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Site-Specific Chemical Labeling of Mitochondrial Respiratory Complex I through Ligand-Directed Tosylate Chemistry

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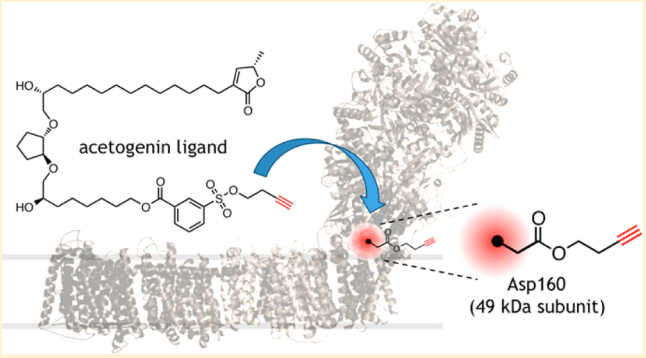
Supporting Information

ABSTRACT: The site-specific chemical modification of NADH-quinone oxidoreductase (complex I) by various functional probes such as fluorophores and microbeads, without affecting the enzyme activity, may allow single-molecule analyses of putative dynamic conformational changes in the enzyme. In an attempt to address this challenge, we performed site-specific alkynylation of complex I in bovine heart submitochondrial particles by means of a ligand-directed tosylate (LDT) chemistry strategy with synthetic acetogenin ligand **1**, which has an alkynylated tosylate in the tail moiety, as a high-affinity ligand against the enzyme. The terminal alkyne was chosen as the tag to be incorporated into the enzyme because this functional group can serve as a "footing" for subsequent diverse chemical modifications via so-called click chemistry (i.e., azide−alkyne [3+2] cycloaddition in water). To identify the position alkynylated by ligand **1**, fluorescent tetramethylrhodamine was covalently attached to the incorporated alkyne by click chemistry after the solubilization of complex I. Detailed proteomic analyses revealed that alkynylation occurred at Asp160 in the 49 kDa subunit, which may be located in the inner part of the putative quinone-binding cavity. The alkynylation was completely suppressed in the presence of an excess of other inhibitors such as bullatacin and quinazoline. While the reaction yield of the alkynylation step via LDT chemistry was estimated to be ∼50%, the alkynylation unfortunately resulted in the almost complete inhibition of enzyme activity. Nevertheless, the results of this study demonstrate that complex I can be site-specifically alkynylated through LDT chemistry, providing a clue about the diverse chemical modifications of the enzyme in combination with click chemistry.



NADH-quinone oxidoreductase (respiratory complex I) couples the transfer of two electrons from NADH to quinone with translocation of four protons across the membrane that drives energy-consuming processes such as ATP synthesis.[1,2] Complex I is the largest of the respiratory chain complexes. The enzyme from bovine heart mitochondria is composed of 45 different subunits with a total molecular mass of ∼1 MDa.[3] Electron density maps were presented for the entire complex I from *Thermus thermophilus*[4] and *Yarrowia lipolytica*[5] at 4.5 and 6.3 Å resolution, respectively. As expected from electron microscopic studies, the enzyme is L-shaped with a membrane and hydrophilic domain. Recently, the structure of the entire *T. thermophilus* complex I was described for the first time at 3.3 Å resolution,[6] providing more useful structural information about the mechanism of coupling between electron transfer and proton translocation in the enzyme. Together with the crystal structure of six membrane subunits of *Escherichia coli* complex I at 3.0 Å,[7] a possible proton translocation pathway was proposed in subunits Nqo12 (NuoL in *E. coli*, ND5 in bovine), Nqo13 (NuoM, ND4), and Nqo14 (NuoN, ND2).[6] The Nqo12, Nqo13, and Nqo14 subunits are homologous to the subunits from the Mrp family of Na^+/H^+ antiporters,[8] which suggests that their mechanisms of ion translocation are related. In this context, it is noteworthy that the "deactive" form of bovine heart complex I elicits Na^+/H^+ antiporter activity.[9] Dynamic conformational movement of the domain has been suggested when the membrane domain translocates protons across the membrane.[6]

Single-molecule analyses are powerful techniques for studying protein conformational dynamics (e.g., a rotary motor protein F_1-ATPase);[10,11] however, these techniques have never been applied to the study of complex I. Site-specific modification of complex I with various functional probes such as fluorophores and microbeads, without affecting the enzyme activity, may allow single-molecule detection or imaging of the enzyme. Various techniques have been used for the site-specific labeling and engineering of native proteins.[12−14] A novel protein labeling methodology termed ligand-directed tosylate (LDT) chemistry, which is based on the principle of affinity labeling (Figure S1 of the Supporting Information), has been applied to various proteins.[14,15] LDT chemistry uses a labeling

