The ND1 Subunit Constructs the Inhibitor Binding Domain in Bovine Heart Mitochondrial Complex I[†]

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ABSTRACT: The inhibitor binding domain in bovine complex I is believed to be constructed by multisubunits, but it remains to be learned how the binding positions of chemically diverse inhibitors relate to each other. To get insight into the inhibitor binding domain in complex I, we synthesized a photoreactive acetogenin [[^{125}I](trifluoromethyl)phenyldiazirinylacetogenin, [^{125}I]TDA], in which an aryldiazirine group serves as both a photoreactive group and a substitute for the γ-lactone ring that is a common toxophore of numerous natural acetogenins, and carried out photoaffinity labeling to identify the labeled subunit using bovine heart submitochondrial particles (SMP). When SMP were UV-irradiated in the presence of [^{125}I]TDA, radioactivity was predominantly incorporated into an ∼30 kDa band on a SDS gel. Blue native gel electrophoresis of the [^{125}I]TDA-labeled SMP revealed that the majority of radioactivity was observed in complex I. Analysis of complex I on a SDS gel showed a predominant peak of radioactivity at ∼30 kDa. Immnoprecipitation of the [^{125}I]TDA-labeled complex I with anti-bovine ND1 antibody indicated that the labeled protein is the ND1 subunit. A variety of complex I inhibitors such as piericidin A and rotenone efficiently suppressed the specific binding of [^{125}I]TDA to ND1, indicating that they share a common binding domain. However, the suppression efficiency of Δlac-acetogenin, a new type of complex I inhibitor synthesized in our laboratory, was much lower than that of the traditional inhibitors. Our results unequivocally reveal that the ND1 subunit constructs the inhibitor binding domain, though the contribution of this subunit has been challenged. Further, the present study corroborates our previous proposition that the inhibition site of Δlac-acetogenins differs from that of traditional inhibitors.

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 45 different subunits with a total molecular mass of about 1 MDa (*2*). Recently, the crystal structure of the hydrophilic domain (peripheral arm) of *Thermus thermophilus* complex I, where all of the known cofactors of the enzyme reside, 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*).

In a historical point of view, detailed studies on the action mechanism of specific inhibitors of respiratory enzymes have provided valuable insights into the function of the enzymes. Many structurally diverse inhibitors of complex I are known (*6*−*8*). With the exception of rhein (*9*) and diphenyleneiodonium (*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 notion. Rather, photoaffinity labeling studies using photoreactive ubiquinones suggested that the inhibitor binding site is not the same as the ubiquinone binding site (*12*, *13*). Earlier studies with two rotenone-derived photoreactive probes located a single inhibitor binding site in the ND1 subunit (*14*, *15*). Recent photoaffinity labeling studies demonstrated that pyridaben and fenpyroximate specifically bind to the PSST and ND5 subunits, respectively (*16*, *17*). On the other hand, mutagenesis studies using the yeast *Yarrowia lipolytica* (*18*, *19*) and *Rhodobacter capsulatus* (*20*) indicated that PSST and 49 kDa

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[1] Abbreviations: AQ, 6-amino-4-(4-*tert*-butylphenethylamino)-quinazoline; CBB, Coomassie brilliant blue; complex I, mitochondrial proton-pumping NADH−ubiquinone oxidoreductase; EM, electron microscopy; EPR, electron paramagnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SMP, submitochondrial particles; TDA, (*t*rifluoromethyl)phenyl*d*iazirinyl*a*cetogenin; [^{125}I]TDA, ^{125}I-labeled TDA; THF, tetrahydrofuran.

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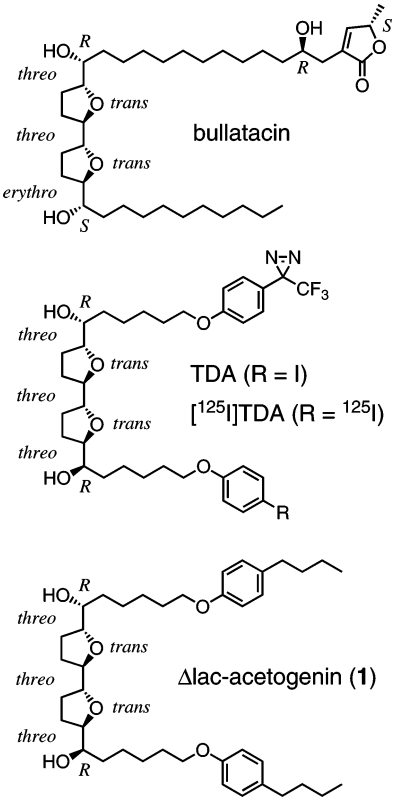


FIGURE 1: Structures of bullatacin, photoaffinity probe TDA (its ^{125}I-labeled derivative, [^{125}I]TDA), and compound **1**. Compound **1** was used as a representative of Δlac-acetogenins.

subunits contribute to the inhibitor binding domain. Therefore, the subunits PSST, 49 kDa, ND1, and ND5 are believed to construct the inhibitor binding pocket in complex I.

