Inhibitory Effects of Galloylglucose on Nicotinamide Adenine Dinucleotide Dehydrogenases of the Aerobic Respiratory Chain of *Escherichia coli*

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The effects of pentagalloylglucose (1,2,3,4,6-penta-*O*-galloyl-β-D-glucose) on the aerobic electron transport system of *Escherichia coli* were studied. The activity of nicotineamide adenine dinucleotide (NADH) reductase was inhibited by pentagalloylglucose, but the activities of succinate dehydrogenase, D-lactate dehydrogenase, and ubiquinol-1 (Q_1H_2) oxidase were not susceptible to the inhibitor. Because the presence of two kinds of NADH dehydrogenase in respiratory chain of *Escherichia coli* has been reported, we examined the effect of galloylglucose independently on both NADH dehydrogenases.

Pentagalloylglucose is potent and specific inhibitor of both NADH dehydrogenases. One of the NADH dehydrogenases (NADH dh II) is more sensitive to the inhibitor than the other (NADH dh I).

Keywords pentagalloylglucose; *Escherichia coli*; NADH dehydrogenase; aerobic respiratory chain; inhibitory effect

Galloylglucoses, which were purified from tannic acid contained in Chinese gall and in Turkish gall, are composed of glucose and gallic acid.[1-3] Recently we have shown that galloylglucose inhibited reduced nicotineamide adenine dinucleotide (NADH) dehydrogenase and the terminal oxidase, cytochrome d complex, of the aerobic respiratory chain of *Photobacterium phosphoreum*.[4] We have also described the inhibitory effects of galloylglucose on succinate dehydrogenase, NADH dehydrogenase, and cytochrome bc_1 complex of the mitochondrial respiratory chain.[5]

The nature of the components of the aerobic respiratory chain of *Escherichia coli* has been studied in many laboratories. Recent investigations suggested that two distinct species of NADH dehydrogenase exist in the membrane of *E. coli*.[6-8] One of the dehydrogenases (NADH dh I) can use both reduced nicotinamide hypoxanthine dinucleotide (d-NADH) and NADH as electron donors, and functions as the coupling site for oxidative phosphorylation. The other dehydrogenase (NADH dh II) uses NADH exclusively, and does not have the coupling site. Another distinction between NADH dh I and II is differential sensitivity to some inhibitors: NADH dh I is significantly more sensitive to 3-undecyl-2-hydroxy-1,4-naphthoquinone (UHNQ), piericidin A, and myxothiazol.

In this report, we studied the effect of pentagalloylglucose (1,2,3,4,6-penta-*O*-galloyl-β-D-glucose) on the aerobic respiratory chain of *Escherichia coli* using the sonicated membrane vesicles. The data presented here indicate that the galloylglucose inhibits both kinds of NADH dehydrogenases of the respiratory chain, and NADH dh II is more sensitive to the inhibitor.

Materials and Methods

Growth of Cells *E. coli* K-12 strain W3110 (our laboratory collection) was grown aerobically to the late exponential phase. It was confirmed spectrophotometrically that both cytochrome o and d complex were contained in this *E. coli*. The medium used was synthetic minimum medium[9] supplemented with 0.3% casamino acid and 0.3% glucose, except that *E. coli* for the assays of D-lactate and succinate-dependent reaction was grown in the medium containing 0.5% sodium lactate and 0.5% sodium succinate, respectively.

Preparation of Sonicated Membrane Containing Two NADH Dehydrogenases (Membrane A) Cells were harvested, washed once in 33 mM Tris–HCl (pH 7.5) containing 5 mM $MgSO_4$, and suspended in 50 mM Tris–HCl (pH 7.5) containing 5 mM $MgSO_4$. The suspension was sonicated with a Tomy Seiko UR-200p ultrasonic disrupter with cooling in an ice bath. The sonicated lysate was centrifuged at $20000 \times g$ for 30 min, and the supernatant was centrifuged at $100000 \times g$ for 1 h. The precipitate obtained was suspended with 50 mM Tris–HCl (pH 7.5) containing 5 mM $MgSO_4$, 1 mM dithiothreitol, and 10% glycerol. The membrane obtained was used for experiments within 3 h.

Preparation of Sonicated Membrane Containing Only NADH dh II (Membrane B) Membrane A was washed twice with 50 mM Hepes (pH 7.5) containing 0.1 M KCl and 5 mM $MgSO_4$ and suspended with 50 mM Tris–HCl (pH 7.5) containing 5 mM $MgSO_4$. Then, the membrane was freeze-thawed twice.

