Mode of Inhibitory Action of Δlac-Acetogenins, a New Class of Inhibitors of Bovine Heart Mitochondrial Complex I†

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ABSTRACT: We have revealed that Δlac-acetogenins, a new class of inhibitors of bovine heart mitochondrial complex I (NADH−ubiquinone oxidoreductase), act differently from ordinary inhibitors such as rotenone and piericidin A [Ichimaru et al. (2005) *Biochemistry 44*, 816−825]. Since a detailed study of these unique inhibitors might provide new insight into the terminal electron transfer step of the enzyme, we further characterized their inhibitory action using the most potent Δlac-acetogenin derivative (compound **1**). Unlike ordinary complex I inhibitors, **1** had a dose−response curve for inhibition of the reduction of exogenous short-chain ubiquinones that was difficult to explain with a simple bimolecular association model. The inhibitory effect of **1** on ubiquinol−NAD^+ oxidoreductase activity (reverse electron transfer) was much weaker than that on NADH oxidase activity (forward electron transfer), indicating a direction-specific effect. These results suggest that the binding site of **1** is not identical to that of ubiquinone and the binding of **1** to the enzyme secondarily (or indirectly) disturbs the redox reaction of ubiquinone. Using endogenous and exogenous ubiquinone as an electron acceptor of complex I, we investigated the effect of **1** in combination with different ordinary inhibitors on the superoxide production from the enzyme. The results indicated that the level of superoxide production induced by **1** is significantly lower than that induced by ordinary inhibitors probably because of fewer electron leaks from the ubisemiquinone radical to molecular oxygen and that the site of inhibition by **1** is downstream of that by ordinary inhibitors. The unique inhibitory action of hydrophobic Δlac-acetogenins may be closely associated with the dynamic function of the membrane domain of complex I.

NADH−ubiquinone oxidoreductase (complex I)[1] is the first energy-transducing enzyme of the respiratory chains of most mitochondria and many bacteria. It catalyzes the oxidation of NADH by ubiquinone, coupled to the generation of an electrochemical proton gradient across the membrane that drives energy-consuming processes such as ATP synthesis and flagella movement (*1*). Complex I is the most complicated multisubunit enzyme in the respiratory chain; e.g., the enzyme from bovine heart mitochondria is composed of 46 different subunits with a total molecular mass of about 1 MDa (*2*). Recently, the crystal structure of the hydrophilic domain (peripheral arm) of complex I from *Thermus thermophilus* was solved at 3.3 Å resolution (*3*). However, our knowledge about the functional and structural features of the membrane arm, such as the ubiquinone redox reaction, proton translocation mechanism, and mode of action of numerous specific inhibitors, is still highly limited (*4*, *5*).

Many structurally diverse inhibitors of complex I are known (*6*−*8*). With the exception of rhein (*9*) and diphenyleneiodonium (DPI) (*10*), which inhibit electron input into complex I, all inhibitors act at the terminal electron transfer step of the enzyme (*6*, *11*). Although these inhibitors are generally believed to act at the ubiquinone reduction site, there is still no hard experimental evidence to verify this possibility. Rather, a recent photoaffinity labeling study using azidoquinone suggested that the inhibitor binding site is not the same as the ubiquinone binding site (*12*). To begin with, both the number and the location of the ubiquinone binding site(s) remain controversial (*3*, *4*, *12*, *13*). On the other hand, mutagenesis studies using the yeast *Yarrowia lipolytica* and *Rhodobacter capsulatus* (*14*−*16*) and photoaffinity labeling studies (*17*, *18*) indicated that PSST, ND5, and 49 kDa subunits contribute to the inhibitor binding domain. Radioligand and fluorescent-ligand binding studies (*11*, *17*, *18*) showed that a certain marker ligand is displaced by numerous competitors (i.e., other complex I inhibitors). These findings are consistent with the model of inhibitor binding sites proposed by Okun et al. (*11*), wherein a wide variety of inhibitors share a common large binding domain with partially overlapping sites. It should, however, be realized that in these studies (*11*, *17*, *18*) the authors demonstrated that the binding of a certain marker ligand to the enzyme is

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[1] Abbreviations: AQ, 6-amino-4-(4-*tert*-butylphenethylamino)-quinazoline; complex I, mitochondrial proton-pumping NADH−ubiquinone oxidoreductase; complex II, succinate−ubiquinone oxidoreductase; complex III, cytochrome bc_1 complex; complex IV, cytochrome *c* oxidase; diethoxy-Q_2, 2,3-diethoxy-5-methyl-6-geranyl-1,4-benzoquinone; DPI, diphenyleneiodonium; EPR, electron paramagnetic resonance; Q_1, ubiquinone-1 (2,3-dimethoxy-5-methyl-6-prenyl-1,4-benzoquinone); Q_2, ubiquinone-2 (2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone); Q_{10}, endogenous ubiquinone-10; Q_nH_2, a reduced form of ubiquinone; SMP, submitochondrial particles.

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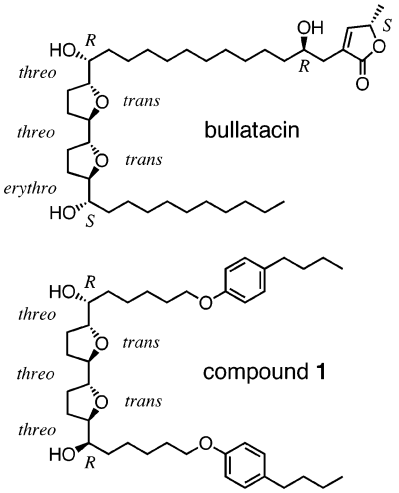

FIGURE 1: Structures of natural bullatacin and a representative Δlac-acetogenin (compound **1**).

completely suppressed in the presence of an excess amount of competitors. Under these experimental conditions, one cannot rule out the possibility that the competitors prevent binding of the marker ligand by inducing structural change in the enzyme, rather than by occupying the same site (*4*). Actually, several studies suggested that complex I undergoes dynamic conformational changes (*19*−*21*). Using a strong inhibitor with intense fluorescence [6-amino-4-(4-*tert*-butylphenethylamino)quinazoline (AQ)], Ino et al. suggested that the apparent competitive behavior among potent complex I inhibitors cannot be explained simply on the basis of competition for the same binding region (*22*). Thus it remains to be learned how binding sites of diverse complex I inhibitors relate to each other.

