aluminium chloride (43.4 g, 326 mmol) at room temperature. To the mixture was added nonanoyl chloride (28.3 g, 179 mmol) dropwise at room temperature, and the reaction mixture was stirred for 3 h. The reaction mixture was then poured on to crushed ice, acidified with dilute HCl and extracted with EtOAc and CH_2Cl_2. The organic layer was washed with dilute HCl, saturated aqueous $NaHCO_3$, and finally with brine, then dried over Na_2SO_4. The organic solvent was removed in vacuo to give crude octyl 2-fluorenyl ketone. The crude product was used in the next reaction without further purification.

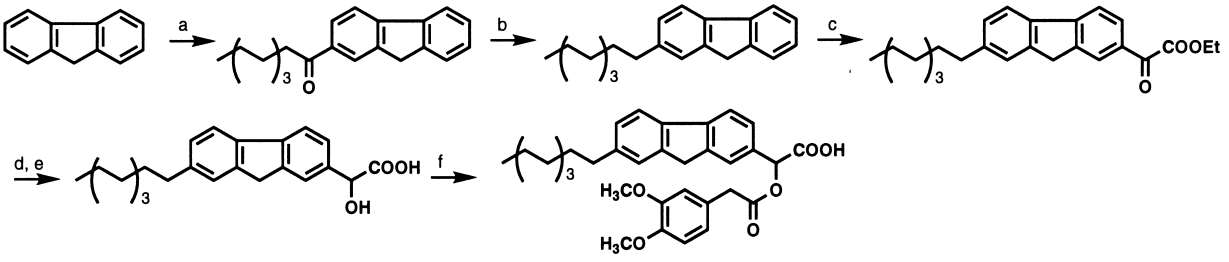

Fig. 2. Reaction conditions for the synthesis of α-DFA. (a) $C_8H_{17}COOH$, $SOCl_2$, $AlCl_3$ in CS_2; (b) H_2NNH_2/H_2O in diethyleneglycol; (c) EtOOCCOCl, $AlCl_3$ in CS_2; (d) aq. NaOH; (e) $NaBH_4$, aq. NaOH in MeOH; (f) 3,4-dimethoxyphenylacetyl chloride, $(Et)_3N$ in CH_2Cl_2.

Table 1
Inhibition of purified GDH activity

Compounds	I_{50} (μM)
FEDA	13 ± 3
α-DFA	Inactive
θ-DFA	19 ± 3
C26	9.7 ± 2
Piericidin A	13 ± 2

The Q_2 reduction activity was measured in reaction medium (2.5 ml) consisting of 5 mM Mops/NaOH (pH 6.5), 0.001% Triton X-100, 10 mM glucose and 20 μM Q_2, with a final protein concentration of 0.16 μg/ml. The I_{50}, i.e., the molar concentration in the reaction medium needed to halve the control enzyme activity, was used as an index of inhibitory potency.

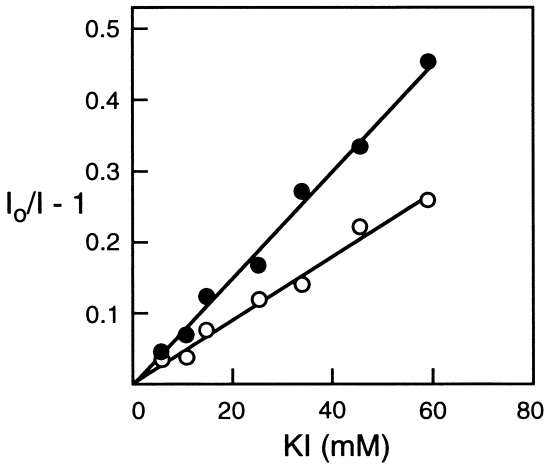

Fig. 3. Stern–Volmer plots for the quenching by KI of fluorenyl fatty acids incorporated into PC vesicles; α-DFA (●) and θ-DFA (○). The molar ratio of the probe to PC was 1:250. The excitation and emission wavelengths were 272 and 315 nm, respectively. I_o and I are the fluorescence intensity in the absence and presence of quencher, respectively.