To find a clue to the inhibitor binding site(s), it is noteworthy that the binding of a radioactive or fluorescent inhibitor to complex I was suppressed by numerous other complex I inhibitors, i.e., competitors (*11*, *16*, *17*). On the basis of these observations, it is believed that 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*, *16*, *17*) the authors showed that the binding of a certain marker ligand to the enzyme is completely suppressed in the presence of a largely 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 (*21*−*23*). Using a strong quinazoline-type inhibitor (AQ) with intense fluorescence, Ino et al. suggested that the apparent competitive behavior among complex I inhibitors cannot be explained simply on the basis of competition for the same binding region (*24*). Thus it remains to be learned how binding positions of chemically diverse 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*). Structure−activity relationship studies for acetogenins showed that the crucial toxophores of these inhibitors are the α,β-unsaturated γ-lactone and hydroxylated tetrahydrofuran (THF) moieties (*25*, *26*). From a synthetic point of view, introduction of a photoreactive group such as aryldiazirine and arylazido groups into the alkyl side chain is not difficult, but this position is remote from the toxophores and may be too flexible to fix the position of photoreactive group in the enzyme (see Figure 1). On the other hand, introduction of some photoreactive group into the toxophore portions is not desirable because such a drastic structural modification may result in a large loss of the activity. It would be ideal if some photoreactive substructure can substitute for the toxophores retaining their functions. In this respect, it is noteworthy that the natural γ-lactone ring itself is not essential for the activity and can be replaced by other substructures such as quinone and heterocyclic rings (*27*−*29*).

In the present study, we designed and synthesized an acetogenin mimic [[^{125}I](*t*rifluoromethyl)phenyl*d*iazirinyl*a*cetogenin, [^{125}I]TDA in Figure 1] that possesses a photoreactive phenyldiazirine in place of the γ-lactone ring. Since [^{125}I]TDA worked as an acetogenin mimic and maintained potent inhibitory activity at the nanomolar level, it became a good candidate for the photoaffinity probe. With [^{125}I]TDA in hand, we carried out photoaffinity labeling to identify the labeled subunit in bovine heart complex I using submitochondrial particles (SMP). Our results demonstrated that the ND1 subunit constructs the inhibitor binding domain. Further, the present study corroborated our previous proposition that the inhibition site of Δlac-acetogenin differs from that of ordinary inhibitors.

EXPERIMENTAL PROCEDURES

Materials. 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. Compound **1** is the same sample as used previously (*30*).

Measurement of Complex I Activity. Bovine heart SMP were prepared by the method of Matsuno-Yagi and Hatefi (*31*) 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 spectrophotometrically with a Shimadzu UV-3000 (340 nm, $\epsilon = 6.2$ mM^{-1} cm^{-1}) at 25 °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.

Measurement of Superoxide Production. Superoxide production was determined at 25 °C by monitoring the superoxide-dependent oxidation of epinephrine to adrenochrome (*32*) 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.

Measurement of Reverse Electron Transfer. Reverse electron transfer (ubiquinol−NAD^+ oxidoreductase activity) was generated by the oxidation of succinate and the hydrolysis of ATP (*33*). The reaction was measured spectrophotometrically by following the reduction of NAD^+ with a Shimadzu UV-3000 (340 nm, $\epsilon = 6.2$ mM^{-1} cm^{-1}) at 25 °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) (*33*).

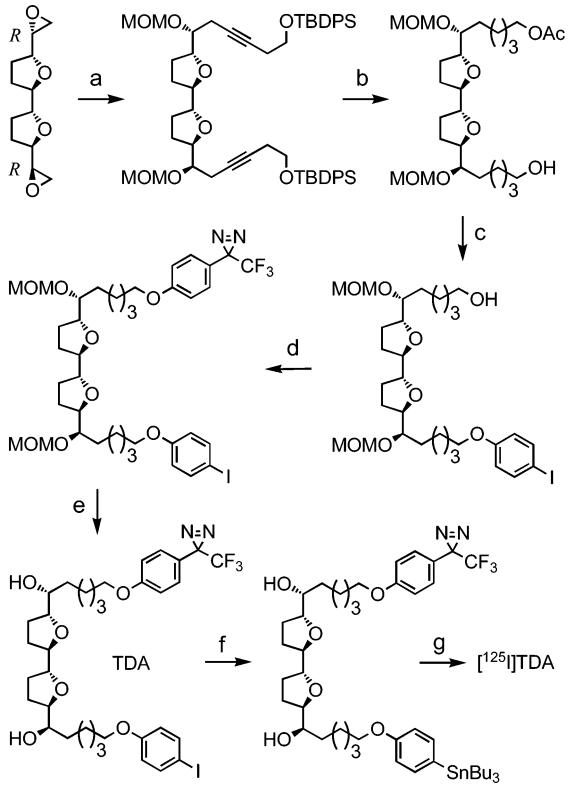
Photoaffinity Labeling of Bovine SMP. Bovine SMP (0.3−0.5 mg of protein/mL) in 100 μL of buffer containing 250 mM sucrose, 1 mM $MgCl_2$, and 50 mM phosphate (pH 7.4) were treated with [^{125}I]TDA (1−110 nM) in 1.5 mL Eppendorf tubes and incubated for 10 min at room temperature. The samples were then irradiated with a long-wavelength UV lamp (Black-Ray model B-100A; UVP) for 10 min on ice at a distance of 10 cm from the light source. When the replacement test was carried out, a competitor (i.e., other inhibitors) was added and incubated for 10 min at room temperature prior to the treatment with [^{125}I]TDA.