Acetogenins isolated from the plant family Annonaceae, such as bullatacin (Figure 1) and rolliniastatin-1, are among the most potent inhibitors of bovine heart mitochondrial complex I (*6*, *8*, *11*). We recently synthesized new acetogenin mimics named Δlac-acetogenins (*23*) that consist of the hydroxylated bis-THF ring and two hydrophobic side chains without a α,β-unsaturated γ-lactone ring, which is a structural feature common to a large number of natural acetogenins (Figure 1). Some Δlac-acetogenins elicited very potent inhibition of bovine complex I at the nanomolar level despite the lack of a γ-lactone ring. An electron paramagnetic resonance (EPR) spectroscopic study on the redox state of iron−sulfur clusters indicated that the inhibition site of Δlac-acetogenins is downstream of the iron−sulfur cluster N2 (*23*), as is the case for other ordinary complex I inhibitors such as rotenone and piericidin A (*6*, *11*). However, several lines of evidence, as summarized below, strongly suggested that the inhibition manner of Δlac-acetogenins is different from that of natural acetogenins as well as ordinary complex I inhibitors (*24*): (i) the profile of the structure−activity relationship of Δlac-acetogenins is entirely different from that of natural-type acetogenins, (ii) double-inhibitor titration shows that the inhibition by Δlac-acetogenins and bullatacin is not additive, (iii) unlike ordinary inhibitors, Δlac-acetogenins do not prevent the specific binding of AQ to complex I, and (iv) the level of superoxide production from complex I induced by Δlac-acetogenins is markedly lower than that induced by ordinary inhibitors.

Thus, Δlac-acetogenins were revealed to be a new type of inhibitor acting at the terminal electron transfer step of complex I. Since a detailed study of the inhibitory action of these unique inhibitors might provide new insight into the terminal electron transfer process, we further characterized their inhibitory effects in the present study. Our results strongly suggest that the binding site of Δlac-acetogenins is not identical to the binding site of ubiquinone and downstream of that of ordinary inhibitors.

EXPERIMENTAL PROCEDURES

Measurement of Complex I Activity. Bovine heart submitochondrial particles (SMP) were prepared by the method of Matsuno-Yagi and Hatefi (*25*) using a sonication medium containing 0.25 M sucrose, 1 mM succinate, 1.5 mM ATP, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 10 mM Tris-HCl (pH 7.4) and stored in a buffer containing 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4) at −84 °C. The NADH oxidase activity in SMP was followed spectrometrically with a Shimadzu UV-3000 (340 nm, $\epsilon = 6.2$ mM^{-1} cm^{-1}) at 30 °C. The reaction medium (2.5 mL) contained 0.25 M sucrose, 1 mM $MgCl_2$, and 50 mM phosphate buffer (pH 7.5). The final mitochondrial protein concentration was 30 μg of protein/mL. The reaction was started by adding 50 μM NADH after the equilibration of SMP with an inhibitor for 4 min. The inhibition of NADH−Q_1 and NADH−diethoxy-Q_2 oxidoreductase activities was determined under the same experimental conditions, except that the reaction medium contained 50 μM Q_1 (or 100 μM diethoxy-Q_2), 0.2 μM antimycin A, and 2 mM KCN.

The content of complex I was calculated as the minimal amount of bullatacin required to completely block the NADH oxidase activity since this compound is the most potent inhibitor of bovine complex I (*6*, *8*, *11*) and binds to the enzyme in a stoichiometric manner (*22*).

Measurement of Reverse Electron Transfer. Reverse electron transfer (ubiquinol−NAD^+ oxidoreductase activity) was generated by the oxidation of succinate and the hydrolysis of ATP (*26*). The reaction was measured spectrometrically by following the reduction of NAD^+ with a Shimadzu UV-3000 (340 nm, $\epsilon = 6.2$ mM^{-1} cm^{-1}) at 30 °C. The reaction medium (2.5 mL) contained 0.25 M sucrose, 7 mM sodium succinate, 6 mM $MgCl_2$, 1 mM KCN, 1 mM NAD^+, and 50 mM Tris-HCl (pH 7.5), and the final protein concentration of SMP was 0.1 mg of protein/mL. The reaction was started by the addition of 2 mM ATP after the equilibration of SMP with an inhibitor for 4 min. The activity was fully sensitive to SF6847 (protonophoric uncoupler) or oligomycin (ATP synthase inhibitor) (*26*).

Measurement of Superoxide Production. Superoxide production was determined at 30 °C by monitoring the superoxide-dependent oxidation of epinephrine to adrenochrome (*27*) with a Shimadzu UV-3000 spectrophotometer (485−575 nm, $\epsilon = 3.0$ mM^{-1} cm^{-1}) in a dual wavelength mode. The reaction medium (2.5 mL) contained 0.25 M sucrose, 1 mM epinephrine, 1 mM EDTA, 1 μM catalase, and 10 mM Tris-HCl buffer (pH 7.5). The reaction was started by adding 100 μM NADH after the equilibration of SMP with the test inhibitor for 4 min. The final protein concentration of SMP was 0.25 mg/mL. Superoxide dismutase (bovine liver) was used at a final concentration of 60 units/mL to give the assay specificity.