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reagent, in which a protein ligand and synthetic tag of choice are connected by an electrophilic tosylate (phenylsulfonate) 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.[14] After nucleophilic substitution, the ligand moiety may dissociate from the binding pocket because of a marked increase in the polarity (hydrophilicity) of the resulting phenylsulfonate anion. Thus, this method provides a powerful way to attach diverse synthetic tags to the target proteins with high site specificity and, in principle, in a traceless manner.[14,15]

LDT chemistry is useful for site-specific chemical labeling; however, if a ligand-binding pocket is deeply buried inside the target protein, such as the complicated membrane protein complex I, the direct incorporation of the tag seems to be impractical because of severe steric interference. Therefore, we were curious to know whether the chemical labeling via LDT chemistry is feasible with complex I in bovine heart submitochondrial particles (SMP). We here chose acetogenin and terminal alkyne as the high-affinity ligand and tag to be incorporated, respectively; we synthesized acetogenin ligand **1** (Figure 1). The terminal alkyne was chosen because this group is able to serve as a "footing" for subsequent diverse chemical modifications via so-called click chemistry. Click chemistry is a convenient method for binding two molecules in water by azide−alkyne [3+2] cycloaddition with a high reaction yield[14,16] (see Figure S1 of the Supporting Information). We performed the alkynylation of complex I via LDT chemistry by incubating bovine SMP with ligand **1**. To identify the alkynylated position, a fluorescent tetramethylrhodamine (TAMRA) tag (Figure 1) was covalently attached to the incorporated alkyne by click chemistry after the solubilization of complex I from SMP. Detailed proteomic analyses revealed that the alkynylation specifically occurred at Asp160 in the 49 kDa subunit with high efficiency. The results of this study demonstrate that complex I can be site-specifically alkynylated through LDT chemistry, providing a clue about the diverse chemical modifications of the enzyme in combination with click chemistry.

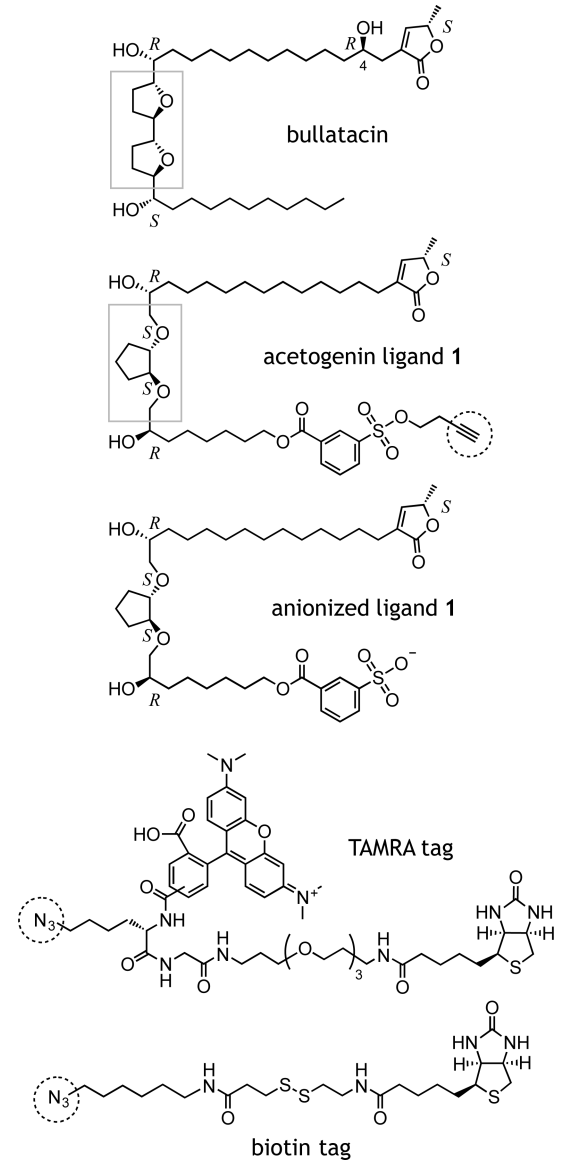

Figure 1. Structures of bullatacin (a natural acetogenin), acetogenin ligand **1**, the anionized form of **1**, and "second" tags (TAMRA and biotin tags). The bis-THF ring and 1,2-cyclopentanediol bis-ether skeleton are boxed with gray lines. The alkyne and azido functional groups are circled with dotted lines.