Electrophoresis. SDS−PAGE was performed according to Laemmli (*34*). Briefly, [^{125}I]TDA-labeled SMP samples were added to 4× sample buffer and incubated at 35 °C for 1 h to prevent protein aggregation. These denatured samples were separated on 10−20% gradient gels or 12.5% isocratic gels (90 × 83 × 1 mm). About 4−6 μg of protein was loaded to each well. Following electrophoresis, gels were fixed, stained with CBB R-250, dried, and exposed to an imaging plate (BAS-MS2040; Fuji Film) for 12−48 h. The migration pattern of radiolabeled proteins was visualized by a Bio-Imaging Analyzer FLA-2000 (Fuji Film). For localization and analysis of labeled protein bands, the gels were cut into 2 mm slices, and radioactivity was quantified using the γ-counting system (Packard, MINAXI γ-5550).

Blue native PAGE (BN-PAGE) was performed according to Schägger and von Jagow (*35*). Radiolabeled SMP samples were solubilized with 1% *n*-dodecyl β-D-maltoside, treated with sample buffer containing Serva Blue G (CBB G-250; Serva), and separated on 5−15% gradient gels (160 × 180 × 1 mm). Ferritin dimer (880 kDa) and monomer (440 kDa) were used as molecular mass markers. The position of complex I was located by activity staining using the NADH-NBT (nitroblue tetrazolium) system (*36*). These blue native gels were also subjected to autoradiography by the same procedure as those for SDS gels.

Isolation of [^{125}I]TDA-Labeled Complex I. [^{125}I]TDA-labeled complex I was isolated by BN-PAGE and electroelution (*37*). Briefly, solubilized [^{125}I]TDA-SMP were concentrated to appropriate protein content using Microcon (Millipore) and separated on 6% isocratic blue native gels (160 × 180 × 2 mm). The complex I band was excised and electroeluted using Centrilutor (Millipore) with an elution buffer containing 25 mM Tricine and 7.5 mM Bistris (adjusted with HCl to pH 7.0) overnight in a cold room. Complex I thus obtained was subjected to SDS−PAGE analysis, western blotting, or immunoprecipitation.

Scheme 1[a]



[a] Reagents and conditions: (a) (i) 4-(*tert*-butyldiphenylsilyloxy)-1-butyne, *n*-BuLi, $BF_3{\cdot}Et_2O$, −78 °C, 30 min, 92%, (ii) chloromethyl methyl ether, $(i\text{-Pr})_2NEt$, CH_2Cl_2, room temperature, overnight, 85%; (b) (i) H_2, 10% Pd/C, EtOH, room temperature, overnight, (ii) $(n\text{-Bu})_4NF$, THF, 0 °C, 1.5 h, 65% (two steps), (iii) acetyl chloride (1.1 equiv), $(Et)_3N$, 4-(dimethylamino)pyridine, CH_2Cl_2, 0 °C, 1 h, 46%; (c) (i) 4-iodophenol, PPh_3, diisopropylazodicarboxylate, THF, room temperature, 1 h, (ii) K_2CO_3, MeOH, room temperature, 3 h, 57% (two steps); (d) 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenol, PPh_3, diisopropylazodicarboxylate, THF, room temperature, 30 min; (e) 4% acetyl chloride in MeOH, CH_2Cl_2, room temperature, 1.5 h, 51% (two steps); (f) hexabutylditin, $Pd(CH_3CN)_2Cl_2$, HMPA, room temperature, overnight, 43%; (g) [^{125}I]NaI (74 TBq/mmol), Chloramine T, aqueous NaH_2PO_4 (0.2 M).