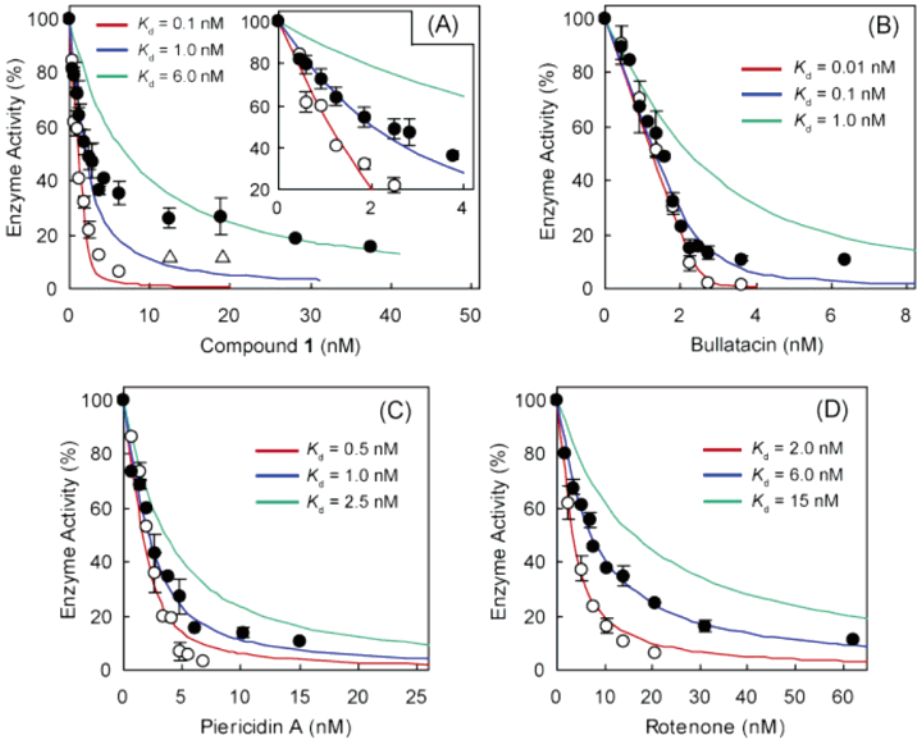

FIGURE 2: Dose−response curves for the inhibition of complex I. The inhibitors used are compound **1** (A), bullatacin (B), piericidin A (C), and rotenone (D). The open and closed circles show the inhibition of NADH oxidase and NADH−Q_1 oxidoreductase activities, respectively. The inset in (A) shows the dose−response curves in the lower concentration range. The open triangles in (A) show the enzyme activity after the addition of 100 nM rotenone or 20 nM piericidin A. Bars show means ± SD of at least three independent measurements. The averaged control NADH oxidase and NADH−Q_1 oxidoreductase activities are 0.55 and 0.58 μmol of NADH min^{-1} (mg of protein)$^{-1}$, respectively. The final mitochondrial protein concentration is 30 μg of protein/mL. The colored lines show the theoretical titration curves that are calculated on the basis of a simple bimolecular association model assuming tentative K_d values (see Supporting Information). The K_d values of the test inhibitors for NADH oxidase and NADH−Q_1 oxidoreductase activities were tentatively assumed to be as follows: for compound **1**, 0.1 and 1.0 nM; for bullatacin, 0.01−0.1 and 0.01−0.1 nM; for piericidin A, 0.5 and 1.0 nM; for rotenone, 2.0 and 6.0 nM, respectively.

Superoxide production was also determined in NADH−Q_1 and NADH−diethoxy-Q_2 oxidoreductase assays under the same experimental conditions, except that the reaction medium contained 100 μM Q_1 (or 100 μM diethoxy-Q_2), 0.8 μM antimycin A (Q_i site inhibitor), and 0.8 μM MOA-stilbene (Q_o site inhibitor).

Measurement of Membrane Potential Formation. The membrane potential generated by the oxidation of NADH was measured at 30 °C by following the changes in absorbance of oxonol VI at 630−601 nm with a Shimadzu UV-3000 in a dual wavelength mode (*28*). The reaction medium (2.5 mL) contained 0.2 M sucrose, 2.5 mM $MgCl_2$, 0.2 μM antimycin A, 2 mM KCN, 2.5 μM oligomycin, 0.1 μM nigericin, 50 μM Q_1, 1 μM oxonol VI, and 50 mM Tricine−KOH (pH 7.5), and the final protein concentration of SMP was 30 μg of protein/mL. The reaction was started by adding 50 μM NADH after the equilibration of SMP with an inhibitor for 4 min. The membrane potential-sensitive oxonol VI signal was transiently enhanced and entirely abolished by the addition of nigericin (K^+/H^+ exchanger) and valinomycin (K^+ ionophore), respectively.

Materials. Compound **1**, AQ, and diethoxy-Q_2 are the same samples as used previously (*22*, *24*, *28*). Piericidin A and bullatacin were generous gifts from Drs. S. Yoshida (The Institute of Physical and Chemical Research, Saitama, Japan) and J. McLaughlin (Purdue University, West Lafayette, IN), respectively. Oxonol VI was obtained from Invitrogen. Other chemicals were commercial products of analytical grade.

RESULTS

Inhibition of NADH−Q_1 Oxidoreductase Activity. In previous studies (*23*, *24*), we examined the effects of Δlac-acetogenins on complex I activity mainly using the NADH oxidase assay in SMP, wherein an endogenous ubiquinone (Q_{10}) serves as an electron acceptor of the enzyme. Since bovine heart complex I is unique in recognizing exogenous short-chain ubiquinones (*28*, *29*), we here examined the inhibitory effect of Δlac-acetogenins on the reduction of exogenous substrates. First, the inhibition of NADH−Q_1 oxidoreductase activity by compound **1** was examined. Compound **1** was used as representative of Δlac-acetogenins throughout the present study since it is the most potent of the Δlac-acetogenins synthesized in our laboratory (*23*, *24*).

The NADH−Q_1 oxidoreductase activity decreased almost linearly with an increase in the concentration of **1** to 50−60% of the control activity (Figure 2A). Interestingly, higher concentrations of **1** were needed to reduce the residual enzyme activity (40−50%), and maximal inhibition (∼95%) was achieved at over 80 nM. This feature is clearly noticeable when the dose−response curves are compared between NADH−Q_1 oxidoreductase and NADH oxidase activities (Figure 2A). The residual enzyme activity was sensitive to piericidin A and rotenone; that is, the residual activity further decreased to ∼10% by the addition of these inhibitors, as shown by open triangles in Figure 2A. It is well established

that 5−10% of NADH−Q_1 oxidoreductase activity is insensitive to ordinary complex I inhibitors due to nonphysiological electron transfer to Q_1 (*28*, *32*, *33*). However, the extent of the residual activity observed for **1** was significantly greater than that expected from nonphysiological electron transfer. The residual activity did not decrease with a prolongation of the incubation period up to 15 min (not shown). On the other hand, the dose−response curve for NADH−Q_1 oxidoreductase activity shifted slightly to a higher concentration range compared with the NADH oxidase assay, indicating a decrease in apparent inhibitor sensitivity. A similar phenomenon has been reported for ordinary inhibitors (*6*, *30*, and *31* and also described below), though the kinetic background remains to be solved.