EXPERIMENTAL PROCEDURES

Materials. Bullatacin was kindly provided by J. L. McLaughlin (Purdue University, West Lafayette, IN). Aminoquinazoline (AQ) was synthesized by the procedures described previously.[17] Protein standards (Precision Plus Protein standards and Kaleidoscope Polypeptide Standards) for SDS−PAGE were purchased from Bio-Rad (Hercules, CA). The Click-iT reaction buffer kit was purchased from Life Technologies (Carlsbad, CA). Other reagents were all of analytical grade.

Synthesis of Acetogenin Ligand 1 and Tags. The procedure used to synthesize acetogenin ligand **1** (Figure 1) is described in the Supporting Information. Fluorescent TAMRA and biotin tags (Figure 1) were synthesized according to the procedures previously reported.[18] The spectral data of the newly synthesized tags are provided in the Supporting Information.

Preparation of Bovine Heart SMP and General Procedures for LDT Chemistry. Bovine heart 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 before being used. The NADH oxidase and NADH-Q_1 oxidoreductase activities in SMP were measured according to the procedures described previously.[20,21] The content of complex I in SMP was roughly estimated as the minimal amount of bullatacin required to completely inhibit the NADH oxidase activity because this inhibitor is the most potent inhibitor of bovine complex I and binds to the enzyme in a stoichiometric manner;[22] the content of complex I in 1.0 mg of SMP proteins was estimated to be 0.10 nmol.

SMP (2.0 mg of protein/mL, 100−200 μL) were incubated with acetogenin ligand **1** in a buffer containing 250 mM sucrose, 1 mM $MgCl_2$, and 50 mM KP_i (pH 7.4) for 24 h at 35 °C. SMP were then collected by ultracentrifugation (200000*g* for 20 min at 4 °C) and denatured in 1% (w/v) SDS (25−50 μL). The proteins, alkynylated by ligand **1** via LDT chemistry, were conjugated with the TAMRA or biotin tag via click chemistry using the Click-iT reaction buffer kit (Life Technologies) according to the manufacturer's instructions.

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The proteins were recovered by precipitation with methanol and chloroform and subjected to further analyses.

Anionized ligand **1** and nonreacted ligand **1** residing in SMP after the alkynylation reaction were removed by washing SMP with a buffer containing 1% (w/v) bovine serum albumin (BSA) several times. Briefly, SMP treated with ligand **1** in a buffer containing 250 mM sucrose, 1 mM $MgCl_2$, and 50 mM KP_i (pH 7.4) were sedimented by ultracentrifugation (200000*g* for 20 min at 4 °C). The pellets were resuspended by homogenization in the same buffer, but with 1% (w/v) BSA. SMP were again sedimented by ultracentrifugation, and the pellets were gently rinsed with a BSA-free buffer. The pellets were then resuspended in a BSA-free buffer, and an aliquot of the suspension was removed for the NADH oxidase activity assay. This washing treatment was repeated three times.

Electrophoresis. TAMRA-attached proteins were separated on a Laemmli-type SDS gel.[23] The migration pattern of fluorescent proteins was visualized by the model FLA-5100 Bio-imaging analyzer (Fuji Film, Tokyo, Japan), using a 532 nm light source and an LPG (575 nm) filter. Data processing and the quantification of fluorescence were conducted using Multi gauge software (Fuji Film). To isolate complex I, SMP treated with ligand **1** were separated by Blue Native-PAGE (BN-PAGE) using a precast 4 to 16% gel system (Life Technologies). Complex I was isolated by electroelution using a model 422 Electro-Eluter (Bio-Rad) and subjected to covalent conjugation with the TAMRA tag via click chemistry.

To analyze the TAMRA-attached 49 kDa subunit of complex I, the subunit was partially purified by SDS−PAGE and electroelution.[24] The purified subunit was digested with lysylendopeptidase (Lys-C), endoprotease Asp-N, or cyanogen bromide (CNBr) in 50 mM Tris-HCl buffer (containing 0.1% SDS), 50 mM NaP_i buffer (containing 0.01% SDS), or 70% formic acid, respectively.[25] The digests were separated on a Schägger-type SDS gel (16.5% T and 6% C, containing 6.0 M urea).[26] To partially digest the TAMRA-attached 49 kDa subunit, it was digested in the 15% Tris-EDTA mapping gel using V8 protease according to the procedure described previously.[24,27]

Enrichment of Biotinylated Proteins. The biotin-attached proteins via click chemistry were captured by immobilized streptavidin on agarose.[28] Briefly, the alkynylated proteins covalently conjugated with the cleavable biotin tag (Figure 1) were recovered by precipitation with methanol and chloroform and solubilized in Tris-buffered saline (TBS buffer, 60 μL) containing 2% (w/v) SDS at 40 °C for 1 h. The solubilized proteins were diluted with TBS buffer (500 μL) containing 1% (w/v) Triton X-100, and the sample was incubated with streptavidin-agarose CL-4B (50 μL suspension, Sigma) 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 TBS buffer without detergent. The biotinylated proteins were eluted in Laemmli's sample buffer containing 2.5% mercaptoethanol, separated on Laemmli-type SDS−PAGE gel, and visualized by CBB staining.

