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Identification of the Binding Position of Amilorides in the Quinone Binding Pocket of Mitochondrial Complex I

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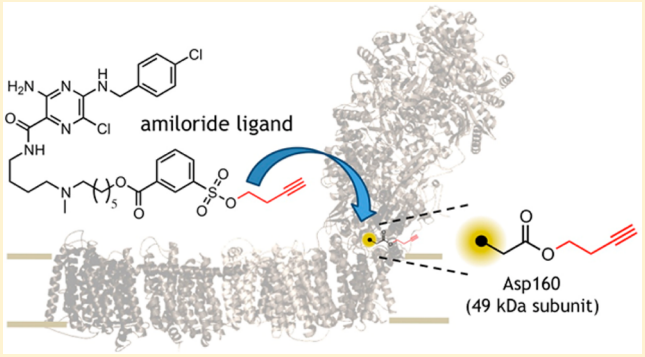
Supporting Information

ABSTRACT: We previously demonstrated that amilorides bind to the quinone binding pocket of bovine mitochondrial complex I, not to the hitherto suspected Na^+/H^+ antiporter-like subunits (ND2, ND4, and ND5) [Murai, M., et al. (2015) *Biochemistry* 54, 2739−2746]. To characterize the binding position of amilorides within the pocket in more detail, we conducted specific chemical labeling [alkynylation (−C≡CH)] of complex I via ligand-directed tosyl (LDT) chemistry using a newly synthesized amide-type amiloride AAT as a LDT chemistry reagent. The inhibitory potency of AAT, in terms of its IC_{50} value, was markedly higher (~1000-fold) than that of prototypical guanidine-type amilorides such as commercially available EIPA and benzamil. Detailed proteomic analyses in combination with click chemistry revealed that the chemical labeling occurred at Asp160 of the 49 kDa subunit (49 kDa Asp160). This labeling was significantly suppressed in the presence of an excess amount of other amilorides or ordinary inhibitors such as quinazoline and acetogenin. Taking into consideration the fact that 49 kDa Asp160 was also specifically labeled by LDT chemistry reagents derived from acetogenin [Masuya, T., et al. (2014) *Biochemistry* 53, 2307−2317, 7816−7823], we found this aspartic acid to elicit very strong nucleophilicity in the local protein environment. The structural features of the quinone binding pocket in bovine complex I are discussed on the basis of this finding.



The proton-translocating NADH-quinone oxidoreductase (complex I) couples electron transfer from NADH to quinone with the translocation of protons across the membrane. The electrochemical proton gradient across the membrane drives energy-consuming processes such as ATP synthesis.[1,2] Complex I is the largest of the respiratory chain enzymes; for example, the enzyme from bovine heart mitochondria is composed of 14 central subunits and many accessory subunits with a total molecular mass of ~1 MDa.[3] The crystal structures of the entire complex I from *Thermus thermophilus*[4] and *Yarrowia lipolytica*[5] provided useful structural information about this enzyme. A recent structural model of bovine complex I determined by single-particle electron cryo-microscopy assigned the location of some accessory subunits.[6] Despite the recent developments in structural biological approaches, the mechanism underlying coupling between electron transfer and proton translocation in complex I has not yet been elucidated.

Pyrazinoyl guanidine chemotype amilorides are well-known inhibitors of Na^+/H^+ and Na^+/Ca^{2+} antiporters and Na^+ channels.[7,8] The structural characteristics of amilorides that are critical to their inhibitory actions greatly vary depending on different ion transporters and species.[7] Amilorides were shown to inhibit bacterial and mitochondrial complex I;[9,10] however, their inhibitory potencies were markedly weaker than those of traditional inhibitors such as rotenone, piericidin A, and acetogenins. Because subunits ND2, ND4, and ND5 (in bovine complex I) in the membrane domain are homologous to subunits of the multidrug resistance protein (Mrp) family Na^+/H^+ antiporters,[11] amilorides are considered to block complex I activity by binding to any or all of the antiporter-like subunits. However, we recently revealed through photoaffinity labeling experiments that amilorides inhibit complex I by occupying the quinone binding pocket rather than by directly blocking proton translocation through the antiporter-like subunits (ND2, ND4, and ND5).[12] We also showed that a photoreactive amiloride (PRA1) possessing a photolabile group in the alkyl side chain moiety labeled accessory subunit B14.5a, indicating that the binding position of amilorides within the pocket is somewhat different form that of traditional inhibitors. Thus, amilorides may become unique molecular tools for characterizing the features of the quinone binding pocket. However, pinpoint identification of the labeled position by photoreactive amilorides (PRA1 and PRA2) within the pocket was unsuccessful because a sufficient amount of the labeled material could not be recovered from the SMP, and this was primarily attributed to the low reaction yield of photoaffinity labeling (<5%).[12]

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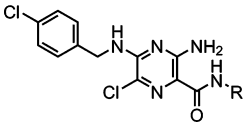
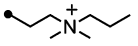
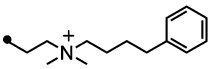
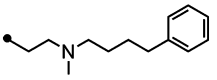
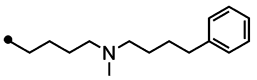
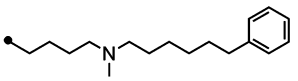
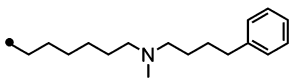
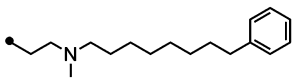
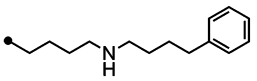
In this study, we used another protein labeling technique termed ligand-directed tosylate (LDT) chemistry, which is based on the principle of affinity labeling, to pinpoint the binding position of amilorides within the quinone binding pocket.[13,14] LDT chemistry uses a labeling reagent, in which a protein ligand and synthetic tag of choice are connected by an electrophilic tosylate (phenylsulfonate ester) group. Because of specific protein−ligand interaction, the LDT reagent specifically binds to the target protein and covalently transfers the synthetic tag to a nucleophilic amino acid residue, such as histidines and lysine, near the ligand binding pocket.[13] We recently demonstrated that Asp160 of the 49 kDa subunit (49 kDa Asp160) specifically reacts with the tosylate group, which was guided into the quinone binding pocket by LDT reagents derived from acetogenin, with a high reaction yield (~50%).[15,16] On the basis of this finding, we expected that if the tosylate group connected to a proper amiloride covalently reacts with a nucleophilic amino acid surrounding the molecule, pinpoint identification of the binding position of the amiloride may become feasible. Therefore, we planned to conduct LDT chemistry using an amiloride ligand.

Prior to the preparation of an amiloride ligand for LDT chemistry, we attempted to produce amiloride derivatives with binding affinities that are as high as possible to satisfactorily conduct LDT chemistry. To achieve this, we explored amide-type amiloride derivatives, which are novel chemotypes distinct from prototypical guanidine-type amilorides such as commercially available EIPA and benzamil (Table 1). On the basis of a structure−activity relationship study, we successfully produced potent amide-type amilorides and subsequently synthesized an amiloride ligand for LDT chemistry, which possesses a terminal alkyne as the tag [AAT (Figure 1)]. Detailed proteomic analyses revealed that the alkynylation occurred at the 49 kDa Asp160. By taking into consideration the fact that the 49 kDa Asp160 was also specifically labeled by LDT chemistry reagents derived from acetogenin,[15,16] we found this aspartic acid to elicit very strong nucleophilicity. The structural features of the quinone binding pocket in bovine complex I are discussed on the basis of this finding.