As references, the dose−response curves of bullatacin, piericidin A, and rotenone for NADH oxidase and NADH−Q_1 oxidoreductase activities are shown in panels B, C, and D of Figure 2, respectively. In all cases, 5−10% of NADH−Q_1 oxidoreductase activity was insensitive to the inhibitors. Compared with the NADH oxidase assay, the dose−response curves of piericidin A and rotenone for the NADH−Q_1 oxidoreductase assay shifted to a higher concentration range, as mentioned above.

To interpret the large residual activity observed for **1**, we tried to analyze the dose−response curve by a theoretical binding model. The curved lines in Figure 2 represent the theoretical titration curves expected from a simple bimolecular association model with the assumption that the extent of inhibition (percent) is equal to the ratio of [enzyme−inhibitor complex]/$[\text{enzyme}]_{\text{total}}$ × 100; conversely, the residual enzyme activity (percent) is equal to the ratio of $[\text{enzyme}]_{\text{free}}/[\text{enzyme}]_{\text{total}}$ × 100 (Figure S1; see Supporting Information). Given a concentration of complex I of 2.5 nM (see Experimental Procedures), almost all measurement points of **1** for the NADH oxidase assay fit a simple binding model tentatively assuming the dissociation constant (K_d) to be 0.1 nM. On the other hand, the first 5−7 points of **1** with the NADH−Q_1 oxidoreductase assay seem to fit the model assuming the K_d value is 1.0 nM (inset in Figure 2A), whereas the points in the higher concentration range markedly deviated from the theoretical titration curve, suggesting heterogeneity of the inhibitor binding (or inhibition manner). It is noteworthy that the dose−response curves of all of the ordinary inhibitors, regardless of whether the NADH oxidase or NADH−Q_1 oxidoreductase assay was used, almost completely fit a simple binding isotherm, as shown in Figure 2B−D.[2]

We previously revealed that bulky diethoxy-Q_2, in which both methoxy groups in the 2- and 3-positions of Q_2 were replaced by an ethoxy group, serves as an efficient electron acceptor in bovine heart mitochondrial complex I, and its electron transfer activity is almost completely (∼95%) inhibited by piericidin A and rotenone (*28*). This finding suggests that the ubiquinone reduction site of bovine complex I is spacious enough to accommodate a bulky exogenous ubiquinone analogue. Taking into consideration that diethoxy-Q_2 or its reduced form (diethoxy-Q_2H_2) serves neither as an electron acceptor nor as an electron donor in succinate−ubiquinone oxidoreductase (complex II) and the cytochrome bc_1 complex (complex III), respectively (*34*), the finding that the bulky ubiquinone can serve as an electron acceptor solely in complex I may reflect a specific feature of the ubiquinone reduction site in the enzyme. Therefore, we also examined the effect of **1** as well as ordinary inhibitors on NADH−diethoxy-Q_2 oxidoreductase activity. The results were similar to those of the NADH−Q_1 assay, i.e., a shift in the dose−response curves of **1**, piericidin A, and rotenone toward a higher concentration range and heterogeneity of the dose−response curve solely for Δlac-acetogenin (Figure S2; see Supporting Information). Taken together, it is certain that the effect of Δlac-acetogenin on the reduction of exogenous short-chain ubiquinones is considerably different from that of ordinary inhibitors.

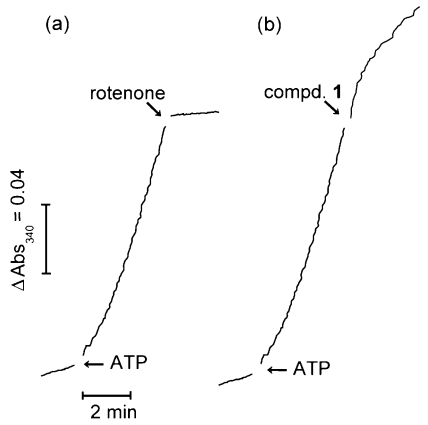

FIGURE 3: Effect of inhibitor on the reverse electron transfer in SMP. The traces (a, rotenone; b, compound **1**) depict the changes of absorbance at 340 nm associated with the reduction of NAD^+. The arrows indicate the addition of 2 mM ATP and 60 nM rotenone or **1**. The final mitochondrial protein concentration is 0.1 mg of protein/mL.

Inhibition of Reverse Electron Transfer (Ubiquinol−NAD^+ Oxidoreductase Activity). It has been established that complex I in intact mitochondria or in coupled SMP operates reversibly; that is, when energy is supplied by aerobic succinate oxidation or by ATP hydrolysis, complex I is able to catalyze the rotenone- and/or uncoupler-sensitive ubiquinol−NAD^+ oxidoreductase activity (*35*, 36). As the above results indicated that **1** perturbs the reduction of ubiquinone in a somewhat different manner from ordinary inhibitors, we next examined the inhibition of the reverse electron transfer by **1** to examine the effect on the oxidation of ubiquinol ($Q_{10}H_2$).

We preliminarily confirmed that the reverse electron transfer was completely suppressed by the addition of SF6847 (protonophoric uncoupler) or oligomycin (ATP synthase inhibitor). The reverse electron transfer was quickly and completely inhibited by the addition of rotenone (trace a in Figure 3) or bullatacin (not shown) at a concentration high enough to exhibit maximal inhibition of NADH oxidation (the forward electron transfer). In contrast, an excess amount of **1** did not quickly inhibit the reverse electron transfer and the inhibitory effect was markedly time-

[2] The K_d values of ordinary inhibitors presumed in the analyses in Figure 2 are lower than those experimentally obtained in ref *11* by a factor of 5−10. This discrepancy may be due to an experimental problem in the literature, wherein the K_d values of various complex I inhibitors were calculated on the basis of the competitive replacement test using two standard radioligands, [^{3}H]dihydrorotenone and [^{3}H]AE F119209. The K_d values of the radioligands were determined experimentally from Scatchard plots of the binding data (Figure 4 in ref *11*). It should be realized that in the experiments the variation in the [bound ligand]/[free ligand] ratio is too biased and narrow (from ∼0.01 to ∼0.1) to accurately estimate the K_d values of the standard ligands.