Assay of Oxidase Activity Ubiquinol-1 (Q_1H_2) oxidase activity was assayed spectrophotometrically as described previously.[10] The activity of NADH oxidase was also measured spectrophotometrically according to the method of Matsushita *et al.*[8]

Assay of NADH-Ubiquinone-1 (Q_1) Reductase Activity NADH-Q_1 dehydrogenase activity was assayed according to the method of Hatefi.[11]

Assay of d-NADH-Q_1 Reductase Activity The activity of d-NADH-Q_1 reductase was measured by the method of Matsushita *et al.*[8]

Assay of Succinate Dehydrogenase Activity Succinate-Q_1 reductase was assayed by the method of Takamiya *et al.*[12] The succinate-phenazine methosulfate (PMS)/3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reductase activity was determined spectrophotometrically by measuring the absorbance change of MTT at 570 nm ($\varepsilon = 17\ mM^{-1}cm^{-1}$).[13]

Assay of D-Lactate Dehydrogenase Activity The D-lactate-PMS/MTT reductase activity was measured by the method of Futai and Kimura.[14]

Determination of Protein Protein concentration was determined by the method of Lowry *et al.*[15] with bovine serum albumin as a standard.

Pentagalloylglucose 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose isolated from Chinese gallotannin was a generous gift from Drs. I. Nishioka and G. Nonaka. This pentagalloylglucose showed a single peak in reverse-phase high performance liquid chromatography (HPLC).

Chemicals Q_1 was a generous gift from Eisai Co., Ltd. Q_1H_2 was prepared by the reduction of Q_1 according to the method of Kita *et al.*[10] The d-NADH was prepared by the reduction of d-NAD (Sigma) with ethanol and alcohol dehydrogenase essentially as described by Dalziel.[16] Myxothiazol was obtained from Boehringer. Other chemical reagents used were of the highest purity commercially available.

Results

***E. coli* W3110 Sonicated Membranes Contain Two Distinct NADH Dehydrogenases** As indicated by Matsushita *et al.*,[8] the inner membrane of *E. coli* GR19N (*cyd*$^-$), contains two kinds of NADH dehydrogenases. To confirm the presence of two components which oxidize NADH in the aerobic respiratory chain of *E. coli* W3110,

TABLE I. The NADH-Q_1 and d-NADH-Q_1 Reductase Activities of Membranes from *E. coli*

Activity	Membrane A	Membrane B
NADH-Q_1	792[a)]	625
d-NADH-Q_1	159	0

a) nmol Q_1/min/mg of protein.

TABLE II. Inhibition of NADH-Q_1 Reductase and d-NADH-Q_1 Reductase Activities by Myxothiazol and Pentagalloylglucose

Activity	Inhibitor	(μM)	Relation activity (%)	
			Membrane A	Membrane B
NADH-Q_1	Myxothiazol	0	100	100
		25	87.2	n.t.
		100	80.5	97.0
	Pentagalloyl-glucose	0	100	100
		20	3.1	5.2
d-NADH-Q_1	Myxothiazol	0	100	—
		37.5	31.5	—
		100	0	—
	Pentagalloyl-glucose	0	100	—
		20	39.5	—

n.t.: not tested. —: not detectable.

we carried out the experiment using membrane prepared by the two different procedures: One according to Matsushita *et al.*[8] except that the destruction of cells was performed by ultrasonication (membrane A), and the other by the washing and freeze-thawing of membrane A (membrane B) (see Materials and Methods).

As shown in Table I, d-NADH-Q_1 reductase activity was detected with membrane A which prepared freshly under mild conditions. After washing and freeze-thawing, d-NADH-Q_1 reductase activity was selectively lost (membrane B). The NADH-Q_1 reductase activity of membrane A did not differ greatly from that of membrane B. The activity of d-NADH-Q_1 reductase in the membrane A is very susceptible to myxothiazol, but that of NADH-Q_1 reductase in the same membrane is inhibited by only about 20% in the presence of 100 μM myxothiazol (Table II). In contrast, the activity of NADH-Q_1 reductase in the membrane B is not inhibited in the same range of concentrations of myxothiazol. These results suggest that about 20% of total NADH-Q_1 and all of total d-NADH-Q_1 reductase activities are due to NADH dh I, and the remainder is due to NADH dh II.

As demonstrated by Matsushita *et al.*,[8] NADH dh I can use NADH and d-NADH as substrates, and NADH dh II can use only NADH as a substrate. In addition, it was also reported that NADH dh I and II can be distinguished on the basis of differential sensitivity to myxothiazol. NADH dh I and NADH dh II are contained in membrane A. Because NADH dh I is very unstable, the selective loss of d-NADH-Q_1 was observed in membrane B.

