Inhibitory Effects of Tannins on the NADH Dehydrogenase Activity of Bovine Heart Mitochondrial Complex I

Kiyoshi KONISHI^{*,a} and Takashi TANAKA^b

Department of Microbiology, Nippon Dental University School of Dentistry at Tokyo,^a 1–9–20 Fujimi, Chiyoda-ku, Tokyo 102–8159, Japan and Nagasaki University School of Pharmaceutical Sciences,^b 1–14 Bunkyo-cho, Nagasaki 852–8521, Japan. Received October 12, 1998; accepted November 25, 1998

NADH-quinone oxidoreductase is classified into two groups, NADH dehydrogenase-1 (NDH-1) and NADH dehydrogenase-2 (NDH-2). Animal mitochondrial complex I is an NDH-1 type enzyme. Previously, we isolated potent inhibitors from plants to both NDH-1 and NDH-2. We have now examined detailed inhibitory effects of three tannins (pentagalloylglucose, sanguin H-11, and oolonghomobisflavan A) on NDH-1 using bovine heart mitochondrial complex I and a subcomplex flavoprotein (containing 3 subunits) derived from complex I. Although many specific inhibitors of NDH-1 (*e.g.* rotenone and piericidin A) have been reported, the reactive sites are at or near to, the ubiquinone-binding site. NADH-ubiquinone-1 oxidoreductase activity of complex I was inhibited by the three tannins, among which sanguin H-11 was the most potent inhibitor. NADH-menadione oxidoreductase activity of complex I was susceptible to the three tannins, but completely resistant to rotenone. The inhibitory effects of tannins were all noncompetitive with respect to NADH, ubiquinone-1, and menadione. The NADH-menadione oxidoreductase of flavoprotein was also inhibited by the three tannins, but not by rotenone, which is consistent with the fact that flavoprotein does not contain a native ubiquinone-binding site. The study of the NADH reduced-minus-oxidized difference spectrum of flavoprotein under steady-state conditions indicated that the inhibitory sites of sanguin H-11 and oolonghomobisflavan A exist between the NADH binding site and the FMN site, and that for pentagalloylglucose exists between FMN and an artificial electron acceptor-binding site. These results suggest that the tannins are potent inhibitors of NADH dehydrogenases, and that the inhibitory mechanisms are novel.

Key words NADH dehydrogenase; tannin; complex I; flavoprotein

NADH-quinone oxidoreductases in the eukaryotic mitochondrial and bacterial respiratory chains can be divided into two groups, depending on whether they have (NADH dehydrogenase-1, NDH-1) or do not have (NADH dehydrogenase-2, NDH-2), an energy-coupling site, in which redox energy is converted to electrochemical proton potential across the membrane.^{1–3} The NDH-1 type NADH dehydrogenase generally contains a non-covalently bound FMN and multiple iron-sulfur (Fe-S) clusters as prosthetic groups, and is composed of many subunits.^{2,4–6} In mammals, it consists of about 40 different subunits⁷ and in bacteria *Paracoccus denitrificans*, of 14 different subunits.^{4,8} On the other hand, NDH-2 is composed of a single polypeptide chain and contains a non-covalent-bound FAD as a prosthetic group.^{2,4} NDH-1 is inhibited by many specific inhibitors such as rotenone, capsaicin, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), 1-methyl-4-phenyl-pyridinium (MPP⁺), and piericidin A.^{5,9} In the mitochondrial membrane which contains complex I, the ubiquinone-reducing activity of the complex is sensitive to rotenone. Purified complex I catalyzes reduction of ubiquinone-1 (Q₁) and rotenone-insensitive reductions of ferricyanide, dichlorophenolindophenol (DCIP), and menadione (K₃). Rotenone inhibits electron transfer between the Fe-S cluster and ubiquinone, as indicated by electron paramagnetic resonance (EPR) studies.¹⁰ Several other inhibitors (piericidin A, HQNO, capsaicin, 1,10-phenanthroline, MPP⁺, and others) were also shown to bind very close to the rotenone-binding site, and near the ubiquinone-binding site.¹¹ Initially, it was believed that the inhibitory site for rhin was on the substrate (NADH) site of the flavin in complex I, and that it was a competitive inhibitor with respect to NADH.¹² However, rhin was later shown to oxidize NADH

directly and apparently inhibits the NADH-dependent electron flow of complex I.¹³ Recently, Majander *et al.* reported that diphenyleneiodinium (DPI) inhibits a site in complex I distinct from those of rotenone and other known NDH-1 specific inhibitors.¹⁴ They also indicated that DPI binds irreversibly to a site between NADH and the Fe-S clusters, and probably reacts with FMN itself.