Table 1: Comparison of the Inhibitory Effect on the Forward (NADH Oxidase) and Reverse (Ubiquinol−NAD^+ Oxidoreductase) Electron Transfer Activities

inhibitor	IC_{50} values (pmol of inhibitor/mg of protein)[a]	
	forward electron transfer[b]	reverse electron transfer[b]
compound **1**	43 ± 4 (1)[c]	180 ± 21 (4)
bullatacin	46 ± 5 (1)[c]	28 ± 4 (1)
piericidin A	81 ± 7 (3)	48 ± 4 (2)
rotenone	130 ± 14 (4)	62 ± 8 (3)

[a] Values are means ± SD of three independent experiments. [b] The figures in parentheses show the ranking in order of inhibitory potency. [c] The IC_{50} values of **1** and bullatacin are regarded as identical.

dependent (trace b in Figure 3). Over 10 min was needed to achieve a final rate under the experimental conditions. To eliminate such a marked time dependency, SMP were preincubated with **1** for 4 min before the reverse electron transfer was started with the addition of ATP. This treatment almost completely eliminated the time dependency and enabled us to reproducibly carry out the inhibitor titration.

Since the reverse electron transfer is much slower than the forward electron transfer, a higher concentration of SMP was used in the former assay (0.1 vs 0.03 mg of protein/mL), resulting in an increase in volume of the membrane lipid phase as well as the concentration of complex I. Accordingly, as the amount of inhibitor trapped in the hydrophobic lipid bilayer differs between the assay systems, a direct comparison of the IC_{50} values between the two, in either molarity or moles of inhibitor per milligram of protein, is complicated. Alternatively, we compared the order of the inhibitory effect of the test compounds in terms of IC_{50} values (moles of inhibitor per milligram of protein), as listed in Table 1. Compound **1** was a more potent inhibitor than rotenone and piericidin A for the forward electron transfer but was the poorest inhibitor for the reverse electron transfer. In terms of inhibitory potency, the ranking of ordinary inhibitors was the same for both electron transfer events; bullatacin > piericidin A > rotenone. This order for the forward electron transfer was observed by different laboratories (*6*, *11*). It is therefore clear that the inhibitory effect of **1** on the reverse electron transfer is significantly weaker than that on the forward electron transfer, indicating a direction-specific effect. More experiments are needed to determine whether this phenomenon is associated with the possibility that different electron transfer pathways operate in the two reactions (*37*).

Kotlyar and Gutman (*38*) claimed that the forward electron transfer exhibits a much higher sensitivity to rotenone than the reverse electron transfer, but this would be due to an underestimation of the inhibitory effect of rotenone on the reverse electron transfer because of the significant time dependency of the inhibition under their experimental conditions (Figure 2 in ref *38*). Actually, we also confirmed a similar time dependency of the inhibition by rotenone with our SMP preparations. However, when SMP were preincubated with rotenone for 4 min before the reverse electron transfer, as described above, the time dependency disappeared and the difference in inhibitor sensitivity was much less significant than that reported in ref *38*.

Effect on Superoxide Production in NADH−Ubiquinone Oxidoreductase Activity. Superoxide is produced by the single electron reduction of oxygen by an electron carrier within the mitochondrial electron transport chain. The reductant of oxygen producing superoxide in complex I is not known, and published results are highly conflicting. One possible cause of this is that the superoxide generation from the enzyme varies greatly depending on the tissue, species, and experimental conditions (*39*−*41*). Lambert and Brand (*42*) suggested that the major site of superoxide production in complex I of intact mitochondria isolated from skeletal muscle of rat, determined as hydrogen peroxide production, is in the region of the ubiquinone reduction site on the basis of the observation that different complex I inhibitors such as rotenone and piericidin A and high concentrations of myxothiazol gave different rates of superoxide production during the forward electron transport. If the main or only site of superoxide production is upstream of the ubiquinone reduction site (i.e., iron−sulfur centers or FMN), then, for a simple linear chain of electron carriers (*3*), the addition of any inhibitor acting at or close to the ubiquinone reduction site should result in the same rate of superoxide production, but this was not the case (*42*). Using bovine heart SMP, we previously showed that the rate of superoxide production induced by an excess amount of Δlac-acetogenin in the NADH oxidase assay is about one-third to one-fourth of that induced by different ordinary inhibitors (ref *24* and reexamined using the present SMP preparations as shown in Figure 4A). This finding indicates that the effect of Δlac-acetogenins on the free radical intermediate in complex I is quite different from that of ordinary complex I inhibitors. Considering that the inhibition site of Δlac-acetogenins is downstream of iron−sulfur clusters (*23*), this result seems to support the idea proposed by Lambert and Brand (*42*), although the difference in the rate of superoxide production induced by ordinary inhibitors was less significant than that determined by these authors.

When a short-chain ubiquinone such as Q_1 is used as an electron acceptor of complex I in place of the endogenous ubiquinone (Q_{10}), the production of superoxide is remarkably enhanced since some of the short-chain ubiquinone added is reduced at nonphysiological ubiquinone reduction site and mediates one electron transfer to molecular oxygen (*43*, *44*). Since Δlac-acetogenin induces significantly less superoxide than ordinary inhibitors with the NADH oxidase assay in which Q_{10} acts as an electron acceptor (Figure 4A), it was of interest to investigate the effect of Δlac-acetogenin on the superoxide production when a short-chain ubiquinone is used as an electron acceptor.