MALDI-TOF MS. Proteins were identified by peptide mass fingerprinting analysis. Proteins were digested in gel with trypsin in a buffer containing 25 mM NH_4HCO_3 at 37 °C overnight. The tryptic digests were desalted with a ZipTip (Millipore) and spotted onto the target plate using α-cyano-4-hydroxycinnamic acid as a matrix.[25] Mass spectrometric analysis was conducted using a Bruker Autoflex III Smartbeam instrument (MALDI-TOF/TOF, Bruker Daltonics). The obtained MS spectra were analyzed according to the previously described procedures using Mascot.[25]

Liquid Chromatography and Mass Spectrometry (LC−MS). The alkynylated site in the 49 kDa subunit was identified by a nano LC (Ultimate 3000, Thermo Scientific) and MS (LTQ Velos orbitrap mass spectrometer, Thermo Scientific, Waltham, MA) system equipped with a monolithic silica capillary column.[29] The alkynylated 49 kDa subunit was enriched by the same procedures described above and digested "in gel" by trypsin, and the digests were extracted with 50% acetonitrile containing 5% formic acid. The tryptic digests were separated by reversed-phase chromatography at a flow rate of 500 nL/min. The gradient was provided by changing the mixing ratio of the two eluents, A being 0.1% (v/v) formic acid and B being 80% acetonitrile containing 0.1% (v/v) formic acid. The gradient was started with 5% elute B, increased to 45% elute B for 60 min, and further increased to 95% elute B to wash the column, and then the ratio was returned to the initial conditions and held for re-equilibration.

The separated peptides were detected via MS with full scan range of m/z 350−1500 in the positive mode followed by 10 data-dependent collision-induced dissociation MS/MS scans. The method used for data-dependent acquisition was set to automatically analyze the top 10 most intense ions observed in the MS scan. An electrospray ionization voltage of 2.4 kV was applied directly to the LC buffer distal to the chromatography column using a microtee. The ion transfer tube temperature on the LTQ Velos ion trap was set to 300 °C.

Collected data were analyzed using Proteome Discoverer version 1.4 (Thermo Scientific) with Mascot (Matrix Science, London, U.K.) for protein identification. The following parameters were employed: Swiss Prot, mammals; precursor ion mass tolerance, 5 ppm; fragment ion mass tolerance, 0.8 Da; trypsin, one missed cleavage. For the trypsin digestion, carbamidomethylation (Cys), oxidation (Met), alkynylation [$C_{15}H_{25}N_5O_2S$ (at Asp, Glu, and His) (see Figure 6A)] were set as a dynamic modification. Data were then filtered at a q value of ≤0.01 corresponding to a 1% false discovery rate.

N-Terminal Sequence Analysis. N-Terminal amino acid residues were determined with a Procise 494 HT protein sequencing system (Applied Life Sciences, Foster City, CA) at the APRO Life Science Institute, Inc. (Tokushima, Japan).

RESULTS

Synthesis of Acetogenin Ligand 1 for LDT Chemistry. In the LDT chemistry strategy, the ligand drives a labeling reagent (tag) to the target protein (Figure S1 of the Supporting Information). We chose acetogenin as a high-affinity ligand against bovine complex I because it is one of the most potent inhibitors of the enzyme. Previous photoaffinity labeling studies revealed that the γ-lactone ring and bis-THF ring moiety of acetogenin, which are essential structural units for the inhibitory action,[30,31] bind to transmembrane helix 4 or 5[32] and the third matrix loop of the ND1 subunit,[33] respectively, indicating that the binding site of acetogenin is located in ND1. We synthesized acetogenin ligand **1**, which possesses an alkynylated phenylsulfonate (tosylate) in its tail moiety. In ligand **1**, the adjacent bis-THF ring skeleton, which is a common structural motif in many natural acetogenins, was replaced with an enantiometric 1,2-cyclopentanediol bis-ether motif to simplify the synthetic task of making the bis-THF ring. We previously demonstrated that the 1,2-cyclopentanediol bis-ether motif and bis-THF ring can be three-dimensionally