Immunoprecipitation. Immunoprecipitation of electroeluted complex I was performed as described previously (*16*, *38*). Briefly, electroeluted complex I was solubilized with 1.25% SDS. To the solubilized complex I solution (60 μL) was added dilution buffer (240 μL, 1.25% Triton X-100, 190 mM NaCl, 6 mM EDTA, 0.1 mM PMSF, 60 mM Tris-HCl, pH 7.4). The resulting mixture (300 μL) was treated with bovine ND1 antibody (5 μL) and rotated overnight in a cold room. Protein A−Sepharose CL-4B (30 μL; GE healthcare; equilibrated with 50 mM Tris-HCl) was added, and the suspension was rotated at room temperature for 2 h. The incubated beads were washed four times with 1 mL of buffer A (0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 5 mM EDTA, 0.1 mM PMSF, 50 mM Tris-HCl, pH 7.4) and then once with 1 mL of buffer B (buffer A without Triton X-100 and SDS). The resulting pellet was treated with SDS−PAGE sample buffer and centrifuged. The supernatant was subjected to electrophoresis and autoradiography.

RESULTS

Synthesis and Characterization of Photoreactive Acetogenin (TDA). Photoreactive (trifluoromethyl)phenyldiaziri-

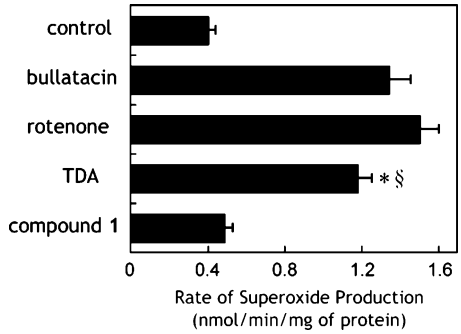


FIGURE 2: Superoxide production from complex I induced by various inhibitors. The rate of superoxide production was measured with NADH oxidase assay in SMP (0.25 mg of protein/mL). The concentration of all inhibitors (1.2 μM) was set high enough to achieve complete inhibition of NADH oxidase activity. Data are the mean of three independent measurements ± SD. *, $p < 0.01$ compared with compound **1**; §, not significant for TDA vs bullatacin.

nylacetogenin (TDA) and its ^{125}I-labeled derivative ([^{125}I]-TDA, 74 TBq/mmol) were synthesized according to the procedures outlined in Scheme 1. The synthetic details and the spectral data for the compounds are described in the Supporting Information. The inhibitory potency of TDA was examined with NADH oxidase activity in SMP (30 μg of protein/mL). The IC_{50} values of TDA and bullatacin were 1.3 (±0.09) and 0.90 (±0.05) nM, respectively, indicating that TDA retains potent inhibitory activity.

We initially designed TDA as a photoreactive derivative of Δlac-acetogenins, a new type of complex I inhibitors developed in our laboratory (*26*, *30*, *39*). One structural feature of Δlac-acetogenins is that they have two hydrophobic side chains attached to the hydroxylated bis-THF ring but have no γ-lactone ring in a terminal of the chain, as shown in Figure 1 taking compound **1** as an example. Unexpectedly, TDA turned out to work as a derivative of *ordinary acetogenins* from the following three observations. First, the most characteristic nature of the inhibitory effects of Δlac-acetogenins is that they induce superoxide production from complex I at a much lower rate than ordinary inhibitors do (*39*). We therefore measured the level of superoxide production from complex I in NADH oxidase assay. The concentrations of all inhibitors were set high enough to achieve complete inhibition of NADH oxidase activity. As shown in Figure 2, the rate of superoxide production induced by TDA was comparable to that of bullatacin and rotenone. The superoxide production induced by compound **1** (IC_{50} = 0.95 nM) was markedly lower than that induced by ordinary inhibitors. Second, the inhibitory effect of Δlac-acetogenins on ubiquinol−NAD^+ oxidoreductase activity (reverse electron transfer) is much weaker than that on NADH oxidase activity (forward electron transfer) (*39*). We confirmed that the reverse electron transfer was quickly and completely inhibited by the addition of TDA (trace A in Figure 3) at a concentration high enough to exhibit maximal inhibition of NADH oxidation. In contrast, an excess amount of **1** did not quickly inhibit the reverse electron transfer, and the inhibitory effect was markedly time-dependent (trace B in Figure 3). Third, double-inhibitor titration showed that the inhibition by a pair of TDA and bullatacin was additive (data not shown), suggesting that the inhibition sites of the two compounds are identical to each other.