Therefore, we measured the rates of superoxide production induced by **1** or ordinary inhibitors using Q_1 or diethoxy-Q_2 as an electron acceptor. To block superoxide production from complex III as well as the electron transfer activity of complex III, we added an excess amount of antimycin A (Q_i site inhibitor) and MOA-stilbene (Q_o site inhibitor) at the same time in this experiment. It should be mentioned that the concentrations of the test inhibitors were set to be high enough to achieve maximal inhibition (~90%) of the electron transfer activity. As shown in Figure 4B, the rates of superoxide production using short-chain ubiquinones, particularly Q_1, in the presence of an inhibitor were markedly greater than those obtained using endogenous ubiquinone (Figure 4A), being consistent with the previous study (*43*). The superoxide production observed with diethoxy-Q_2 was considerably less than that with Q_1 probably because more

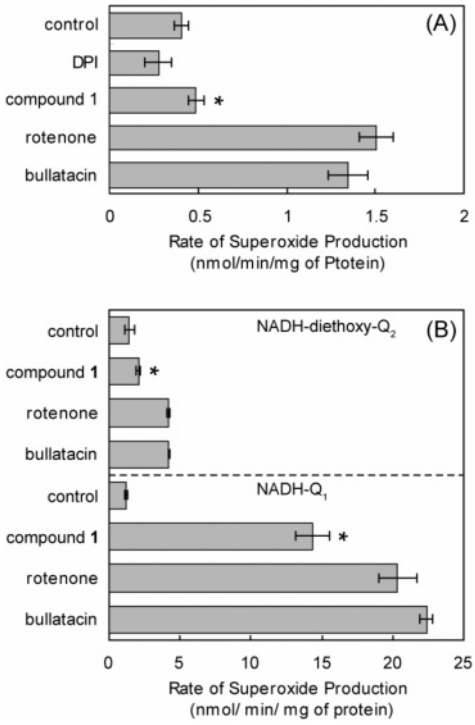

FIGURE 4: Superoxide production from complex I in the presence of inhibitor. (A) Rates of superoxide production in the NADH oxidase assay with SMP. Where indicated, 10 μM DPI, 1.2 μM compound **1**, 1.2 μM rotenone, or 1.2 μM bullatacin was added. The concentrations of all inhibitors were set high enough to achieve complete inhibition of NADH oxidase activity. (B) Rates of superoxide production in NADH−diethoxy-Q_2 (upper) and NADH−Q_1 (lower) oxidoreductase assays with SMP. The concentration of Q_1 and diethoxy-Q_2 was set at 100 μM. Where indicated, 1.2 μM compound **1**, 1.2 μM rotenone, or 1.2 μM bullatacin was added. The concentrations of all inhibitors were set high enough to achieve maximal inhibition (90−95%) of NADH−Q_1 and NADH−diethoxy-Q_2 oxidoreductase activity. The final mitochondrial protein concentration is 0.25 mg of protein/mL. The scale of the horizontal axis in the figure differs between (A) and (B). Bars show means ± SD of at least three independent measurements. *, significant difference ($p < 0.001$) versus rotenone and bullatacin.

hydrophobic diethoxy-Q_2 efficiently partitions into SMP and accepts electrons almost exclusively from the physiological ubiquinone binding site of complex I. Importantly, although the rates of superoxide production induced by ordinary inhibitors were almost comparable regardless of Q_1 or diethoxy-Q_2, the rate induced by **1** was significantly smaller than that induced by ordinary inhibitors, as observed with the NADH oxidase assay (Figure 4A). The difference may not be explained by a difference in the electron transfer process from iron−sulfur clusters (or FMN) to molecular oxygen mediated by the short-chain ubiquinones since the redox status of iron−sulfur clusters (or FMN) should be comparable as the ubiquinone reduction at the physiological binding site is completely blocked in both cases. It is therefore likely that the difference in superoxide production is attributable to that originating from the physiological ubiquinone reduction site. That the degree of difference in superoxide production between **1** and ordinary inhibitors differs depending on the ubiquinone used (Q_1 > diethoxy-Q_2 > Q_{10}) would support this notion. On the other hand, the difference in superoxide production between the control and **1** might be primarily due to the electron leak from the radical intermediate(s) located upstream of the ubiquinone reduction

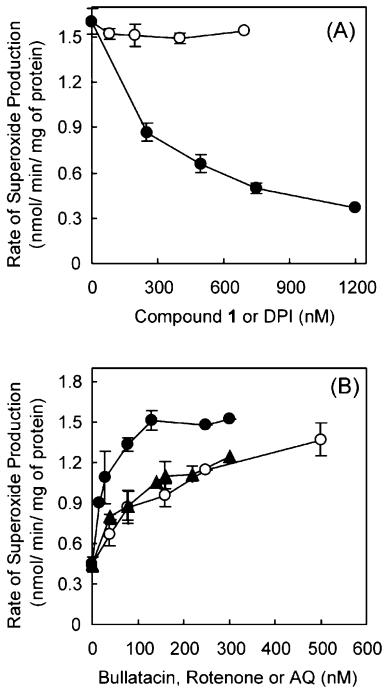

FIGURE 5: Superoxide production in the NADH oxidase assay with SMP. (A) Effect of increasing concentrations of DPI (closed circles) or **1** (open circles) on the superoxide production induced by 130 nM bullatacin. The concentration of bullatacin was high enough to induce maximal superoxide production as well as maximal inhibition of NADH oxidase activity. (B) Rate of superoxide production with increasing concentrations of bullatacin (closed circles), rotenone (open circles), or AQ (closed triangles) in the presence of 200 nM **1**. The concentration of **1** was high enough to induce maximal superoxide production as well as maximal inhibition of NADH oxidase activity. The final mitochondrial protein concentration is 0.25 mg of protein/mL. Bars show means ± SD of three independent measurements.

site. The order of the difference is also Q_1 > diethoxy-Q_2 > Q_{10}.

Effect on Superoxide Production in NADH Oxidase Activity. The previous study (*24*) and the above results certainly revealed that the superoxide production induced by Δlac-acetogenin is significantly less than that by ordinary inhibitors regardless of whether endogenous or exogenous ubiquinone is used as an electron acceptor. On the basis of this finding, we thought that an analysis of the effect of Δlac-acetogenin on superoxide production in combination with ordinary inhibitors may provide a clue as to how the binding sites of complex I inhibitors relate to each other. If the inhibition site of Δlac-acetogenins is upstream of ordinary inhibitors, the rate of superoxide production induced by ordinary inhibitors in NADH oxidation will be reduced in the presence of Δlac-acetogenin since the electron flow to the reaction site, where ordinary inhibitors block the electron transfer event, is reduced by Δlac-acetogenin (*42*, *45*). However, if Δlac-acetogenins act downstream of ordinary inhibitors, the superoxide generation induced by ordinary inhibitors will not be affected since the electron flow into the inhibitor binding site is not restricted by Δlac-acetogenin.