NDH-2 is completely resistant to NDH-1 type-inhibitors which can react on or near the ubiquinone-binding site.⁹ Previously, we studied the inhibitory effects of certain tannins purified from Chinese medicinal plants or teas (green tea, oolong tea, and Assam tea), and related compounds on various NADH-quinone oxidoreductases.¹⁵ Since we discovered that 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose (pentagalloylglucose), sanguin H-11, and oolonghomobisflavan A are potent inhibitors of both types of NADH dehydrogenases (NDH-1 and NDH-2), we considered that the mechanisms of these inhibitors would be unique. In this paper, we examined in detail the inhibitory effects of these tannins on complex I from bovine heart mitochondria, and showed that tannins react near the NADH binding site or the FMN site and that the inhibition mechanisms of these tannins are different from that of DPI.

MATERIALS AND METHODS

Preparation of Complex I from Bovine Heart Mitochondria Isolation of bovine heart mitochondria was carried out according to the published procedure.¹⁶ Complex I was prepared from bovine heart mitochondria by the method of Galante and Hatefi.¹⁷

Preparation of Subfraction (Flavoprotein) from Com-

* To whom correspondence should be addressed.

plex I Flavoprotein (FP) was isolated from complex I. Complex I from bovine heart was suspended in Tris-HCl (pH 7.5) containing 0.25 M sucrose, 5 mM sodium dithionite and 0.2 M KCl. Deoxycholate and cholate were added to concentrations of 1.5% and 1.0%, respectively. Complex I was adjusted to a concentration of 5 mg/ml, and the mixture was incubated for 3 h at 4 °C under a nitrogen atmosphere. Chilled ethanol (4 °C) was added to a concentration of 15% (v/v). The suspension was centrifuged for 30 min at 100000 $\times g$. The supernatant was brought to 30% saturation with ammonium acetate and solid urea was then added to a concentration of 2 M. The obtained supernatant was dialyzed against 50 mM Tris-HCl (pH 8.0) containing 0.25 M sucrose and 5 mM sodium dithionite, and concentrated on a membrane filter (UM-10, Amicon Co., Ltd., Beverly, MA, U.S.A.) with a ultracentrifugation apparatus.

Assay of NADH Dehydrogenase Activity NADH- Q_1 and NADH- K_3 oxidoreductase activities of complex I and FP were assayed according to the method described previously^{17,18)} with slight modifications. A sample (about 40 μ g of protein for the assay of complex I and about 10 μ g of protein for that of FP) was used for each assay. The activities were measured spectrophotometrically by recording the absorbance change of NADH at 340 nm (extinction coefficient; 6.22 $\text{mm}^{-1} \text{cm}^{-1}$) in a 2 ml reaction-mixture containing 50 mM Tris-HCl (pH 7.5) buffer at 25 °C in the presence of electron acceptor (Q_1 or K_3) using a Hitachi spectrophotometer U-320 (Hitachi, Ibaraki, Japan). All activities are expressed as the initial rates of NADH oxidation (μ mol of reduction of NADH per min per mg protein of complex I or FP). Each reaction was initiated by the addition of NADH.

Spectral Measurement The difference spectrum of FP was measured with a Beckman DU-7000 spectrophotometer (Columbia, MD, U.S.A.).

Determination of Protein Concentration The protein concentration was determined by the method of Lowry *et al.*¹⁹⁾ with bovine serum albumin as a standard.

Chemicals Q_1 was a generous gift from Eisai Co., Ltd. (Tokyo, Japan). Pentagalloylglucose was prepared from Chinese gallotannin.²⁰⁾ Sanguin H-11 and oolonghomobisflavan A were isolated from *Sanguisorba officinale* and oolong tea, respectively.¹⁵⁾ Other chemical reagents used were of the highest grade commercially available.

RESULTS AND DISCUSSION

In a previous paper, we reported that pentagalloylglucose, sanguin H-11, and oolonghomobisflavan A inhibited the NADH-ubiquinone oxidoreductase from submitochondrial particles (SMP) and from rat liver mitochondria.¹⁵⁾ Since it was very difficult to prepare complex I from rat liver, we used bovine heart mitochondria as a starting material for the preparation of complex I. Three tannins inhibited the NADH-ubiquinone oxidoreductase from bovine heart SMP in a similar manner to that from rat liver SMP (data not shown). Complex I can use many other substrates as the electron acceptor, for example, potassium ferricyanide, cytochrome c, DCIP, K_3 , and Q_1 . The tannins directly reduced the artificial electron acceptor, ferricyanide (standard oxidation-reduction potential (E'_m) = +420 mV) or cytochrome c (E'_m = +220 mV), and reduced DCIP very slowly (E'_m = +217 mV), but did not

Fig. 1. Inhibition of the Electron Transfer Activity of Complex I by Rotenone

NADH- Q_1 (open circle) and NADH- K_3 (closed circle) oxidoreductase activities were assayed using complex I (40 μ g/ml) from bovine heart. The IC_{50} value of rotenone was about 100 nM (5 nmol/mg of complex I). The concentrations of NADH and Q_1 for substrates were both 100 μ M. The concentration of K_3 was 400 μ M. Results are the mean from two independent experiments.

interact with K_3 (E'_m = +0 mV) or Q_1 (E'_m = +110 mV), indicating that the E'_m values of these inhibitors are approximately above +200 mV. Therefore, we used K_3 and Q_1 as the electron acceptors in our experiments.