Table 1. Inhibitory Activities of Amide-Type Amilorides[a]



Compd.	R	IC_{50} (μM)
AA1		47 ± 6
AA2		12 ± 2
AA3		2.1 ± 0.3
AA4		0.50 ± 0.06
AA5		0.063 ± 0.007
AA6		0.024 ± 0.002
AA7		0.033 ± 0.002
AA8		0.023 ± 0.005
AA9		0.11 ± 0.02

[a]The IC_{50} value is the molar concentration (micromolar) needed to reduce the control NADH oxidase activity in bovine heart SMP by half. Values are means ± the standard deviation of three independent experiments.

EXPERIMENTAL PROCEDURES

Materials. Bullatacin and fenpyroximate were kind gifts from J. L. MacLaughlin (Purdue University, West Lafayette, IN) and Nihon Nohyaku Co. Ltd. (Tokyo, Japan), respectively. Aminoquinazoline and guanidine-type amilorides (**5b** and **5m**) were the same samples that were used in previous studies.[17,18] Protein standards (Precision Plus Protein Standards and Precision Plus Protein Dual Xtra Standards) for SDS−PAGE were purchased from Bio-Rad (Hercules, CA). The Click-iT reaction buffer kit, TAMRA-azido (Figure S1 of the Supporting Information), and membrane potential indicator, oxonol IV, were purchased from Life Technologies (Carlsbad, CA). The cleavable biotin-SS-azido (Figure S1 of the Supporting Information) was synthesized as described previously.[15] Other reagents were all of analytical grade.

Synthesis of Amide-Type Amilorides and AAT. The synthetic procedures for amide-type amilorides and AAT are described in the Supporting Information. All compounds were characterized by ^1H and ^{13}C nuclear magnetic resonance spectroscopy and mass spectrometry.

Preparation of Bovine Heart SMP and Enzyme Assays. Bovine heart submitochondrial particles (SMP) were prepared from isolated bovine heart mitochondria by the method of Matsuno-Yagi and Hatefi[19] and stored in a buffer containing 250 mM sucrose and 10 mM Tris-HCl (pH 7.4) at −80 °C until use.

NADH oxidase activity in SMP was followed spectrometrically with a Shimadzu UV-3000 instrument (340 nm; ε = 6.2 mM^{-1} cm^{-1}) at 30 °C.[20] The reaction medium (2.5 mL) contained 0.25 M sucrose, 1.0 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. NADH-Q_1 oxidoreductase activity was also measured under the same experimental conditions, except that the reaction medium contained 50 μM Q_1, 0.20 μM antimycin A, and 4.0 mM KCN.

Measurement of Membrane Potential Formation. Membrane potential formation was measured at 30 °C by following changes in the absorbance of oxonol IV, an optical indicator for membrane potential, at 601−630 nm with a Shimadzu UV-3000 instrument in dual-wavelength mode.[20] NADH-driven membrane potential formation was measured in reaction medium (2.5 mL) containing 0.2 M sucrose, 2.5 mM $MgCl_2$, 0.20 μM antimycin A, 2.0 mM KCN, 2.5 mM

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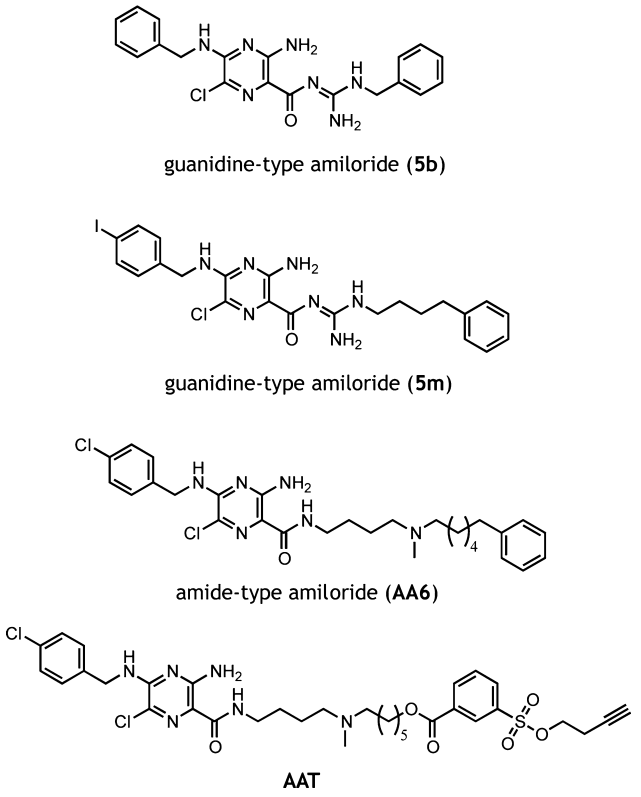

Figure 1. Structures of the compounds used in this study. Guanidine-type amilorides **5b** and **5m** were synthesized in the previous study.[18]

oligomycin, 0.10 μM nigericin, 50 μM Q_1, 1.0 μM oxonol IV, and 50 mM Tricine-KOH (pH 7.5). The final protein concentration was set to 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. Uncoupler SF6847 was used at a final concentration 0.1 μM to confirm the complete disappearance of membrane potential.

ATP-driven membrane potential formation was also measured in the same reaction medium, but without Q_1 and oligomycin, and the reaction was started by adding 4 mM ATP.

General Procedure for LDT Chemistry and Estimation of the Labeling Yield. SMP (2.0 mg of protein/mL, 100−200 μL) were incubated with AAT in a buffer containing 250 mM sucrose, 1.0 mM $MgCl_2$, and 50 mM KP_i (pH 7.4) for 24 h at 35 °C. SMP were then collected by ultracentrifugation (20000*g*, 20 min, 4 °C) and denatured in 1.0% (w/v) SDS (25−50 μL). The proteins alkynylated by AAT via LDT chemistry were conjugated with a TAMRA or biotin-SS-azido tag via Cu^+-catalyzed click chemistry using the Click-iT reaction buffer kit (Life Technologies) according to the manufacturer's instructions. Proteins were recovered by precipitation with methanol and chloroform and subjected to subsequent analyses.

The content of complex I in SMP was roughly estimated as the minimal amount of bullatacin required to completely inhibit the NADH oxidase activity; the content of complex I in 1.0 mg of SMP proteins was estimated to be 0.10 nmol.[15,20] The procedure for estimating the amount of TAMRA-attached 49 kDa subunit in the SDS−PAGE gel was modified from that used in a previous study;[15] the amount of TAMRA-attached 49 kDa subunit in the gel was quantified by a Bioimaging analyzer using small polyacrylamide gel pieces containing varying amounts of the TAMRA-azido tag (0, 0.4, 0.8, 1.2, 1.6, and 2.0 pmol/gel piece) as calibration standards. The SDS−PAGE gel containing the TAMRA-attached 49 kDa subunit, together with the gel pieces containing TAMRA-azido, were set and analyzed on the same fluorescent glass stage (FLA Fluor Stage 4046, Fuji Film). The amount of TAMRA-attached 49 kDa subunit was estimated by interpolating the fluorescence intensity of the band to the calibration plot. Typically, the reaction yield was estimated to be ∼25% when SMP (2.0 mg of protein/mL) were labeled (alkynylated) with 5.0 μM AAT. We note that the previously reported reaction yields of LDT chemistry using acetogenin ligands AL1[15] and AL2,[16] which were estimated using carbonic anhydrase II having only one cysteine as a model protein, were considerably overestimated (∼50%). The reaction yields for AL1 and AL2 re-estimated by the method presented here were ∼30%.