As a control, the superoxide production induced by bullatacin in combination with DPI, which is known to inhibit the electron input into complex I acting at FMN (*10*), was examined (Figure 5A). In this experiment, the concentration of bullatacin (130 nM) was set high enough to achieve

maximal superoxide production as well as maximal inhibition of the NADH oxidase activity. Expectedly, the rate of superoxide production induced by bullatacin was reduced by DPI in a concentration-dependent manner. At very high concentrations of DPI, the rate was the same as that with DPI alone, as shown in Figure 4A. In contrast, the superoxide production induced by bullatacin was not affected in the presence of **1** even at markedly excess concentrations (Figure 5A). It should be noted that **1** exhibited maximal inhibition of NADH oxidase activity at about 50 nM under the experimental conditions. Similar results were observed for rotenone (Figure S3; see Supporting Information). In these experiments, it is unlikely that **1** displaced bullatacin (or rotenone) from the enzyme since if these inhibitors were to be replaced by an increasing amount of **1**, the rate of superoxide production would gradually decrease to the level induced by **1** alone, but this was not the case.

Next we measured the rate of superoxide production induced by increasing the concentration of bullatacin, rotenone, or AQ in the presence of **1** (200 nM). In this experiment, 200 nM **1** was enough to induce maximal superoxide production. As shown in Figure 5B, **1** did not significantly affect the concentration dependency of superoxide production induced by these inhibitors. For a reference, the titrations obtained without **1** are shown in Figure S4 (see Supporting Information). Under the experimental conditions, it is also likely that the ordinary inhibitors did not displace **1** from the enzyme since the binding affinity of **1** in the NADH oxidase assay is comparable to or slightly higher than that of the inhibitors (Figure 2), and a high concentration of **1** (200 nM) was used. Actually, using AQ with intense fluorescence, we have revealed that **1** and AQ do not displace each other in bovine complex I (*24*). It is therefore certain that both **1** and the ordinary inhibitor bind to the enzyme at the same time under the experimental conditions. Taken together, the above results indicate that the effect of ordinary inhibitors on the free radical intermediate in the forward electron transfer is not influenced by Δlac-acetogenin. The most straightforward explanation for this finding is that the binding site of Δlac-acetogenin is downstream of that of the ordinary inhibitors.

Effect on Proton Translocation by Complex I. The proton translocation by complex I is tightly coupled, either directly or indirectly (*19*, *46*−*48*), with the ubiquinone redox reaction. Since the present study revealed that the effects of Δlac-acetogenin on the redox reaction of both endogenous and exogenous ubiquinones are considerably different from those of ordinary inhibitors, it may not be strange to consider the possibility that Δlac-acetogenin disturbs the tight coupling between the ubiquinone reduction and the proton translocation, i.e., a so-called decoupling effect, which occurs in complexes III and IV (cytochrome *c* oxidase) due to chemical modification by *N*,*N*′-dicyclocarbodiimide (DCCD) (*4*, *49*, *50*). We investigated therefore the effect of **1** on the membrane potential formation induced by complex I, which was monitored as changes in absorbance of oxonol VI and compared it with the effect on the electron transfer activity using bullatacin as a reference. To eliminate the contribution of the proton pumping activity of complexes III and IV, we measured the membrane potential generated by NADH−Q_1 oxidoreductase activity. In preliminary experiments, we confirmed that the oxonol VI signal generated by NADH

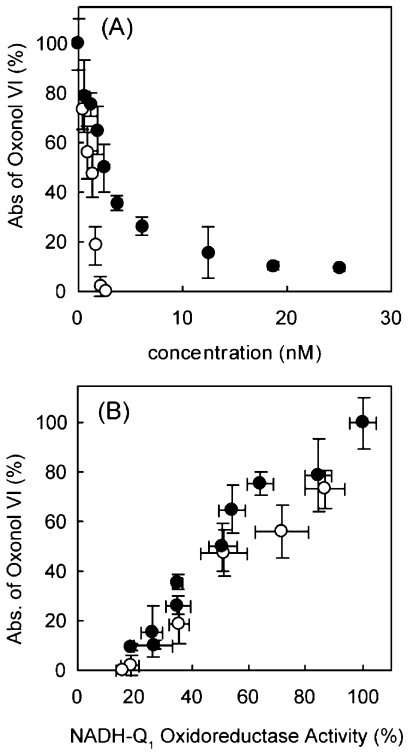

FIGURE 6: Dose−response curves for the inhibition of membrane potential formation by complex I. The NADH−Q_1 oxidoreductase assay was used to monitor the membrane potential formation. (A) Inhibition of membrane potential formation by **1** (closed circles) and bullatacin (open circles), respectively. (B) Correlation between the changes in electron transfer activity and in membrane potential formation for **1** (closed circles) and bullatacin (open circles). The data on the electron transfer activity were taken from Figure 2A,B.

oxidation was transiently enhanced and entirely abolished by the addition of nigericin (K^+/H^+ exchanger) and valinomycin (K^+ ionophore), respectively. For both **1** and bullatacin, the relationship between the membrane potential formation and the concentration of inhibitor was comparable to that seen in the inhibition of electron transfer activity (Figure 6A vs Figure 2A,B). Namely, for bullatacin, an almost linear dose−response curve was observed, and the nonphysiological electron transfer (5−10% of the control activity) generated no membrane potential (*33*). In contrast, high concentrations of **1** were needed to abolish residual (∼40%) membrane potential. To see the correlation between the changes in electron transfer activity and in membrane potential formation, the two parameters were plotted in Figure 6B. The relationship between the two parameters was almost the same for **1** and bullatacin. Therefore, it is reasonable to conclude that the decoupling effect of Δlac-acetogenin is negligibly small, if any. Yagi (*51*) reported that the proton translocation and electron transfer activity in the NADH−Q_1 oxidoreductase assay in bovine complex I are inhibited in parallel by DCCD.