To a solution of crude 2-fluorenyl ketone (21 g) in diethyleneglycol (150 ml) was added hydrazine monohydrate (4.1 g, 82 mmol). The reaction mixture was heated at 200°C for 30 min. After cooling the solution to about 90°C, KOH (1.5 g, 27 mmol) dissolved in a small amount of water was added dropwise and then the solution was heated again to 200°C and stirred for 10 min. Excess hydrazinehydrate and water formed in the reaction was distilled off, and the reaction mixture was refluxed for 3 h. After cooling, the solution was poured into water and neutralized with dilute HCl. The reaction mixture was extracted with EtOAc and dried over Na_2SO_4. The residue was purified by silica gel column chromatography (hexane/EtOAc, 19:1) to give 2-nonylfluorene in 45% yield. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.88 (3H, t, J = 7.0 Hz, CH_2CH_3), 1.1–1.4 (12H, br, nonyl H), 1.65 (2H, m, nonyl H), 2.66 (2H, t, J = 7.5 Hz, nonyl H), 3.84 (2H, s, fluorene C9-H), 7.2–7.7 (7H, fluorene H).

2-Nonylfluorene (6.5 g, 22.3 mmol) was reacted with ethyl oxalylchloride (3.4 g, 24.9 mmol) by Friedel–Crafts acylation as described above. The reaction mixture was purified by silica gel column chromatography (hexane/EtOAc, 19:1) to give crude material, which was crystallized from the same solvent mixture to obtain ethyl α-oxo-7-nonyl-2-fluoreneacetate in 64% yield. Subsequently, ethyl α-oxo-7-nonyl-2-fluoreneacetate (0.30 g, 0.76 mmol) was hydrolyzed by refluxing in methanol/H_2O (1:1) mixture containing NaOH (0.45 g, 11.2 mmol) for 2 h to obtain α-oxo-7-nonyl-2-fluoreneacetic acid in quantitative yield. ^{1}H-NMR (DMSO-d_6, 300 MHz) δ 0.83 (3H, t, J = 6.7 Hz, CH_2CH_3), 1.1–1.4 (12H, m, nonyl H),

Table 2
GDH activity reconstituted into PC vesicles containing various amount of α-DFA or θ-DFA.

Molar ratio (Probe:PC)	Residual enzyme activity (%)							
	Q_1		Q_2		PB		DB	
	α-DFA	θ-DFA	α-DFA	θ-DFA	α-DFA	θ-DFA	α-DFA	θ-DFA
1:4000	62	93	55	83	58	95	62	86
1:400	35	56	34	51	32	55	39	61
1:100	5	15	12	19	7	20	13	21

GDH activity was determined using Q_1 (100 μM), Q_2 (20 μM), PB (100 μM) or DB (20 μM) as an electron acceptor. The control enzyme activity measured in 100% PC vesicles were 0.71, 3.0, 0.37 and 3.2 mmol Q/min per mg of protein for Q_1, Q_2, PB and DB, respectively. Absorbance at 275 nm for Q_1 and Q_2 and at 279 nm for PB and DB, respectively, was measured with a millimolar extinction coefficient of 12.3. The molar ratio of the probe to PC was set as 1:100, 1:400 or 1:4000. Averaged residual enzyme activity (%) from two separate preparations is shown.

1.59 (2H, m, nonyl H), 2.64 (2H, t, $J=8.2$ Hz, nonyl H), 3.99 (2H, s, fluorene C9-H), 7.1–8.2 (6H, fluorene H).

To a solution of α-oxo-7-nonyl-2-fluoreneacetic acid (0.21 g, 0.58 mmol) in methanol (10 ml) was added $NaBH_4$ (0.039 g, 0.87 mmol) dissolved in 0.2 M aqueous NaOH dropwise at 0°C. The reaction mixture was stirred for 2 h at room temperature and neutralized with dilute HCl. After removal of methanol in vacuo, the reaction mixture was extracted with EtOAc. The organic layer was washed with dilute HCl and brine, then dried over Na_2SO_4. The crude material so obtained was purified by silica gel column chromatography (hexane/EtOAc, 7:3) to give α-hydroxy-7-nonyl-2-fluoreneacetic acid in 85% yield. ^{1}H-NMR (DMSO-d_6, 300 MHz) δ 0.84 (3H, t, $J=6.3$ Hz, CH_2CH_3), 1.1–1.4 (12H, m, nonyl H), 1.59 (2H, m, nonyl H), 2.62 (2H, t, $J=7.5$ Hz, nonyl H), 3.86 (2H, s, fluorene C9-H), 5.10 (1H, s, C*H*COOH), 7.2–7.8 (6H, fluorene H).