Electrophoresis. TAMRA-attached proteins were separated on a Laemmli-type SDS gel.[21] The migration patterns of fluorescent proteins were visualized by the model FLA-5100 (Fuji Film, Tokyo, Japan) or Typhoon FLA9500 (GE Healthcare, Buckinghamshire, U.K.) bioimaging analyzer using a 532 nm light source and LPG (575 nm) filter. Data processing and the quantification of fluorescence were conducted using Multi Gauge (Fuji Film) and Image Quant (GE Healthcare), respectively.

To analyze the TAMRA-attached 49 kDa subunit, the subunit was partially purified by SDS−PAGE and electro-elution.[15,16] The purified subunit was digested by lysylendopeptidase (Lys-C) or endoprotease Asp-N in 50 mM Tris-HCl buffer (containing 0.1% SDS) or 50 mM NaP_i buffer (containing 0.01% SDS[15,16]), respectively. The digests were separated on a Schägger-type SDS gel (16.5% T, 6.0% C containing 6.0 M urea).[22]

Enrichment of the Alkynylated Protein. SMP (2.0 mg of proteins/mL, total of 1.0 mg) were alkynylated with 5.0 μM AAT, denatured in a 50 mM Tris-HCl buffer (pH 8.0) containing 1% (w/v) SDS, and conjugated with a cleavable biotin-SS-azido via Cu^+-catalyzed click chemistry. The proteins were recovered by methanol/chloroform precipitation and solubilized in Tris-buffered saline (TBS buffer, 120 μL) containing 2.0% (w/v) SDS at 40 °C for 1 h. The solubilized proteins were diluted with TBS buffer (1.0 mL) containing 1.0% (w/v) Triton X-100, and the sample was incubated with streptavidin-agarose CL-4B (50 μL suspension, Sigma-Aldrich) at 4 °C for 16 h. The resin was washed twice with TBS buffer containing 0.5% (w/v) Triton X-100 and once with a TBS buffer without detergent. The biotinylated proteins were eluted in Laemmli's sample buffer containing 2.5% mercaptoethanol, separated on a Laemmli-type SDS−PAGE gel, visualized by CBB staining, and subjected to a mass spectrometric analysis.

Mass Spectrometry. Alkynylated protein was identified and characterized using the Bruker Autoflex III Smartbeam instrument (MALDI-TOF/TOF) and LTQ Velos Orbitrap mass spectrometer equipped with Ultimate 3000 nano-LC (LC−MS, Thermo Scientific), respectively, under the same experimental and data processing conditions that have been previously reported.[15,16]

RESULTS

Structure−Activity Relationship of Novel Amide-Type Amilorides. We previously performed structural modifications of commercially available amilorides to obtain strong derivatives as mitochondrial complex I inhibitors.[18] Although we successfully produced some potent amilorides, we never

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obtained amilorides with activities similar to those of potent ordinary inhibitors such as rotenone, piericidin A, and acetogenins, the IC_{50} values of which are at one-digit nanomolar levels.

To further improve the inhibitory potency, prominent structural modifications, including the prototypical pyrazinoyl guanidine skeleton, which is considered a critical structural unit as inhibitors of various antiporters, may be needed. A search of the literature revealed that some amide-type amiloride derivatives, which are novel chemotypes distinct from the prototypical guanidine type and possess a positively charged quaternary amine center in the alkyl amine portion, exhibit strong inhibition of the epithelial sodium channel.[23,24] Therefore, we herein synthesized amide-type derivatives and evaluated their inhibitory activities with bovine complex I. The pyrazinoyl moiety was fixed as 3-amino-6-chloro-5-(4-chlorobenzyl)amino-pyrazine because this skeleton was shown to be favorable for inhibitory activity.[18]

In the initial trial, we synthesized simple amide-type amilorides that possess a quaternary amine in their side chains (**AA1**−**AA3**). The inhibitory activity increased with elevations in the hydrophobicity of the alkyl side chain (**AA1** < **AA2** < **AA3**). We synthesized a tertiary amine derivative (**AA4**) corresponding to **AA3** to examine the importance of a positively charged quaternary amine. Unexpectedly, the inhibitory activity of **AA4** was significantly more potent than that of **AA3**, indicating that the presence of the positive charge is not necessarily favorable for the inhibitory action.

As greater hydrophobicity of the side chain moiety was found to be favorable for the inhibitory activity among the amilorides synthesized to date,[18] we next prepared tertiary amine derivatives (**AA5** and **AA6**) with an alkyl chain longer than that of **AA4**. The inhibitory activity markedly increased with elevations in the hydrophobicity of the side chain (**AA4** < **AA5** < **AA6**). The activity of **AA6** (IC_{50} = 24 nM) was approximately 1000-fold more potent than that of commercially available amilorides (such as EIPA and benzamil), and approximately 10-fold more potent than that of the most potent derivative produced in the previous study.[18] This result suggested that the tertiary amine nitrogen plays a specific role as a hydrogen bond acceptor against the enzyme. The activity of secondary amine derivative (**AA9**) was slightly lower than that of the corresponding tertiary amine (**AA5**), indicating the advantage of the presence of a tertiary amine in the side chain.

To examine the role of the tertiary amine, we changed the position of the amine nitrogen by fixing the total number of carbon atoms at 10 (**AA6**−**AA8**). Their activities were similar irrespective of the different positions of the nitrogen atom in the side chain. This is probably because the side chains of these compounds are sufficiently flexible to spatially place the tertiary amine nitrogen in the required position. Further increases in the total number of carbon atoms in the side chain moiety did not enhance the inhibitory activity (data not shown).

Synthesis of an Amiloride Reagent for LDT Chemistry. The structure−activity relationship study discussed above showed that amide-type amilorides possessing a tertiary amine in the side chain elicit potent inhibitory activities against mitochondrial complex I. By using this type of amiloride as a template, we designed and synthesized the tosylate derivative AAT (Figure 1), which was expected to function as a reagent for LDT chemistry. A terminal alkyne (−C≡CH) was incorporated into the tosylate group as a tag because it can be used as a "footing" for subsequent Cu^+-catalyzed click chemistry (i.e., azide−alkyne [3+2] cycloaddition in water[25]) to visualize and/or purify the labeled protein or peptides. AAT was found to be the strongest derivative among the numerous amilorides synthesized in our laboratory (IC_{50} = 6.7 ± 0.3 nM), indicating that this compound is a promising reagent for LDT chemistry.

Characterization of the Inhibitory Action of Amide-Type Amilorides. Proton translocation by complex I is tightly coupled with the quinone redox reaction. The driving force for proton translocation must be transmitted from the Fe−S cluster N2/quinone coupling site to the gates of the proton channels in the membrane domain; however, this energy-transducing mechanism has not yet been elucidated in detail.[2] Because the previous findings strongly suggested that the binding position of amilorides within the quinone binding pocket is considerably different from that of ordinary inhibitors,[12] there is the possibility that our synthetic amilorides may inhibit the quinone redox reaction and proton translocation to different extents (the so-called decoupling effect). The decoupling effect was claimed by Batista et al. for the action of EIPA against complex I in *Rhodothermus marinus* membrane vesicles.[26]

We investigated the effects of guanidine- and amide-type amilorides on membrane potential formation induced by complex I in SMP, which was monitored as changes in the absorbance of oxonol VI, and compared them with the effects on the electron transfer activity of the enzyme (i.e., NADH-Q_1 oxidoreductase activity) using bullatacin as a reference. In preliminary experiments, we confirmed that the oxonol VI signal generated by NADH oxidation was transiently enhanced and entirely abolished by the addition of nigericin (K^+/H^+ exchanger) and valinomycin (K^+ ionophore), respectively. To determine the relationship between changes in electron transfer activity and membrane potential formation, these two parameters are plotted in Figure 2A. The relationship between the inhibition of electron transfer activity and membrane potential formation was comparable for **AA6** and bullatacin. Nonphysiological electron transfer (5−10% of control activity) generated no membrane potential.[27] In contrast, **5b** and **5m** markedly diminished the membrane potential, even in the concentration range that elicited the partial inhibition of electron transfer activity; the membrane potential was completely abolished even though 50−60% of electron transfer activity remained. This result indicates that some effect of **5b** and **5m** other than electron transfer inhibition is responsible for this phenomenon, possibly an uncoupling effect due to its protonophoric ability.