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superimposed; hence, the former can substitute for the latter while a very potent inhibitory activity is maintained.[34] Additionally, we note that the OH group at position 4 of bullatacin is not essential for the inhibitory activity.[30,31]

The inhibitory effects of ligand **1** and bullatacin (a natural acetogenin) were examined with the NADH oxidase activity in SMP (30 μg of protein/mL). The IC_{50} values of ligand **1** and bullatacin were 3.2 ± 0.20 and 0.90 ± 0.15 nM, respectively, which indicated that ligand **1** is slightly less active presumably because of steric bulkiness but still maintains a potent inhibitory effect at the nanomolar level. Therefore, we conclude that ligand **1** can be used for LDT chemistry as a promising ligand.

LDT Chemistry with Bovine SMP. Bovine SMP (2.0 mg of protein/mL, equivalent to ~200 nM complex I) were incubated with ligand **1** (50 nM to 10 μM) at 35 °C for 24 h, followed by solubilization with 1% SDS. The fluorescent TAMRA tag was then attached to the incorporated alkyne via click chemistry using a commercially available reaction kit (Click-iTR reaction buffer kit). If both LDT chemistry and subsequent click chemistry occur successfully, the terminal alkyne transferred from ligand **1** to complex I can be visualized as a fluorescent band(s) on the SDS gel.

As shown in Figure 2, the alkyne was specifically incorporated into the ~50 kDa protein in a concentration-dependent manner. The saturation of the fluorescence increase in this band was achieved at 1.0 μM ligand **1**. At high concentrations of ligand **1**, the ~25 kDa protein was also slightly alkynylated (Figure 2A). To ascertain suitable conditions for LDT chemistry with SMP, we also varied the incubation period by fixing the ligand concentration at 1.0 μM. As LDT chemistry proceeded slowly, SMP had to be incubated with ligand **1** for at least 13−15 h (data not shown).

Identification of the Alkynylated Subunit of Complex I. To establish whether the fluorescent ~50 kDa band is a component of complex I, SMP treated with ligand **1** were separated by BN-PAGE (Figure 3A), and complex I was isolated by electroelution. The isolated complex I was reacted with the TAMRA tag under click chemistry conditions and analyzed on a Laemmli-type SDS gel. As shown in Figure 3B, fluorescence was observed only in the band corresponding to the 49 kDa subunit (confirmed by MS as described below).

We succeeded in enriching the alkynylated proteins using an immobilized streptavidin. SMP (2.0 mg of protein/mL) were treated with 5.0 μM ligand **1**, followed by solubilization with 1% SDS, and the cleavable biotin tag (Figure 1) was then attached via click chemistry. The biotinylated proteins were captured and released by streptavidin-agarose (Figure 3C). The major ~50 kDa band was identified as the 49 kDa subunit by MALDI-TOF MS analysis of its tryptic digests [41% coverage (Figure S2 and Table S1 of the Supporting Information)]. We also observed a faint CBB-stained band in the ~25 kDa region, which corresponds to the minor fluorescent band observed in Figure 2A. This band was identified as the ADP/ATP carrier by MALDI-TOF MS analysis of its tryptic digests [43% coverage (Figure S2 and Table S1 of the Supporting Information)]. This minor labeling of the ADP/ATP carrier was not examined in further detail.

While natural acetogenins bind to the ND1 subunit, as mentioned above, the terminal alkyne group of ligand **1** specifically labeled the 49 kDa subunit. This is probably because the alkyl tail moiety of ligand **1** is inherently flexible and fairly polar because of the presence of the ester and sulfonate groups,