α-DFA was prepared by reacting α-hydroxy-7-nonyl-2-fluoreneacetic acid (2.0 g, 5.5 mmol) and 3,4-dimethoxyphenylacetyl chloride (2.1 g, 10.7 mmol) under the same reaction conditions as described for FEDA. The product was purified by recrystallization twice from hexane/EtOAc mixture. ^{1}H-NMR (DMSO-d_6, 300 MHz) δ 0.85 (3H, t, $J=6.4$ Hz, $CHCH_3$), 1.1–1.4 (12H, m, nonyl H), 1.61 (2H, m, nonyl H), 2.64 (2H, t, $J=7.5$ Hz, nonyl H), 3.72 (2H, s, CH_2Ph), 3.82 (3H, s, OCH_3), 3.83 (3H,s, OCH_3), 3.90 (2H, s, fluorene C9-H), 5.90 (1H, s, C*H*COOH), 6.81 (1H, dd, $J=7.2$, 1.9 Hz, phenyl H), 6.89 (1H, d, $J=8.2$ Hz, phenyl H), 6.94 (1H, d, $J=1.8$ Hz, phenyl H), 7.2–7.9 (6H, fluorene H). Anal. calcd for $C_{34}H_{40}O_6$: C, 74.97; H, 7.40. Found: C, 74.94; H, 7.39.

2.2.3. θ-(3,4-Dimethoxyphenyl)acetyloxy-7-ethyl-2-fluorenenonanoic acid (θ-DFA)

θ-DFA was synthesized by the same method used for the preparation of α-DFA, except that commercially available 2-acetylfluorene was used as a starting material, and 8-methoxycarbonyloctanoyl chloride was used in place of ethyl oxalylchloride in reaction step c. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.27 (3H, t, $J=7.6$ Hz, CH_2CH_3), 1.21–1.29 (8H, m, nonanoyl H), 1.58 (2H, m, nonanoyl H), 1.7–2.0 (2H, br, nonanoyl H), 2.30 (2H, t, $J=7.5$ Hz, nonanoyl H), 2.70 (2H, q, $J=7.6$ Hz, CH_2CH_3), 3.58 (2H, s, $PhCH_2$), 3.77 (2H, s, fluorene C9-H), 3.83 (6H, s, $2OCH_3$), 5.79 (1H, t, $J=7.4$ Hz, nonanoyl H), 6.78 (3H, m, phenyl H), 6.79 (2H, s, phenyl H), 7.1–7.7 (6H, fluorene H). Anal. calcd for $C_{34}H_{40}O_6$: C, 74.97; H, 7.40. Found: C, 74.97; H, 7.36.

2.3. Fluorescence quenching studies

The PC vesicles containing α-DFA or θ-DFA were prepared as follows. PC dissolved in chloroform and α-DFA or θ-DFA dissolved in tetrahydrofurane were mixed to attain a molecular ratio of probe to PC of 1:250. The PC vesicles were prepared by first removing the solvents in a rotary evaporator and then drying in vacuo overnight. The thin lipid film thus obtained was suspended in 50 mM Tris–HCl (pH 7.5) and vortexed at a final PC concentration of 20 mg/ml. The suspension was sonicated with a TOMY UR-200P sonicator in an ice-cooled bath under Ar gas. The resulting suspension was centrifuged at $20\,000\times g$ for 30 min at 4°C to remove undispersed PC. The amount of PC was calculated from phosphorus content by a modified Bartlett method [13].

Fluorescent quenching studies were carried out according to the method of Lala et al. [10]. Fluorescent measurements of fluorenyl fatty acid incorporated in PC vesicles were performed on a Shimadzu RF-5000 at 30°C with excitation and emission wavelengths of 272 and 314 nm, respectively. Excitation and emission slits were 5 and 10 nm, respectively. A 4.0 M solution of KI containing 0.1 mM $Na_2S_2O_3$ was used for iodide quenching studies. Small aliquots (<5 μl) of the KI solution were added successively to 2.5 ml of PC vesicle preparations (52 μM PC) and the emission spectra were recorded.

2.4. Enzyme preparation and assays

The membrane-bound GDH was purified from *E. coli*, which overproduced the enzyme in the membrane, and stored in a buffer containing 50 mM Mops/NaOH (pH 6.5) and 0.1% Triton X-100 at −78°C, as described previously [3]. The holo-enzyme was prepared by incubating the apo-enzyme with 5 mM $MgSO_4$ and 2.5 μM PQQ in a buffer contain-

ing 50 mM Mops/NaOH (pH 6.5) and 0.1% Triton X-100 for at least 20 min at 25°C before the assays. The Q_2 reduction activity was measured in a Shimadzu UV3000 spectrophotometer at 30°C by following absorbance at 275 nm with a millimolar extinction coefficient of 12.3. The reaction medium, in a final volume of 2.5 ml, contained 5 mM Mops/NaOH (pH 6.5) and 0.001% Triton X-100, and final protein concentration was 0.16 μg/ml. The final concentration of Triton X-100 was set at this low level since Triton X-100 inhibits the Q reductase activity at high concentrations (>0.02%) [14]. After incubating purified GDH with 20 μM Q_2 and various concentrations of inhibitor for 2 min, the enzyme reaction was started by addition of 25 μl of 1.0 M glucose, making the final concentration 10 mM. Protein concentration was determined according to Bradford [15] using bovine serum albumin as a standard.