Prototypical guanidine-type amilorides are in an equilibrium state between protonated (guanidine portion) and non-protonated forms at physiological pHs because their pK_a values generally range from 8 to 9.[7] Roberts and Hirst reported the significant uncoupling effect of EIPA due to its protonophoric ability in the mitochondrial membrane,[28] as reported previously.[29] In contrast, Batista et al. claimed that EIPA elicits no protonophoric activity with *R. marinus* membrane vesicles in the concentration range of 0−20 μM.[26]

To assess the protonophoric ability of prototype amilorides, we next examined the effect of **5b** and **5m** on membrane potential formation induced by ATP hydrolysis with ATP synthase. We confirmed that the oxonol VI signal generated by ATP hydrolysis was completely abolished by the addition of SF6847 (a potent protonophore[30]). As shown in Figure 2B, **5b** and **5m** both markedly abolished membrane potential

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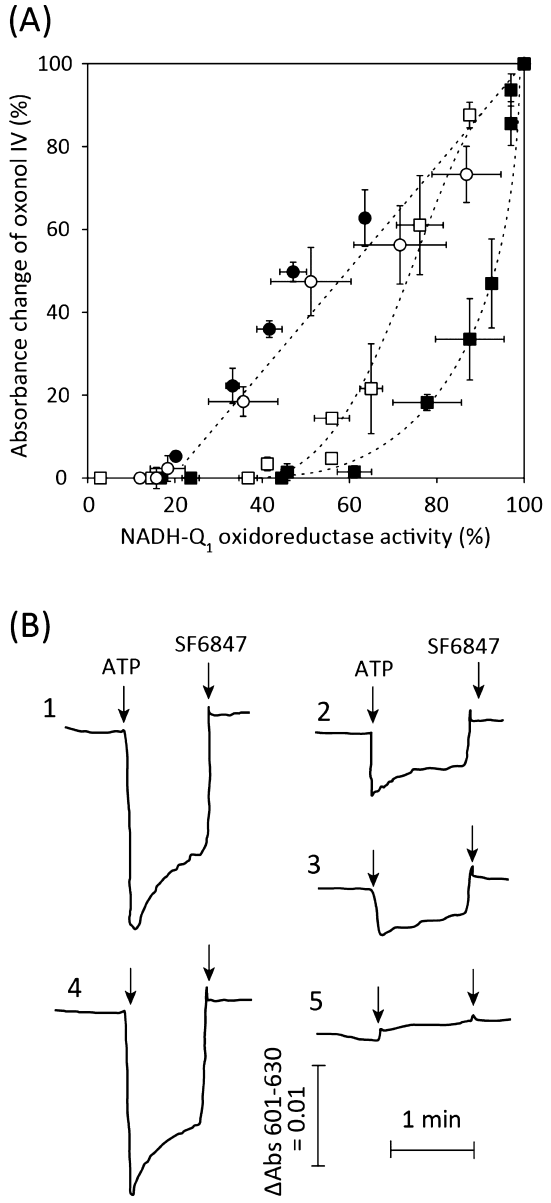

Figure 2. Effects of amilorides on transmembrane potential formation with SMP. (A) Relationship between changes in electron transfer activity (NADH-Q_1 oxidoreductase activity) and NADH-driven membrane potential formation by complex I. Both measurements were taken in the presence of various concentrations of bullatacin (○), amide-type amiloride **AA6** (●), and guanidine-type amilorides **5b** (■) and **5m** (□). The protein concentration in both measurements was set to 30 μg/mL. The data for bullatacin were taken from ref 20. Data are means ± the standard deviation of three independent measurements. (B) Effects of amilorides on ATP-driven membrane potential formation. Measurements were taken in the same buffer without NADH, Q_1, and oligomycin. The arrows indicate the addition of 4.0 mM ATP and 0.20 μM SF6847. The traces are as follows: 1, control; 2, 1.0 μM **5m**; 3, 6.0 μM **5b**; 4, 50 nM **AA6**; 5, 3.0 μM oligomycin.

formation at a concentration that inhibited the electron transfer activity of complex I by approximately 70−80%. However, amide-type **AA6** did not affect the membrane potential even at a concentration that inhibited complex I activity by approximately 80%. Taken together, we conclude that guanidine-type amilorides, but not amide-type amilorides, elicit significant protonophoric ability under the experimental conditions used in this study.

Specific Alkynylation of Bovine Complex I via LDT Chemistry. Bovine SMP (2.0 mg of protein/mL, equivalent to ~200 nM complex I) were incubated with AAT at 35 °C for 24 h and then solubilized with 1% SDS. To visualize the incorporated alkyne, a fluorescent TAMRA-azido tag was conjugated with the alkyne via Cu^+-catalyzed click chemistry.[15] As shown in Figure 3, a fluorescent band was exclusively

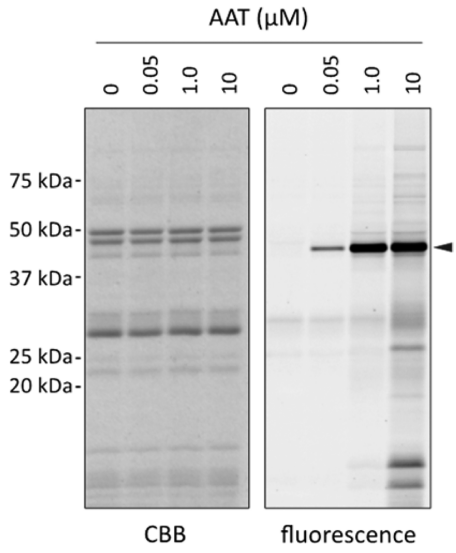

Figure 3. Alkynylation of complex I in SMP via LDT chemistry. SMP (2.0 mg of protein/mL) were alkynylated via LDT chemistry using AAT at 35 °C for 24 h, denatured by 1.0% (w/v) SDS, and then conjugated with a fluorescent TAMRA-azido tag (Figure S2 of the Supporting Information) via Cu^+-catalyzed click chemistry, as described in Experimental Procedures. The proteins in SMP were separated on a 12.5% Laemmli-type SDS gel and subjected to CBB staining and fluorescent gel imaging. Approximately 30 μg of SMP proteins was loaded onto each well.

observed at ~50 kDa, indicating that the terminal alkyne was successfully transferred from AAT to the ~50 kDa protein. The saturation of fluorescence intensity in the ~50 kDa band was almost achieved at ~1.0 μM AAT. We note that the presence of 100 μM NADH during the LDT chemistry reaction did not affect the extent of labeling (data not shown).