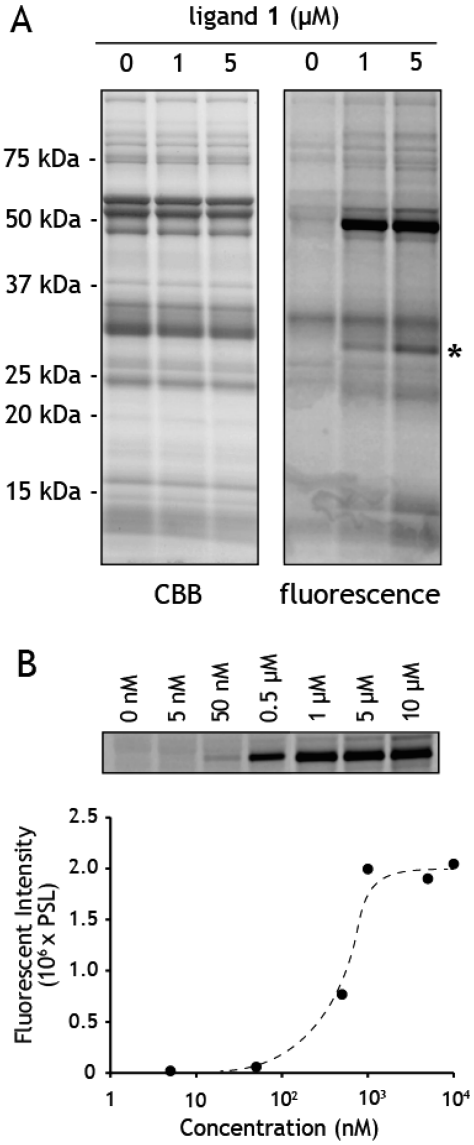

Figure 2. LDT chemistry using ligand **1** with bovine heart SMP. (A) Bovine heart SMP (2.0 mg of protein/mL) were alkynylated via LDT chemistry using ligand **1** at 35 °C for 24 h and denatured with 1% (w/v) SDS, followed by the attachment of the fluorescent TAMRA tag (Figure 1) via click chemistry. The proteins in SMP were separated on a 12.5% Laemmli-type SDS gel and subjected to fluorescent gel imaging. A minor labeled protein is marked with an asterisk. (B) Concentration dependency of the alkynylation reaction against the ~50 kDa protein. The values represent an average of two independent experiments. A fluorescent image of the ~50 kDa band at the given concentrations is shown.

which allowed the tail moiety to extend from the membrane domain (ND1) into the hydrophilic domain.

On the other hand, our previous photoaffinity labeling studies indicated that structurally diverse complex I inhibitors share a large binding pocket composed of the hydrophilic 49 kDa/PSST subunits and the hydrophobic ND1 subunit.[20,21,33] We examined the effects of other complex I inhibitors on the specific alkynylation of the 49 kDa subunit. An excess amount (100-fold molar excess) of bullatacin and aminoquinazoline completely suppressed the labeling (Figure 4). These results strongly suggest that the alkynylated region may comprise a

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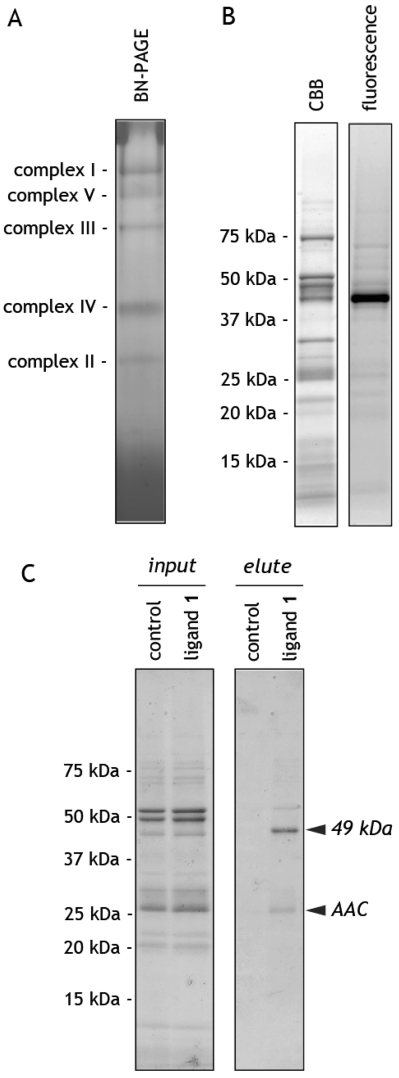

Figure 3. Analysis of complex I alkynylated by ligand **1**. (A) BN-PAGE of SMP treated with ligand **1**. SMP (2.0 mg of protein/mL) were alkynylated with 1.0 μM ligand **1** at 35 °C for 24 h and separated on a 4 to 16% BN gel. The complex I band was then isolated by electroelution. (B) Analysis of the TAMRA-attached complex I. The isolated complex I, which was alkynylated by ligand **1**, was denatured in 50 mM Tris-HCl containing 1% (w/v) SDS and subjected to the covalent attachment of the TAMRA tag via click chemistry. The proteins were separated on a 12.5% Laemmli-type SDS gel. (C) Enrichment of the alkynylated proteins. SMP (2.0 mg of protein/mL) alkynylated by 5.0 μM ligand **1** were denatured as described in the legend of Figure 2 and biotinylated with the cleavable biotin tag (Figure 1) via click chemistry. The biotinylated proteins were enriched using streptavidin-agarose, as described in Experimental Procedures. SMP not treated with ligand **1** were used as a control. The enriched ~50 and ~25 kDa proteins were identified as the 49 kDa subunit and ADP/ATP carrier, respectively, by MALDI-TOF MS.