To interpret the kinetic data obtained with the tannins and NDH-1, it is important to establish whether the inhibition is fully reversible or not, as expected for an equilibrium interaction with a binding site. Rotenone, which is a specific and potent inhibitor of NDH-1, binds very tightly, and its inhibitory effect is dependent of the concentration of the protein of the enzyme.⁹⁾ Restoration of rotenone inhibition was not observed after dilution of the reaction mixture containing enzyme and rotenone by 100-fold with buffer. DPI is a completely irreversible inhibitor of NDH-1, and its IC_{50} value is also affected by the enzyme concentration.¹⁴⁾ However, the inhibitory effects of the tannins were not dependent on the protein concentration of complex I, and only depended on the absolute concentration of inhibitors, and the inhibitory effect completely disappeared by a 1000-fold dilution or a dialysis against a 1000-fold volume of 10 mM Tris-HCl (pH 7.5) buffer in order to remove the inhibitors (data not shown). These results clearly indicate that the tannins are completely reversible inhibitors.

Since a native electron mediator between complex I and III (cytochrome bc_1 complex) is ubiquinone-10, the physiological activity is NADH-ubiquinone oxidoreductase. The binding sites of NDH-1 specific inhibitors such as rotenone, capsaicin, HQNO, MPP⁺, or piericidin A, are in or near the binding site of ubiquinone, and these inhibitors strongly inhibit the activity of NADH-ubiquinone oxidoreductase.^{5,9,11)} However, when an artificial electron mediator (K_3 , DCIP, or ferricyanide) is used as an electron acceptor of NDH-1 activity, electrons do not pass through the ubiquinone binding site and NADH-artificial electron acceptor oxidoreductase activities are not susceptible to NDH-1 specific inhibitors.⁹⁾ As indicated in Fig. 1, NADH- Q_1 oxidoreductase from bovine heart complex I was inhibited by rotenone, but NADH- K_3 oxidoreductase activity was not affected at up to 100 μ M of rotenone. The residual activity (about 20%) of NADH- Q_1 oxidoreductase did not disappear in the presence of a high con-

Table 1. Inhibitory Effects of Tannins on NADH Dehydrogenase Activities of Complex I

Compound ^{a)}	Relative activity (%)	
	NADH-Q ₁	NADH-K ₃
Pentagalloylglucose	1.2	8.7 ^{b)}
Sanguin H-11	0.3	1.3
Oolonghomobisflavan A	3.7	4.8

a) Activities were assayed in the presence of 50 μM of inhibitors. The data are the mean from three independent experiments. b) In the presence of 100 μM pentagalloylglucose, only 4.0% of activity was detected.

Fig. 2. Double-Reciprocal Plot of Reduction Rate of Complex I

NADH-Q₁ oxidoreductase activity of complex I was measured in the presence or absence of pentagalloylglucose. From the results in the absence of inhibitor, K_m for Q₁ and V_{max} were calculated as 71.4 μM and 2.3 $\mu\text{mol/min/mg}$ protein, respectively.

centration of rotenone (20 μM). Three tannins inhibited almost completely both NADH-Q₁ and NADH-K₃ oxidoreductase activities (Table 1). These results suggest that the inhibitory sites for tannin differ from the known specific inhibitors such as rotenone, and may exist near the NADH binding site (electron donor site) of complex I.

Pentagalloylglucose was a noncompetitive inhibitor with respect to NADH, or Q₁ in NADH-Q₁ oxidoreductase activity, and to K₃ in NADH-K₃ oxidoreductase activity (results for Q₁ only are shown in Fig. 2). The other types of inhibition activity assayed were all noncompetitive, and parameters are indicated in Table 2. Sanguin H-11 was the most potential inhibitor.