Identification of the Alkynylated Site by AAT in the 49 kDa Subunit. To isolate the alkynylated ~50 kDa protein, bovine SMP (2.0 mg of protein/mL) were incubated with 5.0 μM AAT, which gave complete saturation of the alkynylation, followed by conjugation with a cleavable biotin-SS-azido tag via Cu^+-catalyzed click chemistry, and the biotinylated protein was enriched using immobilized streptavidin. Figure 4 shows the results of the enrichment procedure, indicating that the alkynylated protein was successfully enriched by the immobilized avidin. The major ~50 kDa band, which corresponds to the fluorescent band in Figure 3, was identified as the 49 kDa subunit by MALDI-TOF MS (41% sequence coverage).

To identify the region alkynylated by AAT, the 49 kDa subunit was conjugated with a fluorescent TAMRA-azido tag, separated on a Laemmli-type SDS gel, partially purified by electroelution, and exhaustively digested with Lys-C and Asp-N. The digests were resolved on a Schägger-type Tricine gel to provide major fluorescent bands at ~8 and ~12 kDa, respectively (Figure 5A). Because these digestion patterns were almost identical to those previously obtained for specific

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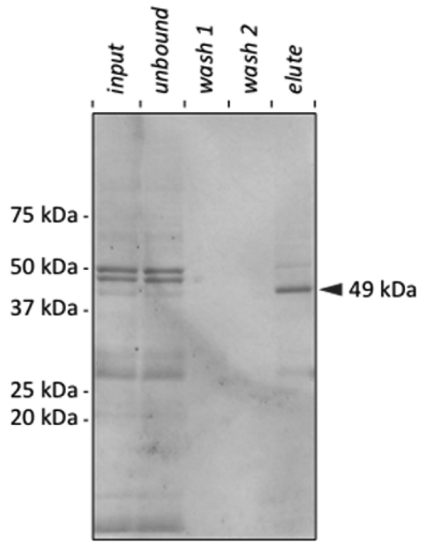

Figure 4. Enrichment of the alkynylated protein in SMP. SMP (2.0 mg of protein/mL) were alkynylated by 5.0 μM AAT and conjugated with a cleavable biotin-SS-azido tag (Figure S2 of the Supporting Information) via Cu^{+}-catalyzed click chemistry. The biotinylated proteins were enriched using streptavidin-agarose, as described in Experimental Procedures. Specific capture/release procedures were monitored by SDS−PAGE (shown as input, unbound, wash, and elute). The enriched ~50 kDa protein was identified as the 49 kDa subunit by peptide mass fingerprinting using MALDI-TOF MS.

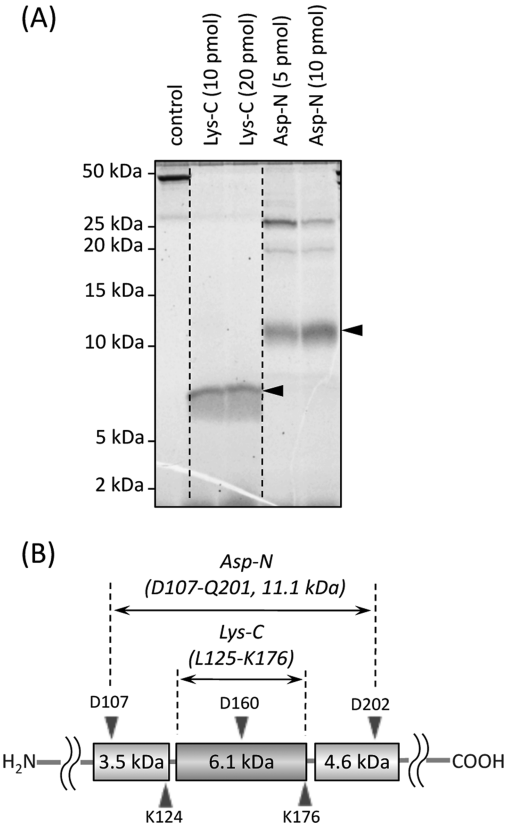

Figure 5. Localization of the alkynylated site in the 49 kDa subunit by limited proteolysis. (A) SMP (2.0 mg of protein/mL) were alkynylated by 5.0 μM AAT and conjugated with a fluorescent TAMRA-azido tag via Cu^{+}-catalyzed click chemistry, followed by partial purification of the 49 kDa subunit by SDS−PAGE and electroelution. The isolated 49 kDa subunit was digested by Lys-C or Asp-N. The digests were separated on a 16.5% Schägger-type SDS gel. Data are representative of three independent experiments. (B) Schematic presentation of the digestion by Lys-C or Asp-N. Residue numbers refer to the mature sequence of the bovine 49 kDa subunit (SwissProt entry P17694).

chemical labeling by acetogenin ligands,[15,16] the Lys-C and Asp-N digests may be assigned to the regions of Leu125−Lys176 (6.1 kDa) and Asp107−Gln201 (11.1 kDa), respectively (Figure 5B).

Furthermore, to pinpoint the alkynylated site in the 49 kDa subunit, the tryptic digests of the enriched 49 kDa subunit were extensively characterized by LC−MS/MS, setting the specific chemical modification [$C_{15}H_{25}N_5O_2S$ (Figure 6A)] as a variable protein modification. Among the 26 49 kDa-derived sequences [80% sequence coverage (Figure S2 of the Supporting Information)], a triply charged ion with an m/z value of 1216.27 (z = 3) was detected as the candidate peptide, which corresponds to the calculated mass of the alkynylated peptide Lys147−Arg174 (1103.20 + 113.05 = 1216.25). The fragment ion spectra of the peptide confirmed the sequence L^{147}LNH-IMAVTTHALDIGAMTPFFWMEER^{174} with alkynylation at Asp160 (Figure 6B). The chemical modification of Asp160 may explain why endoprotease Asp-N failed to cleave the peptide bond between Leu159 and Asp160, resulting in a large digest with an apparent molecular mass of ~12 kDa (Figure 5A).

Effects of Various Inhibitors on the Specific Labeling of 49 kDa Asp160. SMP (2.0 mg of protein/mL) were alkynylated with AAT (0.1 μM) in the presence of various complex I inhibitors, including guanidine- and amide-type amilorides [**5m** and **AA6**, respectively (Figure 1)]. As shown in Figure 7, 10 μM bullatacin, aminoquinazoline, and fenpyroximate, the inhibitory potencies of which (in terms of IC_{50} values) are stronger than that of AAT, almost completely suppressed the specific alkynylation of 49 kDa Asp160. On the other hand, ~20 and ~50% of the fluorescence intensity were retained in the presence of 30 μM (300 molar-fold) **AA6** and **5m**, respectively. We did not use higher concentrations of **AA6** and **5m** because high concentrations of hydrophobic chemicals nonspecifically disturb the enzyme and/or membrane environment. These differences between ordinary inhibitors and amilorides may be attributable to differences in their binding affinities for the enzyme; the IC_{50} values of **AA6** and **5m** are approximately 5- and 70-fold larger than that of AAT, respectively. On the other hand, by taking into consideration the fact that the binding region of guanidine-type amiloride PRA2 does not cover 49 kDa Asp160,[12] we are unable to exclude the possibility that the binding position of amide-type amilorides within the quinone binding pocket is somewhat different from that of guanidine-type amilorides.