part of the putative inhibitor/ubiquinone-binding pocket in complex I.[6]

Identification of the Alkynylated Site in the 49 kDa Subunit. To roughly localize the alkynylated site, the 49 kDa subunit isolated from SMP treated with ligand **1** was partially digested with V8 protease. This partial digestion provided several fluorescent bands on a 15% Tris-EDTA SDS−PAGE mapping gel, which converged on ~13 kDa bands (fragments B and C, Figure 5A). The N-terminal sequence of fragments B

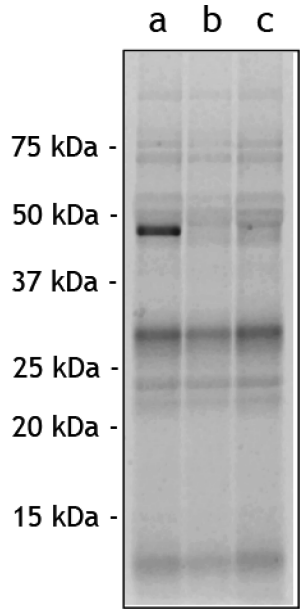

Figure 4. Effects of other complex I inhibitors on the alkynylation of the 49 kDa subunit. SMP (2 mg of protein/mL) were incubated with 0.1 μM ligand **1** in the absence (a) or presence of 10 μM bullatacin (b) or aminoquinazoline (c). SMP treated with ligand **1** were denatured, attached by the fluorescent TAMRA tag, and analyzed on a 12.5% Laemmli-type SDS gel. Data are representative of three independent experiments.

and C, transferred onto a PVDF membrane, was determined to be H_2N-Q^{116}ASYL in common, indicating that the former and latter are the Gln116−Glu224 and Gln116−Glu213 regions, respectively (Figure 5C). Because the N-terminal sequence of fragment A was determined to be H_2N-T^{25}AHWK, the alkyne was incorporated into the Gln116−Glu213 region.

Next, the 49 kDa subunit was subjected to exhaustive digestion by Lys-C and Asp-N. Major fluorescent bands were observed at ~8 and ~12 kDa, respectively (Figure 5B). A careful examination of the predicted cleavage sites for the proteases in the region determined by the V8 partial digestion (Gln116−Glu213) strongly suggested that the Lys-C and Asp-N digests are peptides Lys125−Lys176 (6.1 kDa) and Asp107−Gln201 (11.1 kDa), respectively.

We could not detect modified peptides in the tryptic digests of the 49 kDa subunit, which was enriched as described in the legend of Figure 3, by peptide mass fingerprinting analysis via MALDI-TOF MS. This result suggests that the alkynylated site may be located in a region that cannot be detected by this analysis. Therefore, we reinvestigated the tryptic digests of the enriched 49 kDa subunit by LC−MS. The sequence coverage of LC−MS analysis of the nonalkynylated (control) 49 kDa subunit was 76%, including 27 peptides (Figure S3 of the Supporting Information). The fragment ion spectra of the modified 49 kDa subunit were compared to those of the control subunit.

We detected the fragment ion spectra of triply charged precursor ions, at m/z 1113.87 and 1226.93, from the tryptic digests of the control and modified 49 kDa subunit, respectively, which suggested that the peptide L^{147}LNHIMA-VTTHALDIGAMTPFFWMFEER^{174} as a candidate sequence (Figure 6B). The mass precursor ion from the modified peptide (m/z 1226.93) matches the calculated mass of the control peptide plus 339 Da (m/z 113; z = 3) (Figure 6A). Several b-