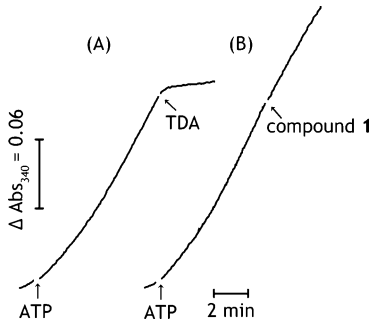


FIGURE 3: Effect of inhibitor on the reverse electron transfer in SMP. The traces (A, TDA; B, compound **1**) depict the changes of absorbance at 340 nm associated with the reduction of NAD^+. The arrows indicate the addition of ATP (2 mM) and the inhibitors (TDA, 40 nM; compound **1**, 60 nM). The final mitochondrial protein concentration is 0.1 mg of protein/mL.

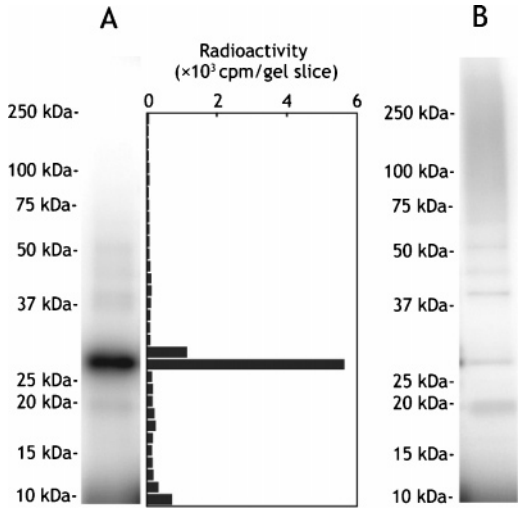


FIGURE 4: Labeling of bovine SMP with [^{125}I]TDA. SMP (0.3 mg of protein/mL) were photoaffinity labeled with [^{125}I]TDA (3 nM) as described in Experimental Procedures. (A) An autoradiograph of SDS−PAGE analysis (a 10−20% gel) of [^{125}I]TDA-labeled SMP is shown. (B) An autoradiograph, when the radiolabeled SMP were dissociated in SDS at 100 °C for 5 min, is shown. Data shown are representative of three independent measurements.

Consequently, we used [^{125}I]TDA as a photoreactive derivative of ordinary acetogenins in all of our experiments. The aryldiazirine group in TDA may serve as a substitute for the γ-lactone ring functionally, as observed for the quinone and heterocyclic derivatives (*27*−*29*). Moreover, although the length of the spacer portion linking the hydroxylated bis-THF and aryldiazirine group seems to be significantly shorter than that of potent acetogenin such as bullatacin, we previously showed that acetogenin derivatives that have a short spacer retain potent inhibitory effect (*40*).

Photoaffinity Labeling of Submitochondrial Particles. For the photoaffinity labeling study, SMP were used to ensure the intactness of complex I since the isolated enzyme is less sensitive to inhibitors (*16*). UV irradiation of SMP (0.3 mg of protein/mL) in the presence of [^{125}I]TDA (3 nM) resulted in one solely labeled region with an apparent molecular mass of approximately 30 kDa on a SDS gel (Figure 4, panel A). Nearly complete labeling (>90%) was established within 10 min of UV irradiation. As shown in Figure 5, a good correlation was found between the incorporation of the radioactivity into the 30 kDa band and the inhibition of NADH oxidase activity. It is therefore obvious that the photo-

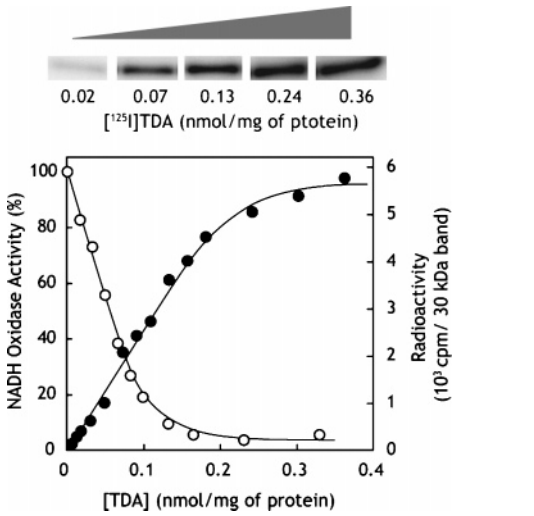


FIGURE 5: Correlation between the incorporation of radioactivity into the 30 kDa band (closed circles) and inhibition of the NADH oxidase activity in SMP (open circles). The values represent the average of two independent measurements. An autoradiograph of the 30 kDa band at given concentrations is shown on the top.