DISCUSSION

In this study, we produced novel amide-type amilorides, the inhibitory potencies of which with bovine complex I are markedly greater than those of prototypical guanidine-type amilorides. Using amide-type amilorides as a template, we succeeded in preparing the reagent AAT, which is suitable for LDT chemistry. To further characterize the binding position of amilorides in the quinone binding pocket of complex I, we conducted specific alkynylation (−C≡CH) of the enzyme via LDT chemistry. Detailed proteomic analyses unambiguously revealed that the alkynylation occurs at 49 kDa Asp160. According to a sequence alignment for the bovine 49 kDa subunit and *T. thermophilus* Nqo4 subunit, 49 kDa Asp160

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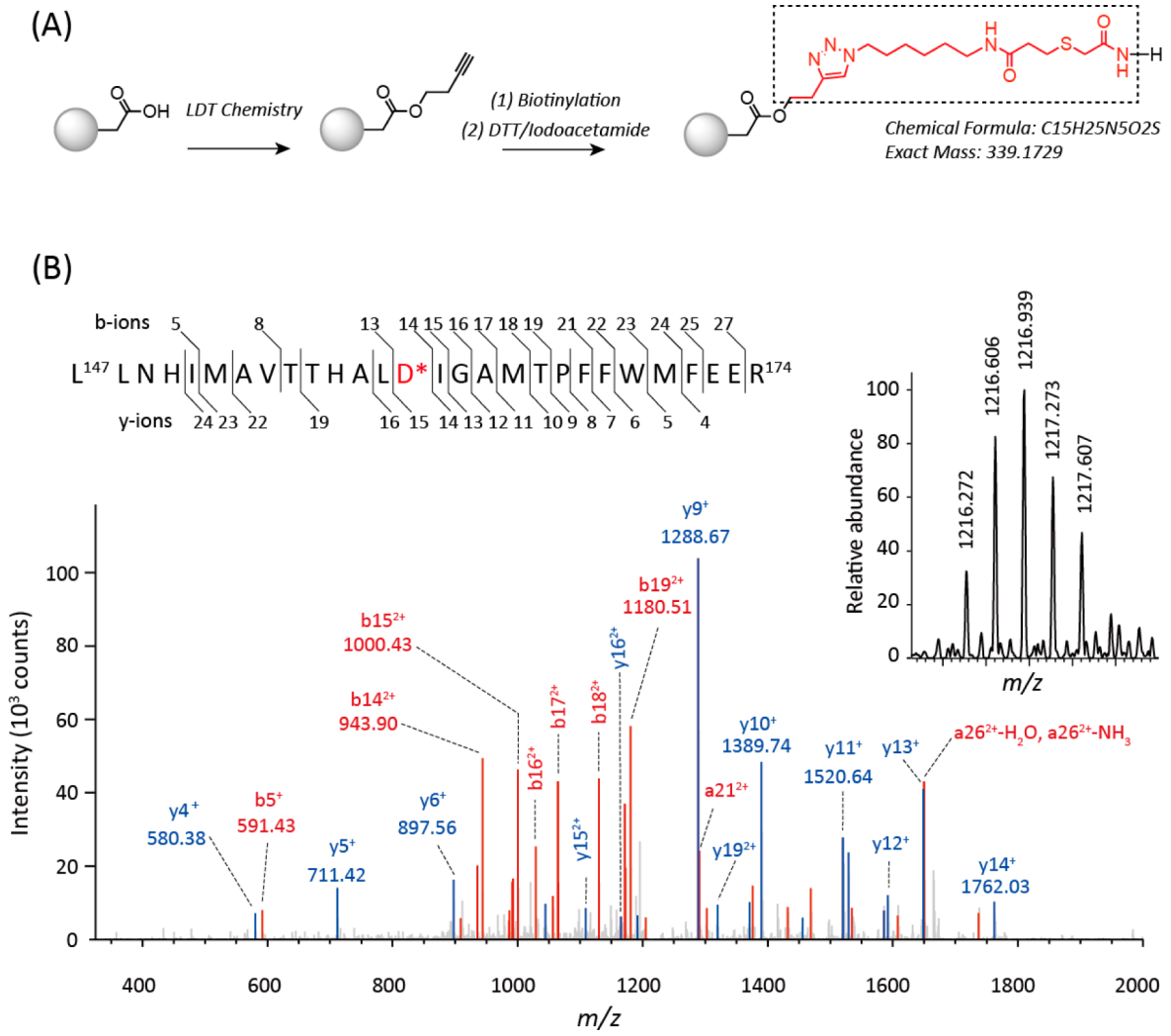

Figure 6. Identification of the alkynylated site in the 49 kDa subunit. The alkynylated 49 kDa subunit was enriched as described in Experimental Procedures or the legend of Figure 4, followed by "in-gel" digestion with trypsin. The digests were extensively characterized using an Orbitrap mass spectrometer. (A) Predictable formation of a stable adduct by alkynylation via LDT chemistry, biotinylation via Cu^{+}-catalyzed click chemistry, and carbamidomethylation. (B) Fragment ion spectra of the alkynylated peptide L^{147}LNHIMAVTTHALD*IGAMTPFFWMFEER^{174}, in which D* corresponds to an *O*-alkynylated aspartic acid with a mass shift of +339.458. The b- and y-ions identified were mapped onto the amino acid sequence. The inset shows the precursor ion spectra. The residue numbers refer to the mature sequence of the bovine 49 kDa subunit (SwissProt entry P17694), and oxidation (+15.999) occurred at Met152.

(Asp139 in Nqo4) is located in the inner part of the putative quinone binding cavity.[4] The mutagenesis study of conserved amino acids in the NuoD subunit of *Escherichia coli* complex I revealed that Asp325 (49 kDa Asp160 in bovine) is important but not essential for the enzyme activity because Asp325Ala/Glu variants still retain 50−60% activity.[31] The results of this study seem to be difficult to reconcile with the previous photoaffinity labeling study, in which the terminal end of the alkyl side chain of guanidine-type amilorides was located in the N-terminal region of the 49 kDa subunit, which does not cover Asp160.[12] Although the reason for this discrepancy remains unclear, the unique reactivity of 49 kDa Asp160 may be involved in LDT chemistry within the quinone binding pocket, as discussed below. In addition, we are unable to exclude the possibility that the binding position of amide-type AAT within the quinone binding pocket may be slightly different from that of guanidine-type amilorides.

We previously demonstrated that LDT reagents derived from acetogenins, which have a tosylate (phenylsulfonate ester) group in their alkyl side chain, also specifically label 49 kDa Asp160.[15,16] It should be, however, realized that the photoaffinity labeling studies previously showed that amiloride and acetogenin occupy somewhat different positions inside the quinone binding pocket of bovine complex I.[12,32−34] These findings lead to the question of why 49 kDa Asp160 elicits such a strong nucleophilicity against the tosylate group of different types of LDT reagents. To answer this question, we will consider the reaction between the carboxy group of Asp and Glu residues (as a nucleophile) and tosylate group (as a carbon electrophile) as follows.