There are two apparent K_ms for NADH in membrane A (data not shown), the values of which are about 87 and 20 μM. However, only one component (low affinity for NADH) was observed in the membrane B. For the d-NADH-Q_1 reductase activity of membrane A, a single K_m was detected (12 μM, not shown). These kinetic values are

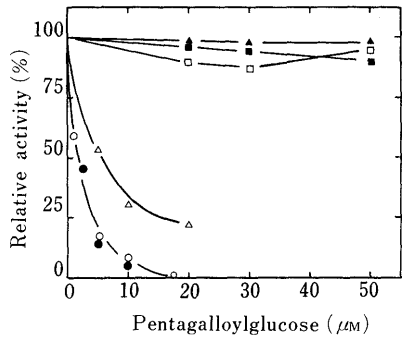

Fig. 1. Inhibition of the Electron Transfer Activity of Membrane of *E. coli* by Pentagalloylglucose

Membrane A was used for d-NADH-Q_1 reductase activity assay (△). NADH-Q_1 reductase activity (○) and NADH oxidase activity (●) assays were carried out using membrane B. Succinate-dependent reductase and lactate-dependent reductase were assayed with sonicated membrane prepared from cells grown with succinate- and lactate-containing medium, respectively. Other membranes were grown with medium containing glucose. (□), Q_1H_2 oxidase; (▲), succinate dehydrogenase; (■), D-lactate dehydrogenase.

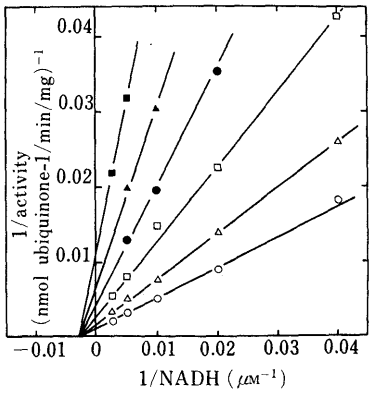

Fig. 2. Double-Reciprocal Plot of the Effect of Pentagalloylglucose on NADH-Q_1 Reductase Activity in Sonicated Membrane (Membrane B)

The concentrations of pentagalloylglucose were 0 (○), 1 (△), 3 (□), 15 (●), 25 (▲), and 50 μM (■).

in agreement with the data reported previously[8] (K_ms for NADH are 14.7 and 50 μM, and K_m for d-NADH is 9.7 μM).

Inhibitory Effect of Pentagalloylglucos in the Respiratory Chain Membrane B was used for the assays of NADH-Q_1 reductase and NADH oxidase in the following experiments. As indicated in Fig. 2, the activities of D-lactate dehydrogenase, succinate-Q_1 oxidoreductase, succinate dehydrogenase, and Q_1H_2 oxidase were not inhibited by pentagalloylglucose. Two dehydrogenases, cytochrome o complex and cytochrome d complex of the respiratory chain, were resistant to the inhibitor. On the other hand, NADH oxidase and NADH-Q_1 reductase activities were most sensitive to pentagalloylglucose among the respiratory components studied in this paper. The activity of d-NADH-Q_1 reductase is less sensitive. From these results, NADH dh I and II are both sensitive to pentagalloylglucose, and the inhibitor is more effective against NADH dh II.

We studied the kinetics of inhibition of NADH-Q_1 reductase activity by pentagalloylglucose at various concentrations of NADH. The result is illustrated in a double-reciprocal plot in Fig. 2, indicating clearly that pentagalloylglucose is a noncompetitive inhibitor of NADH-Q_1

reductase activity ($K_i = 1.6\,\mu M$). Kinetic data were determined for the other substrates (data not shown). The K_i values for Q_1 in membrane A and in membrane B were calculated as 2.5 and 3.3 μM, respectively. The K_i value of the inhibitor for d-NADH (6.0 μM, in membrane A) is about four times that for NADH. All inhibitions were noncompetitive with respect to substrate.

Discussion

It was indicated by Matsushita *et al.*[8] that there are two distinct NADH dehydrogenases in the respiratory chain of the inner membrane of *E. coli*. We have confirmed that. It was suggested that NADH dh II is probably the same enzyme as the one encoded by the *ndh* gene that was cloned and sequenced by Young *et al.*[17,18] This *ndh* gene product is a single polypeptide with a molecular weight of about 47 kilodaltons. In contrast, the properties of NADH dh I are scarcely known because of the relative instability of its activity. Another difference between NADH dh I and II is their substrate specificity; dh I reacts with NADH and d-NADH, but dh II reacts only with NADH.

In addition, the sensitivity of the two NADH dehydrogenases to quinone analogues is different. The NADH-Q_1 reductase activity of NADH dh I is significantly more sensitive to inhibitors, such as UHNQ, piericidin A, and myxothiazol.[8] Each of these materials is a potent inhibitor of complex I or III in the mitochondrial electron transport system. However, no potent inhibitor of NADH dh II has been known. We have now found that pentagalloylglucose is a specific and potent inhibitor of the two NADH dehydrogenases of the respiratory chain of *E. coli* membrane. Other components of the respiratory chain, such as D-lactate dehydrogenase, succinate dehydrogenase, cytochrome o complex, and cytochrome d complex, are not susceptible to pentagalloylglucose. This inhibitor will be a useful tool for the study of the respiratory chain of *E. coli* because of its specific inhibition of the two routes of electron flow from NADH.

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