2.5. Reconstitution of PC vesicles with glucose dehydrogenase

Reconstitution of PC vesicles with purified GDH was performed by the octylglucoside dilution method [2]. Sonicated PC vesicles (3.5 mg of PC) containing various amounts of α-DFA or θ-DFA were mixed with purified GDH (10 μg of protein), and octyl β-D-glucopyranoside was added to a final concentration of 1.25%; the final volume was brought to 400 μl with 50 mM potassium phosphate (P_i) buffer (pH 7.5). The molar ratio of fluorenyl fatty acid to PC was set to 1:100, 1:400 or 1:4000. The mixture was incubated on ice for 20 min and then diluted into 20 ml of 50 mM P_i buffer (pH 6.5, buffer A) that had been equilibrated to 25°C. The reconstituted vesicles were collected by centrifugation at $110\,000 \times g$ for 3 h, and the precipitate and centrifuge tube were rinsed for three times with small portions of buffer A. Then, the precipitate was suspended in 150 μl of buffer A. By this method, 55–65% of the added GDH was reconstituted into the vesicles irrespective of the molar ratio of the probe to PC.

The holo-enzyme was prepared by incubating the reconstituted apo-enzyme with 2 μM PQQ for at least 20 min at 25°C. The small portion of reconstituted vesicles was added to 1 ml of reaction medium consisting of 50 mM P_i buffer (pH 6.0) and 5 mM $MgSO_4$ to give a final protein concentration of 0.4 μg/ml. After addition of the indicated amounts of short-chain Q analogue, the enzyme reaction was started by adding 25 μl of 1.0 M glucose, making the final concentration 10 mM.

3. Results and discussion

3.1. Effects of fluorenyl inhibitors on purified GDH

The naturally occurring inhibitor capsaicin (Fig. 1), the pungent principal of red peppers, and particularly its synthetic analogues, inhibit Q reduction activity of mitochondrial NADH-Q oxidoreductase (complex I) [16]. In our previous study [4], we showed that some of the capsaicin analogues are also potent competitive inhibitors of Q reduction activity of GDH in *E. coli*. Interestingly, wide chemical modifications of the functional groups of capsaicins, such as the methoxy groups on the benzene ring and the polar amide bond unit, did not markedly affect their inhibitory potencies [4]. That is, the structural requirements of capsaicins for inhibition of GDH are fairly loose. This property of the inhibitory action of capsaicins seemed to be useful to modify their structures and then to attach them to the bulky fluorenyl group while maintaining the inhibitory activity.

In preliminary experiments, to screen for a proper capsaicin mimic structure, several capsaicin mimics were attached to the 2-position of fluorene (structures not shown) and their inhibitory potencies were examined with purified GDH. Considering its synthetic facility as well as its inhibitory potency, we selected FEDA (Fig. 1) as the best structure to be incorporated into the fatty acid. As shown in Table 1, the inhibitory potency of this compound was comparable to those of known potent inhibitors (e.g., synthetic capsaicin (C26, Fig. 1) and piericidin A).

On the basis of the above findings, we synthesized fluorenyl fatty acids containing capsaicin mimic (FEDA) in the molecule, α-DFA and θ-DFA (Fig. 1). The attachment of a hydrophobic tail in the 7-position of the fluorenyl group helps in better aligning the fluorenyl moiety in the lipid bilayer and can overcome the problems associated with looping back of the probe moiety to the membrane–water interface [10,11]. To determine whether the capsaicin mimic can inhibit GDH activity even if it is attached to

the fatty acid, the effects of α-DFA and θ-DFA on purified GDH activity were investigated (Table 1). θ-DFA appeared to retain its inhibitory activity, although the potency was slightly reduced compared to that of FEDA. Unexpectedly, α-DFA elicited no inhibition up to the solubility limit (ca. 110 μM). This is probably due to the proximity of the polar carboxyl group to the capsaicin mimic moiety, preventing the inhibitor moiety from fitting into the Q reduction site. However, this property of α-DFA might be convenient for the purpose of the present study since even if a portion of the α-DFA once incorporated into PC vesicles would be released back to the bulk medium, inhibition by free α-DFA is negligible with the reconstituted system described later.