Weerapana et al. investigated the labeling profiles of numerous proteins and amino acids by three carbon electrophiles [phenylsulfonate ester (SE; tosylate), α-chloroacetamide (CA), and α,β-unsaturated ketone (UK) (see Figure S3 of the Supporting Information)], which elicit the highest levels of reactivity against nucleophilic amino acids in proteins.[35] A very restricted reactivity profile was observed for the CA and UK probes; these probes selectively labeled cysteine residues in

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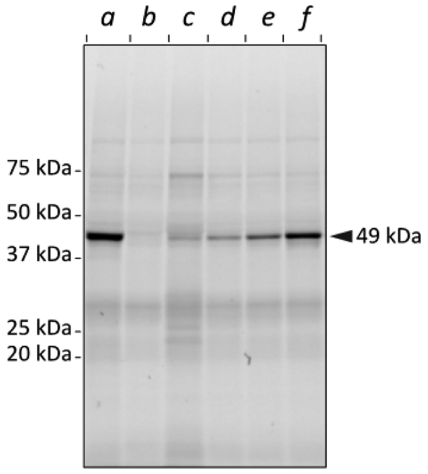

Figure 7. Effects of complex I inhibitors on the alkynylation of the 49 kDa subunit by ATT. SMP (2.0 mg of protein/mL) were alkynylated with 0.10 μM AAT in the absence (*a*) or presence of bullatacin (10 μM, *b*), aminoquinazoline (10 μM, *c*), fenpyroximate (10 μM, *d*), **AA6** (30 μM, *e*), or **5m** (30 μM, *f*). SMP treated with AAT were denatured, attached by a fluorescent TAMRA-azido tag via Cu^{+}-catalyzed click chemistry, and separated on a 12.5% Laemmli-type SDS gel. Data are representative of three independent experiments.

proteins. In contrast, the SE probe reacted with several nucleophilic amino acids, including Asp, Glu, Cys, Tyr, and His residues. They investigated whether the distinct proteome labeling profiles of the SE, CA, and UK probes can be discerned from their reactivities with amino acids in aqueous solution.[35] Of particular interest was the markedly low reactivity of the SE probe to the carboxy group in solution, which contrasted sharply with the large number of Asp and Glu labeling sites in the proteins reported for this probe. Thus, the *O*-alkylation of Asp and Glu residues (i.e., the reaction between the carboxy group and SE) to form an ester product is generally negligible in a solution because of the essentially weak nucleophilicity of the carboxy group. Although the reason why nucleophilic substitution proceeds between SE incorporated into LDT reagents and the carboxy groups in target proteins remains largely unknown, the so-called "proximity-induced effect" has been proposed, in which the frequency of nucleophilic attack by the carboxy group in a local protein microenvironment increases because of the high binding affinity of the ligand moiety for the target protein.[13] Looking at our results from this point of view, the LDT reagents derived from amide-type amiloride and acetogenin may be remarkably concentrated in the quinone binding pocket because of their high binding affinities, which enhances the nucleophilic reactivity of 49 kDa Asp160 to the SE moiety. We also cannot neglect that the position of SE incorporated into our LDT reagents is in a portion that is not critical for their inhibitory actions; therefore, the SE moiety may be free from restrictive interactions (or contact) with the enzyme such as hydrogen bonding. This flexibility of SE within the quinone binding pocket may also increase the frequency of interactions with nucleophilic 49 kDa Asp160.

There are a substantial number of nucleophilic amino acids within the quinone binding (or accessing) cavity of bovine complex I. Even if regions are limited to those participating in the binding of various inhibitors, examples include His55, His59, and Glu173 in 49 kDa,[36] E58 and His61 in PSST,[37] and Asp199 and Glu202 in ND1.[32−34] Therefore, another issue is why only 49 kDa Asp160 reacted to the SE group of our LDT reagents. This cannot be explained solely by the enrichment of LDT reagents in the quinone binding pocket as described above. Two factors may affect the attack of nucleophilic amino acids to the SE group within the pocket: one is whether the residue is located in the position to which SE can spatially approach and the other is whether the nucleophilicity of the residue is masked by nearby residue(s) through strict interactions such as hydrogen bonding and electrostatic interactions. Therefore, we next consider these two factors on the basis of the X-ray crystallographic structure of complex I. The X-ray structure of *Y. lipolytica* mitochondrial complex I suggests that the deeper access of ubiquinone or inhibitor to the pocket (closer to the Fe−S cluster N2) is blocked by the tip of loop β_1−β_2 (49 kDa); hence, they cannot reach several nucleophilic residues of the 49 kDa subunit (e.g., Asp143, Tyr144, and Asp458),[5] which is distinct from the features of the quinone binding pocket of the *T. thermophilus* complex I.[4] Nevertheless, because Asp196 (Asp160 in bovine) is located in the relatively spacious area of the quinone binding pocket, the SE group of LDT reagents may reach this residue. On the other hand, the mutagenesis study showed that Asp325 in the NuoD subunit of *E. coli* complex I (49 kDa Asp160 in bovine) is not essential for enzyme activity, as discussed above.[31] Therefore, 49 kDa Asp160 may be free from strict interactions arising from nearby residue(s). Thus, 49 kDa Asp160 appears to meet the requirements listed above. The reason why acidic residues lined on the loop connecting TMH5 and TMH6 of ND1 (e.g., Glu206, Glu208, and Glu210 in *Y. lipolytica* complex I), which may comprise the internal surface of the quinone-accessing cavity, did not react with the SE group of our LDT reagents is currently unclear. It is noted that Zickermann et al. demonstrated significant differences in the orientations of these acidic residues on the loop between bacterial and mitochondrial complex I.[4,5] Further structural information about the quinone binding pocket is needed to elucidate the mechanism responsible for the very strong nucleophilicity of 49 kDa Asp160.

In conclusion, we prepared the very potent amide-type amiloride AAT as a LDT chemistry reagent. The tosylate (SE) group incorporated into AAT specifically reacted with 49 kDa Asp160 within the quinone binding pocket. The results of the competition test suggested that the binding position of amide-type amilorides within the pocket is somewhat different from that of prototypical guanidine-type amilorides. By taking into consideration the fact that 49 kDa Asp160 was also specifically labeled by LDT chemistry reagents derived from acetogenin,[15,16] we found this aspartic acid to elicit very strong nucleophilicity in the local protein environment.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Syntheses of **AA1**−**AA9** and AAT, structures of TAMRA-azido and biotin-SS-azido tags (Figure S1), LC−MS analysis of the labeled 49 kDa subunit (Figure S2), and structures of SE, CA, and UK (Figure S3). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00385.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CA, α-chloroacetamide; CBB, Coomassie brilliant blue R250; complex I, proton-translocating NADH-quinone oxidoreductase; LDT, ligand-directed tosyl; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SE, phenylsulfonate ester; SMP, submitochondrial particles; TMH, transmembrane helix; UK, α,β-unsaturated ketone.