DISCUSSION

To obtain new insight into the terminal electron transfer step of bovine mitochondrial complex I, we herein characterized the inhibitory action of Δlac-acetogenins in more detail. The present study indicates that the inhibitory

effects of Δlac-acetogenin on the redox reaction of both endogenous and exogenous ubiquinones are considerably different from those of ordinary inhibitors. Notably, the inhibition of the reduction of exogenous ubiquinones by **1** is difficult to explain with a simple bimolecular association model. Taking into consideration that Q_1 and diethoxy-Q_2 are efficient electron acceptors from the physiological quinone reduction site (*28*), these results strongly suggest that the binding site of Δlac-acetogenin is not identical to that of ubiquinone and that the binding of Δlac-acetogenin to the enzyme *secondarily* (or *indirectly*) affects the reduction of the ubiquinones. On the basis of Lineweaver−Burk plots of the kinetic data on NADH−Q_1 oxidoreductase activity, we previously suggested that Δlac-acetogenins inhibit complex I in a competitive manner against Q_1 (*23*). However, this idea has to be revised since the problem inherent to the studies employing Michaelis−Menten-type kinetics (*6*, *31*, *32*, *37*, *52*) is that the physicochemical properties of the ubiquinones, the inhibitors, and the membrane-bound enzyme as well as the complexity of underlying catalytic mechanism make interpretation of the kinetic data difficult and ambiguous. In particular, hydrophobic ubiquinones and inhibitors remarkably accumulate in the hydrophobic lipid phase of the inner mitochondrial membrane, making the actual concentrations at the reaction sites difficult to determine.

We analyzed the rate of superoxide production as an index to obtain a clue as to the inhibition site of Δlac-acetogenin. Our results clearly showed that the superoxide production induced by Δlac-acetogenin, regardless of whether endogenous or exogenous ubiquinone is used, is significantly less than that induced by ordinary inhibitors (Figure 4). The difference in the superoxide production induced by different ordinary inhibitors in the NADH oxidase assay is less significant than that reported by Lambert and Brand (*42*) (see Figure 4A in the present study and Figure 4 in ref *24*). It is reasonable to interpret our results to mean that different ordinary inhibitors induce superoxide at almost the same rate, as reported by Genova et al. (*43*). While the aim of the present study was not to identify the major site of superoxide generation in complex I, our results may provide information about the superoxide generated from ubisemiquinone. When the ubiquinone reduction in complex I is fully blocked by Δlac-acetogenin or ordinary inhibitors, the extent to which electrons accumulate in the cofactors located in the peripheral arm should be comparable. Therefore, the difference in superoxide generation may not be attributable to a difference in the electron leak from the cofactors to molecular oxygen. Taking into consideration the finding that the effect of Δlac-acetogenin on the reduction of ubiquinone is considerably different from that of ordinary inhibitors, it is highly likely that blocking of the reduction of ubiquinone by Δlac-acetogenin induces less electron leak from ubisemiquinone to molecular oxygen than the ordinary inhibitors do. Genova et al. (*43*) claimed that the ubisemiquinone radical is not a candidate for a direct electron donor to molecular oxygen in the presence of an ordinary complex I inhibitor since rotenone remarkably diminishes the formation of ubisemiquinone radical as revealed by EPR spectroscopy (*53*). However, one cannot exclude the possibility that destabilized ubisemiquinone, which may be EPR-undetectable under the experimental conditions, and/or the residual ubisemiquinone serves as an electron mediator to molecular oxygen (*42*, *45*). Yano et al. (*54*) showed that two ubisemiquinones, SQ_{Nf} and SQ_{Ns}, in complex I exhibit markedly different specificities to complex I inhibitors.

Importantly, Δlac-acetogenin did not influence the effect of ordinary inhibitors on the free radical intermediate producing superoxide in the NADH oxidase assay (Figure 5). On the basis of the scenario that the major site of superoxide production in the presence of an ordinary inhibitor is the ubiquinone reduction site, we interpret the results to mean that the inhibition site of Δlac-acetogenin is downstream of that of the ordinary inhibitors. It should be mentioned that even if the major site of superoxide production was upstream of the ubiquinone reduction site (i.e., FMN or iron−sulfur clusters), we might reach the same conclusion because our results indicate that the action site of Δlac-acetogenin does not exist between the radical intermediate and ordinary inhibitors in a simple linear chain of electron carriers.

Currently, there is little direct evidence for any mechanism of proton transduction in complex I, but it became recognized that some transmembraneous subunits may be implicated in the proton translocation. For instance, on the basis of single particle analysis of electron micrographs of *Escherichia coli* complex I, Sazanov's group (*55*, *56*) proposed a model of the arrangement of subunits in the bacterial complex I in which the transmembraneous subunits NuoL (ND5 in bovine) and NuoM (ND4) are located at the distal end of the membrane arm, spatially separated from the peripheral arm where all the known cofactors are located. Taking into consideration the proposed quinone binding sequence (L-X_3-H-X_3-T/S) (*57*) as well as the functional importance of these subunits (*12*, *18*), the authors also suggested that these subunits are involved in the proton pumping through long-range conformational interaction with the peripheral arm (*55*, *56*). On the other hand, structure−activity studies of a series of Δlac-acetogenins revealed that large and symmetrical hydrophobicity of both alkyl side chains is crucial for exhibiting potent inhibitory effect (*23*, *58*). This means that Δlac-acetogenins bind to the hydrophobic membrane arm of complex I, and the physicochemical properties of the side chain decide the precise location of the hydrophilic bis-THF ring moiety at or close to the membrane interface. The present study strongly suggests that the inhibition site of Δlac-acetogenin is downstream of that of ordinary inhibitors. At first, this notion seems to be inconsistent with the generally accepted idea that ordinary inhibitors such as rotenone and piericidin A inhibit the *terminal* electron transfer step of complex I (i.e., the ubiquinone reduction), which might take place in the membrane arm. Nevertheless, taking into consideration the proposed dynamic function of the membrane domain (*55*, *56*), it may not be strange that there are diverse chemicals that disturb the function of the membrane domain differently depending on their structural specificity.

In conclusion, Δlac-acetogenins appear to be unique molecular probes with which to investigate the terminal electron transfer step of bovine complex I. To fully understand the inhibitory action of these inhibitors, identification of the binding site is necessary. To this end, a photoaffinity-labeling study is currently underway in our laboratory.

SUPPORTING INFORMATION AVAILABLE

Figures S1−S4 as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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