3.2. Fluorescence quenching studies

The excitation and emission maxima of both α-DFA and θ-DFA appeared at 272 and 313 nm, respectively, in methanol. The emission spectra of the two compounds on excitation at 272 nm were also recorded in other organic solvents such as *n*-hexane, chloroform, *n*-octanol and ethanol. Variations in solvent polarity led to practically no change in the emission maxima, as reported for other types of fluorenyl fatty acids [10]. Similar excitation and emission spectra of the two compounds were observed in PC vesicles.

The orientation of the fluorescent probes in membranes has been studied by following the depth-dependent quenching of their fluorescence in membranes [10,11]. To determine the orientation of fluorenyl fatty acids in PC vesicles, we examined their fluorescence quenching in PC vesicles using the water-soluble quencher iodide. This quencher is highly soluble in the aqueous environment relative to the apolar environment of the lipid bilayer. Consequently, high concentrations of the quencher are required to quench fluorescent chromophores embedded in the lipid bilayer. Taking advantage of this low solubility in the lipid bilayer, iodide has been used to assess the transverse location of fluorescent probes in the membrane [6,10,11]. The Stern–Volmer plots [6] for iodide quenching of the fluorescence of α-DFA and θ-DFA in PC vesicles are shown in Fig. 3. The plots were linear passing through the origin. As expected, iodide quenched fluorescence of the shallower chromophore (i.e., α-DFA) more efficiently than that of the deeper chromophore (i.e., θ-DFA). This result indicated that a fluorenyl group and thus the attached capsaicin mimic moiety of θ-DFA is aligned deeper in the bilayer compared to that of α-DFA. The quenching efficiencies of α-DFA and θ-DFA measured in 10% methanol/water (v/v) were almost identical within experimental error (data not shown). Therefore, the difference in quenching efficiencies of the vesicles is essentially due to that in depth of the fluorenyl group.

3.3. Effects of fluorenyl fatty acids on reconstituted GDH

The inhibition of Q reduction activity of GDH reconstituted in PC vesicles containing the fluorenyl fatty acid in various molar ratios relative to PC was examined using different short-chain Q analogues as electron acceptors; Q_1, Q_2, PB and DB (Table 2). The 100% enzyme activity was determined with GDH reconstituted in 100% PC vesicles (i.e., without fluorenyl fatty acid). Interestingly, although free α-DFA did not inhibit Q reduction activity of purified GDH (Table 1), this compound elicited inhibition with the reconstituted system. This is probably because the proximity of the carboxyl group to the capsaicin mimic was relieved, at least in part, since the polar carboxyl anion was aligned in the membrane–water interface when α-DFA was incorporated into the bilayer membrane.

For both reconstituted systems, the extent of inhibition increased with increases in the molar ratio of the fluorenyl fatty acid to PC. However, the extent of inhibition was greater in the reconstituted system containing α-DFA than that containing θ-DFA irrespective of short-chain Q. These results strongly suggested that the Q reduction site in *E. coli* GDH is located close to the membrane surface rather than in the hydrophobic membrane interior, supporting the topological model proposed by Yamada et al. [3], which predicted that the binding site for Q is located in a loop in the periplasmic side of the N-terminal transmembrane region. It should be noted that the Q reaction sites of other respiratory enzymes, such as complex I in *Rhodobacter capsulatus* [17], cyto-

chrome *bo* in *E. coli* [18,19] and cytochrome bc_1 complex (Q_o and Q_i centers) in bovine heart mitochondria [20,21], are supposed to be located close to the membrane surface.

If our conclusion is correct, in one view, the difference in depth-dependent inhibition between the α-DFA- and θ-DFA-incorporated vesicles (Table 2) would be somewhat less than that predicted from the difference in the attached positions of the inhibitor moiety. There are two possible reasons for this. First, considering the lack of inhibitory activity of free α-DFA described in the previous section, α-DFA may be inherently disadvantageous for inhibition even with the reconstituted system. Second, the capsaicin mimic moiety of θ-DFA could still be capable of looping back to the membrane surface because of conformational flexibility of the fatty acyl chain. Although an alkyl tail (ethyl group in this case) was attached to the 7-position of the fluorene to overcome this problem, complete resolution might be difficult especially for flexible lipid probes [22]. Thus, taking into account these factors, the difference in the two systems can be regarded as significant.

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