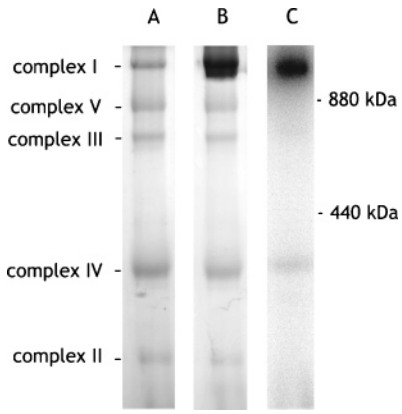


FIGURE 6: Localization of the labeled 30 kDa band in SMP. SMP (0.5 mg of protein/mL) were photoaffinity labeled by 6 nM [125I]-TDA. (A) Separation of the five oxidative phosphorylation enzyme complexes from [125I]TDA-labeled SMP by BN-PAGE (a 5−15% gradient gel). (B) Complex I band on the gel was identified by the activity staining with NADH-NBT. (C) An autoradiograph of the gel is shown. Data shown are representative of three independent measurements.

cross-linking of the 30 kDa band is responsible for the inhibition of complex I. Although the titration curve for the radiolabeling shifted to a slightly higher concentration range than that for the inhibition of the enzyme activity, this is rationalized by that a cross-linking yield may not be 100% under the experimental conditions. Even at a higher concentration of [125I]TDA (110 nM), radioactivity in other regions on a SDS gel was less than 5% of that in the 30 kDa region (data not shown), indicating that the extent of nonspecific labeling is negligibly small. When the radiolabeled SMP were dissociated in SDS at 100 °C for 5 min, extensive aggregation of the labeled material took place (Figure 4, panel B). This observation suggests that the labeled protein is highly hydrophobic (*15*).

Identification of the Labeled 30 kDa Protein. SMP (0.5 mg of protein/mL) cross-linked with 6 nM [125I]TDA were subjected to BN-PAGE and resolved into five oxidative phosphorylation enzyme complexes (Figure 6, panel A). The complex I band was identified by the activity staining using a NADH−NBT system (Figure 6, panel B). Major radioactivity was detected in the complex I band (Figure 6, panel C), indicating that complex I was predominantly radiolabeled by [125I]TDA.

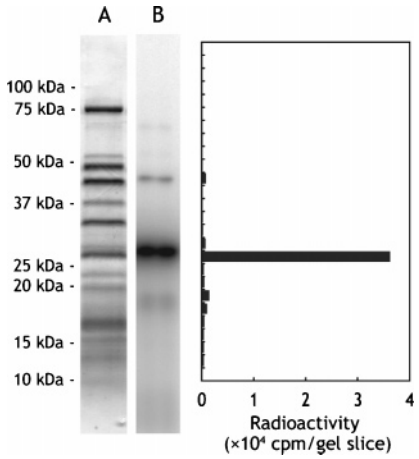


FIGURE 7: Analysis of the photoaffinity-labeled subunit in the isolated complex I. (A) SDS−PAGE (a 10−20% gel) analysis of [125I]TDA-labeled complex I isolated by BN-PAGE shown in Figure 6. (B) An autoradiograph of the gel is shown. Data shown are representative of three independent measurements.

Next, the radiolabeled complex I band was isolated by electroelution and subjected to the SDS−PAGE for further separation. As shown in Figure 7, we detected a major radioactivity peak around 30 kDa. The radioactivities of other regions were less than 5% of that of 30 kDa. Among the subunits of bovine complex I, those that migrate at approximately 30 kDa include ND1, ND2, and IP 30 kDa subunits. Among these subunits, the ND1 subunit is presumed to be the putative [125I]TDA-binding subunit as shown by western blotting analysis of both SMP and electroeluted complex I (Figure S2; see Supporting Information). The molecular mass of the detected ND1 signal and disappearance of this signal by boiling were consistent with the 30 kDa band of the autoradiogram shown in Figure 4.

To identify the radiolabeled protein, we carried out immunoprecipitation of the radiolabeled complex I with anti-bovine ND1 antibody. The control experiment was performed under the same experimental conditions, but without ND1 antibody. Figure 8 shows that the radiolabeled subunit is the ND1 that resides in the membrane segment of complex I. In contrast to the previous work (*17*), NADH did not enhance the specific labeling of the ND1 subunit by [125I]-TDA over a concentration range from 10 to 500 μM (data not shown).

Suppression of Specific Labeling of ND1 by Other Complex I Inhibitors. Previous photoaffinity labeling studies showed that specific labeling of PSST (*16*) and ND5 (*17*) subunits by pyridaben and fenpyroximate derivatives, respectively, is completely blocked by numerous other inhibitors (i.e., competitors). In these studies, the concentrations of other inhibitors were in largely excess of that of the photoaffinity probes (>200-fold). Under such experimental conditions, one cannot exclude the possibility that a large amount of hydrophobic chemicals induces some side effect-(s) on the enzyme, as pointed out in the introduction.