Complex I is a large and very complex membrane enzyme. In order to define the detailed mechanism of the inhibitory effect of tannins, we studied a subcomplex of the intact enzyme. Numerous attempts to resolve the structure-function relationships within the mammalian coupling site 1, have resulted in the isolation of several types of soluble NADH dehydrogenase originating from complex I. The soluble three-subunit iron-sulfur FP fraction appears to be the simplest catalytic component fragment among known subfractions of complex I.^{21,22)} The FP subcomplex was isolated from complex I by detergents and urea in combination with ammonium acetate. FP contained FMN, 51, 24, and 10 kDa subunits, and some nonheme irons and acid-labile sulfurs (data not shown). FP showed NADH oxidase activities with a vari-

Table 2. Inhibitor Constants for Tannins on NADH Dehydrogenase Activities of Complex I and FP

Compound	Complex I (μM)			FP (μM)	
	K_i^{NADH}	$K_i^{\text{Q}_1}$	$K_i^{\text{K}_3}$	K_i^{NADH}	$K_i^{\text{K}_3}$
Pentagalloylglucose	0.9 ^{a)}	1.1	6.0	0.31	0.25
Sanguin H-11	0.5	0.4	0.8	0.06	0.05
Oolonghomobisflavan A	1.7	1.9	3.1	0.15	0.25

a) K_i^{NADH} value is the inhibition constant of compounds for NADH (variable concentrations) in the presence of a saturated concentration of another substrate (Q₁; 100 μM), and calculated from double-reciprocal plots at various concentrations of inhibitors as in Fig. 2 (for pentagalloylglucose). $K_i^{\text{Q}_1}$ value was also calculated from NADH-Q₁ oxidoreductase assays in the presence of 200 μM NADH. $K_i^{\text{K}_3}$ value was estimated from NADH-K₃ oxidoreductase assay in the presence of 200 μM NADH.

Fig. 3. Reduced-Minus-Oxidized Difference Spectra of FP

The spectra of FP (440 $\mu\text{g/ml}$) were measured in 50 mM Tris-HCl (pH 7.5). Trace A, reduction by 40 μM NADH; trace B, reduction by sodium dithionite; trace C, reduction by 40 μM NADH in the presence of 20 μM pentagalloylglucose; trace D, reduction by 40 μM NADH in the presence of 20 μM sanguin H-11; trace E, reduction by 40 μM NADH in the presence of 20 μM oolonghomobisflavan A.

ety of electron acceptors. Specific activity of the NADH-K₃ oxidoreductase of FP was 68 $\mu\text{mol/min/mg}$ of protein, and was about 30-fold higher than that of complex I. The NADH-Q₁ activity also appeared in FP, but the specific activity was about one-third of that of NADH-K₃. NADH-K₃ activity of FP was not inhibited by rotenone (50 μM). The ratio of activities of NADH-K₃ (completely resistant to rotenone) and the rotenone-insensitive part (about 20%, in Fig. 1) of NADH-Q₁ oxidoreductases of complex I, is similar to that of FP (about 3:1) as mentioned above. These findings suggest that the rotenone-insensitive NADH-Q₁ oxidoreductase activity in FP was not produced artificially during the preparation of FP, but is present in the original complex I. Pentagalloylglucose, oolonghomobisflavan A, and sanguin H-11 inhibited completely both activities of FP (data not shown), and the inhibition patterns of these inhibitors were all noncompetitive (Table 2). Sanguin H-11 was the most potent inhibitor among the three tannins. The sensitivity of FP to tannins was higher than that of complex I (Table 2). Furthermore, the K_i^{NADH} value of complex I to oolonghomobisflavan A was about twice that to pentagalloylglucose, but this ratio was reversed in FP. These discrepancies in the activities between complex I and FP have not been explained yet.

To locate the inhibitory site in FP, we carried out spectrophotometrical assay of the flavin of FP. In the absence of inhibitor, the reduced-minus-oxidized difference spectrum

was monitored at short intervals (10 s) after the addition of NADH. The shape of NADH reduced-minus-oxidized difference spectra of FP did not change between 20 s and 2 min, indicating steady state reduction during this period. As indicated in Fig. 3, about 20% of FMN was reduced in the presence of NADH in comparison with that reduced by sodium dithionite. The shape of the spectrum of NADH reduced enzyme in the presence of pentagalloylglucose was similar to that of the control enzyme, indicating that electrons are transferred to FMN and therefore, the inhibition site of pentagalloylglucose is downstream of FMN in terms of electron flow. On the other hand, in the presence of sanguin H-11 or oolonghomobisflavan A, the obvious peak in the difference spectrum was not detected, indicating that the electrons were not transferred to FMN and hence, the reaction sites of these two tannins may be present between the NADH-binding site and FMN. These results suggest that the inhibition sites of sanguin H-11 and oolonghomobisflavan A occur at a different site to the known inhibitors of the NDH-1 type enzyme which do not react with FP.

In summary, our results demonstrate that these three tannins are not only unique inhibitors of NDH-1 in mitochondria, but also that they might be useful tools to examine the electron flow mechanism around the sites of NADH and FMN.

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