■ REFERENCES

(1) Brandt, U. (2006) Energy converting NADH:quinone oxidoreductase (complex I). *Annu. Rev. Biochem. 75*, 69−92.
(2) Hirst, J. (2013) Mitochondrial complex I. *Annu. Rev. Biochem. 82*, 551−575.
(3) Carroll, J., Fearnley, I. M., Skehel, J. M., Shannon, R. J., Hirst, J., and Walker, J. E. (2006) Bovine complex I is a complex of 45 different subunits. *J. Biol. Chem. 43*, 32724−32727.
(4) Baradaran, R., Berrisford, J. M., Minhas, G. S., and Sazanov, L. A. (2013) Crystal structure of the entire respiratory complex I. *Nature 494*, 443−448.
(5) Zickermann, V., Wirth, C., Nasiri, H., Siegmund, K., Schwalbe, H., Hunte, C., and Brandt, U. (2015) Mechanistic insight from the crystal structure of mitochondrial complex I. *Science 347*, 44−49.
(6) Vinothkumar, K. R., Zhu, P., and Hirst, J. (2014) Architecture of mammalian respiratory complex I. *Nature 515*, 80−84.
(7) Kleyman, T. R., and Cragoe, E. J., Jr. (1988) Amiloride and its analogs as tools in the study of ion transport. *J. Membr. Biol. 105*, 1−21.
(8) Putney, L. K., Denker, S. P., and Barber, D. L. (2002) The changing face of the Na^+/H^+ exchanger, NHE: Structure, regulation, and cellular actions. *Annu. Rev. Pharmacol. Toxicol. 42*, 527−552.
(9) Nakamaru-Ogiso, E., Seo, B. B., Yagi, T., and Matsuno-Yagi, A. (2003) Amiloride inhibition of the proton-translocating NADH-quinone oxidoreductase of mammals and bacteria. *FEBS Lett. 549*, 43−46.
(10) Stolpe, S., and Friedrich, T. (2004) The *Escherichia coli* NADH-ubiquinone oxidoreductase (complex I) is a primary proton pump but may be capable of secondary sodium antiport. *J. Biol. Chem. 279*, 18377−18383.
(11) Mathiesen, C., and Hägerhäll, C. (2002) Transmembrane topology of the NuoL, M, and N subunits of NADH:quinone oxidoreductase and their homologues among membrane-bound hydrogenases and *bona fide* antiporters. *Biochim. Biophys. Acta 1556*, 121−132.
(12) Murai, M., Murakami, S., Ito, T., and Miyoshi, H. (2015) Amilorides bind to the quinone binding pocket of bovine mitochondrial complex I. *Biochemistry 54*, 2739−2746.
(13) Tsukiji, S., Miyagawa, M., Takaoka, Y., Tamura, T., and Hamachi, I. (2009) Ligand-directed tosyl chemistry for protein labeling *in vivo*. *Nat. Chem. Biol. 5*, 341−343.
(14) Takaoka, Y., Ojida, A., and Hamachi, I. (2013) Protein organic chemistry and applications for labeling and engineering in live-cell systems. *Angew. Chem., Int. Ed. 52*, 4088−4106.
(15) Masuya, T., Murai, M., Ifuku, K., Morisaka, H., and Miyoshi, H. (2014) Site-specific chemical labeling of mitochondrial respiratory complex I through ligand-directed tosylate chemistry. *Biochemistry 53*, 2307−2317.
(16) Masuya, T., Murai, M., Morisaka, H., and Miyoshi, H. (2014) Pinpoint chemical modification of Asp160 in the 49 kDa subunit of bovine mitochondrial complex I via a combination of ligand-directed tosyl chemistry and click chemistry. *Biochemistry 53*, 7816−7823.
(17) Ino, T., Nishioka, T., and Miyoshi, H. (2003) Characterization of inhibitor binding sites of mitochondrial complex I using fluorescent inhibitor. *Biochim. Biophys. Acta 1605*, 15−20.
(18) Murai, M., Habu, S., Murakami, S., Ito, T., and Miyoshi, H. (2015) Production of new amilorides as potent inhibitors of mitochondrial respiratory complex I. *Biosci., Biotechnol., Biochem.*, DOI: 10.1080/09168451.2015.1010479.
(19) Matsuno-Yagi, A., and Hatefi, Y. (1985) Studies on the mechanism of oxidative phosphorylation. *J. Biol. Chem. 260*, 14424−14427.
(20) Murai, M., Ichimaru, N., Abe, M., Nishioka, T., and Miyoshi, H. (2006) Mode of inhibitory action of Δlac-acetogenins, a new class of inhibitors of bovine heart mitochondrial complex I. *Biochemistry 45*, 9778−9787.
(21) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680−685.
(22) Schägger, H. (2006) Tricine-SDS-PAGE. *Nat. Protoc. 1*, 16−21.
(23) Hunt, T., Atherton-Watson, H. C., Collingwood, S. P., Coote, K. J., Czarnecki, S., Danahay, H., Howsham, C., Hunt, P., Paisley, D., and Young, A. (2012) Discovery of a novel chemotype of potent human ENaC blockers using a bioisostere approach. Part 1: Quaternary amines. *Bioorg. Med. Chem. Lett. 22*, 929−932.
(24) Hunt, T., Atherton-Watson, H. C., Collingwood, S. P., Coote, K. J., Czarnecki, S., Danahay, H., Howsham, C., Hunt, P., Paisley, D., and Young, A. (2012) Discovery of a novel chemotype of potent human ENaC blockers using a bioisostere approach. Part 2: α-Branched quaternary amines. *Bioorg. Med. Chem. Lett. 22*, 2877−2879.
(25) Wang, Q., Chan, T. R., Hilgraf, R., Fokin, V. V., Sharpless, K. B., and Finn, M. G. (2003) Bioconjugation by copper(I)-catalyzed azide-alkyne [3+2] cycloaddition. *J. Am. Chem. Soc. 125*, 3192−3193.
(26) Batista, A. P., Marreiros, B. C., and Pereira, M. M. (2011) Decoupling of the catalytic and transport activities of complex I from *Rhodothermus marinus* by sodium/proton antiporter inhibitor. *ACS Chem. Biol. 6*, 477−483.
(27) Di Virgilio, F., and Azzone, G. F. (1982) Activation of site I redox-driven H^+ pumping by exogenous quinones in intact mitochondria. *J. Biol. Chem. 257*, 4106−4113.
(28) Roberts, P. G., and Hirst, J. (2012) The deactive from of respiratory complex I from mammalian mitochondria is a Na^+/H^+ antiporter. *J. Biol. Chem. 287*, 34743−34751.
(29) Davies, K., and Solioz, M. (1992) Assessment of uncoupling by amiloride analogues. *Biochemistry 31*, 8055−8058.
(30) Miyoshi, H., Nishioka, T., and Fujita, T. (1987) Quantitative relationship between protonophoric and uncoupling activities of analogs of SF6847 (2,6-di-*t*-butyl-4-(2′,2′-dicyanovinyl)phenol). *Biochim. Biophys. Acta 891*, 293−299.
(31) Sinha, P. K., Castro-Guerrero, N., Patki, G., Sato, M., Torres-Bacete, J., Sinha, S., Miyoshi, H., Matsuno-Yagi, A., and Yagi, T. (2015) Conserved amino acid residues of the NuoD segment important for structure and function of *Escherichia coli* NDH-1 (complex I). *Biochemistry 54*, 753−764.
(32) Murai, M., Ishihara, A., Nishioka, T., Yagi, T., and Miyoshi, H. (2007) The ND1 subunit constructs the inhibitor binding domain in bovine heart mitochondrial complex I. *Biochemistry 46*, 6409−6416.

DOI: 10.1021/acs.biochem.5b00385
Biochemistry 2015, 54, 3677−3686

(33) Sekiguchi, K., Murai, M., and Miyoshi, H. (2009) Exploring the binding site of acetogenin in the ND1 subunit of bovine mitochondrial complex I. *Biochim. Biophys. Acta 1787*, 1106−1111.
(34) Nakanishi, S., Abe, M., Yamamoto, S., Murai, M., and Miyoshi, H. (2011) Bis-THF motif of acetogenin binds to the third matrix-side loop of ND1 subunit in mitochondrial NADH-ubiquinone oxidoreductase. *Biochim. Biophys. Acta 1807*, 1170−1176.
(35) Weerapana, E., Simon, G. M., and Cravatt, B. (2014) Disparate proteome reactivity profiles of carbon electrophiles. *Nat. Chem. Biol. 4*, 405−407.
(36) Shiraishi, Y., Murai, M., Sakiyama, N., Ifuku, K., and Miyoshi, H. (2012) Fenpyroximate binds to the interface between PSST and 49 kDa subunits in mitochondrial NADH-ubiquinone oxidoreductase. *Biochemistry 51*, 1953−1963.
(37) Murai, M., Sekiguchi, K., Nishioka, T., and Miyoshi, H. (2009) Characterization of the inhibitor binding site in mitochondrial NADH-ubiquinone oxidoreductase by photoaffinity labeling using a quinazoline-type inhibitor. *Biochemistry 48*, 688−698.

DOI: 10.1021/acs.biochem.5b00385
Biochemistry 2015, 54, 3677−3686