We examined the suppression effect of other inhibitors on the specific labeling of the ND1 subunit by [125I]TDA (3 nM) over a wide concentration range. Bullatacin and

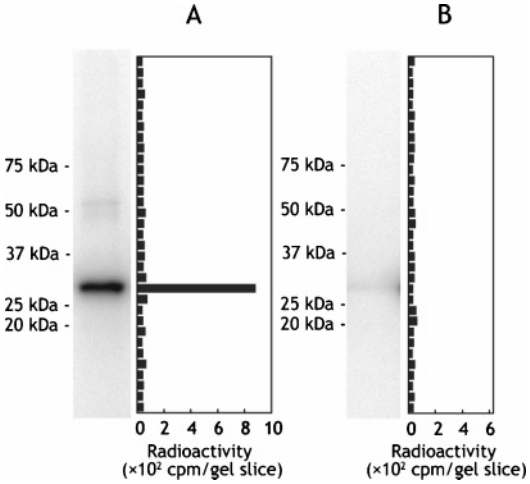


FIGURE 8: Identification of the labeled 30 kDa subunit. (A) [^{125}I]-TDA-labeled complex I isolated by BN-PAGE (Figure 6) was subjected to immunoprecipitation with bovine ND1 antibody. An autoradiograph of the SDS−PAGE gel (a 10−20% gel) is shown. (B) A control experiment was carried out under the same experimental conditions without ND1 antibody. Data shown are representative of three independent measurements.

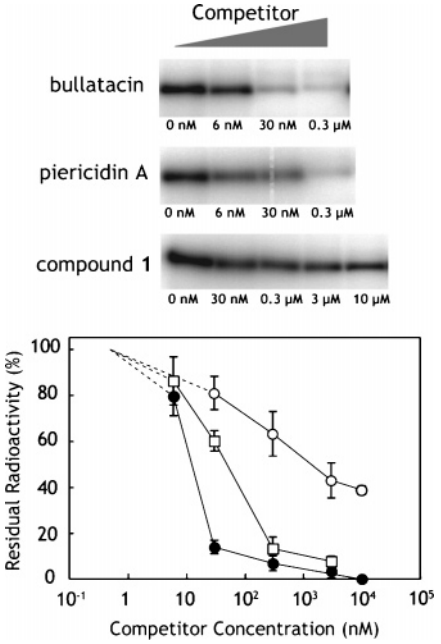


FIGURE 9: Effects of various complex I inhibitors on the specific labeling of the ND1 subunit by [^{125}I]TDA. A competitor was added to SMP (0.3 mg of protein/mL) at given concentrations and incubated for 10 min prior to the treatment with [^{125}I]TDA (3 nM): bullatacin (closed circles), piericidin A (open squares), and compound **1** (open circles). Data are the mean of three independent measurements ± SD. An autoradiograph of the ND1 band at given concentrations is shown on the top.

piericidin A suppressed the radiolabeling in a concentration-dependent manner (Figure 9). Other inhibitors (rotenone, AQ, fenpyroximate, and pyridaben) also suppressed the radiolabeling (Figure S3; see Supporting Information). As expected, a natural acetogenin analogue bullatacin suppressed the radiolabeling most efficiently. In the case of other inhibitors, higher concentrations relative to [^{125}I]TDA were required to suppress the radiolabeling. Typically, a 10−100-fold excess was needed to replace most of [^{125}I]TDA. This is because [^{125}I]TDA is an irreversible inhibitor whereas other inhibitors are reversible inhibitors. These results indicate that [^{125}I]TDA and numerous other inhibitors share the same binding domain in complex I.

Suppression of Specific Labeling of ND1 by Δlac-Acetogenin. On the basis of several lines of circumstantial evidence, as summarized in the Discussion, we previously proposed that Δlac-acetogenins are a new type of inhibitor that acts downstream of the iron−sulfur cluster N2 but does not share a common binding domain with ordinary inhibitors (*39*). Verification of this proposition must be helpful to get insight into the function of the membrane arm of complex I. Therefore, we examined the effect of Δlac-acetogenin on the specific labeling of the ND1 by [^{125}I]TDA (3 nM) using compound **1** as a representative of Δlac-acetogenin. Compared with ordinary inhibitors, much higher concentrations of **1** were needed to suppress the [^{125}I]TDA binding (Figure 9). About 40% of the radiolabeling was still retained in the presence of 10 μM compound **1** (about 3300-fold). It should be mentioned that the maximum inhibition of NADH oxidase activity by **1** was achieved at 20−30 nM under the experimental conditions. Our finding therefore indicates that Δlac-acetogenin and ordinary inhibitors do not share a common binding domain.

DISCUSSION

We synthesized a photoreactive acetogenin derivative ([^{125}I]TDA) and carried out photoaffinity labeling with bovine heart SMP. Our results indicate that the binding subunit of [^{125}I]TDA is the ND1 that resides in a membrane fragment of the enzyme and has eight putative transmembrane helices. Earlier studies with two rotenone-derived photoaffinity probes ([^{3}H]arylazidoamorphigenin in ref *14* and [^{3}H]-dihydrorotenone in ref *15*) showed that the ND1 subunit in isolated bovine complex I is the binding subunit of rotenone. However, this observation was challenged because of (i) low specific radioactivity of [^{3}H]arylazidoamorphigenin (0.02 Ci/mmol) for detecting a specific binding site, (ii) an inefficient labeling yield by [^{3}H]dihydrorotenone due to low reactivity of the arylketone group, and (iii) low inhibitor sensitivity of the isolated enzyme (*16*). On the other hand, it is notable that a photoreactive pyridaben derivative binds to the ND1 at excessively high concentrations (*16*), but this phenomenon has no relation with the inhibition of the electron transfer event (i.e., nonspecific binding). Furthermore, the photoaffinity labeling study using a fenpyroximate derivative provided no evidence for the contribution of the ND1 subunit to the inhibitor binding (*17*). With all things combined, there has been no strong experimental evidence that verifies whether the ND1 is one of the subunits which construct the inhibitor binding domain. This situation may be associated with that the photoaffinity probes hitherto studied have a photoreactive group at a remote position from a toxophore moiety of the inhibitors. Using an acetogenin derivative [^{125}I]-TDA in which the aryldiazirine group serves as a substitute for the γ-lactone ring as well as a photoreactive group, we proved that ND1 is the binding subunit of acetogenin. Taking into consideration that a proton-pumping activity of bovine complex I is inhibited by covalent binding of *N*,*N*′-dicyclohexylcarbodiimide (DCCD) to ND1 (*41*), this subunit must be concerned with proton translocation in the enzyme.

On the basis of the results of the replacement test performed in the previous photoaffinity labeling studies (*16*,

17), it is believed that a wide variety of inhibitors share a common large binding domain with partially overlapping sites. Although there are few structural similarities between acetogenins and other complex I inhibitors including synthetic agrochemicals such as pyridaben, our results showed that the specific binding of an acetogenin derivative [^{125}I]-TDA to the ND1 subunit was efficiently blocked by a number of complex I inhibitors. The present study strongly supports the concept of a common inhibitor binding domain in bovine complex I. Considering that PSST (*16*) and ND5 (*17*) subunits were labeled by photoreactive pyridaben and fenpyroximate derivatives, respectively, it is likely that PSST, which is located at the junction of the peripheral and membrane arms (*42*, *43*), ND5, and ND1 may be close to each other and construct a common inhibitor binding domain. The location of ND5 seems to be in disagreement with that deduced from structural model of *Escherichia coli* complex I based on low-resolution EM analyses (*44*, *45*). Some segment of the ND5 subunit may be localized close to PSST and/or ND1subunits, but this part is not recognized by the low-resolution EM analyses. Alternatively, we cannot rule out the possibility that there could be an additional inhibitor (or ubiquinone) binding site in ND5.

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, as is the case for other ordinary complex I inhibitors (*6*, *11*). However, the inhibition manner of Δlac-acetogenins is fairly different from that of ordinary complex I inhibitors in several points: (i) double-inhibitor titration shows that the inhibition by a pair of Δlac-acetogenin and natural acetogenin (bullatacin) is not additive (*26*, *30*), (ii) unlike ordinary inhibitors, Δlac-acetogenins do not prevent the specific binding of AQ to complex I (*30*), (iii) the inhibitory effect of Δlac-acetogenins on reverse electron transfer in complex I is much weaker than that on forward electron transfer (*39*), and (iv) the level of superoxide production from complex I induced by Δlac-acetogenins, regardless of whether endogenous or exogenous ubiquinone is used, is markedly lower than that induced by ordinary inhibitors (*30*, *39*). The present study reveals that Δlac-acetogenin (**1**) does not efficiently block the specific binding of [^{125}I]TDA to the ND1 subunit, indicating that Δlac-acetogenin does not share a common binding domain with ordinary inhibitors. This must be a cause for the unique inhibitory action of Δlac-acetogenins. It should be realized, however, that at excessively high concentrations compound **1** partly suppressed the binding of [^{125}I]TDA to the ND1. It is therefore likely that the binding of **1** affects the structure of the binding domain for ordinary inhibitors as a secondary effect. The kinetic studies and remarkable direction-specific effect of the inhibition by Δlac-acetogenins suggest that these inhibitors do not directly block the ubiquinone reduction by acting as a competitive inhibitor against ubiquinone (*39*). It is obvious that the essential components of the proton translocating machinery must reside in the transmembraneous subunits (*3*). In view of the proposed dynamic function of the membrane domain (*44*, *45*), it is not strange that there are diverse chemicals that disturb the function of the membrane domain differently depending on their structural specificity.

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SUPPORTING INFORMATION AVAILABLE

Syntheses of TDA and [^{125}I]TDA and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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