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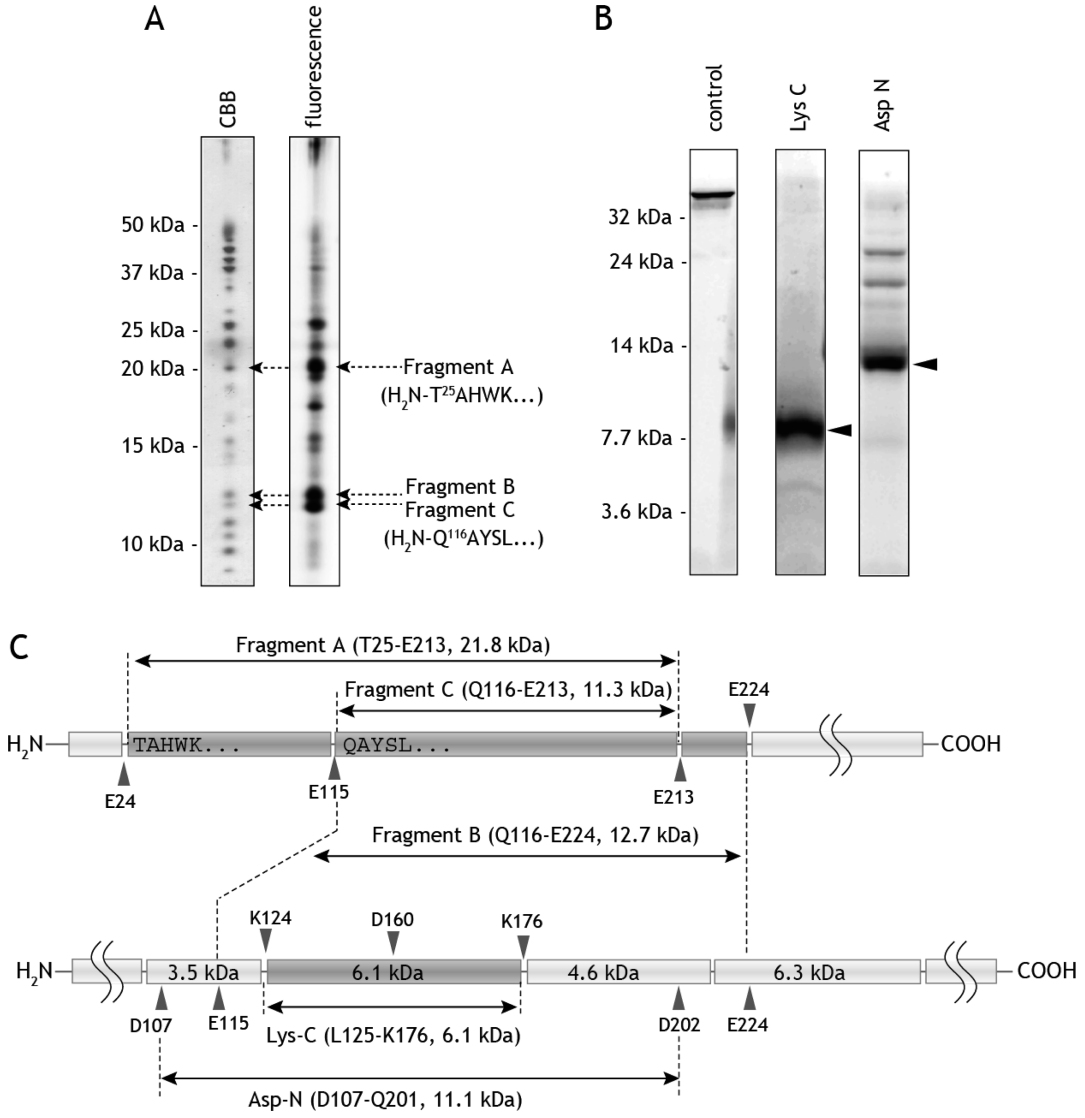

Figure 5. Proteomic analysis of the 49 kDa subunit. Complex I in SMP, which was treated with ligand **1**, was purified by BN-PAGE and covalently attached to the TAMRA tag via click chemistry, followed by SDS−PAGE on a 12.5% Laemmli-type SDS gel. (A) The CBB-stained gel piece of the 49 kDa subunit was subjected to in gel partial digestion with V8 protease.[21] The N-terminal sequences of the CBB-stained bands corresponding to the fluorescent bands were determined by Edman degradation. (B) The 49 kDa subunit was exhaustively digested with Lys-C or Asp-N. Each digest was analyzed on a Schägger-type Tricine gel (16% T and 6% C, containing 6.0 M urea). (C) Schematic presentation of the partial and exhaustive digestion of the 49 kDa subunit. The predicted cleavage sites are denoted with arrows and indicated by their residue numbers in the mature sequences of the bovine 49 kDa subunit (SwissProt entry P17694).

and y-ions (y_1−y_{14} and b_1−b_{13}) were detected in both fragment spectra, whereas the spectra of the modified peptide contained several b- and y-ions (y_{15}−y_{28} and b_{14}−b_{28}) that were not detected in the control peptide. These results strongly suggest that the alkynylation occurred at Asp160 (Figure 6B). It should also be noted that the y_{15} ion (m/z 1908), corresponding to DIGAMTPFFWMFEER, was detected in the control peptide only.

To corroborate the results of MS/MS analysis, we examined the extracted ion chromatograms of the tryptic digests of the control and modified 49 kDa subunits (Figure 6C). The peptide with an m/z value of 1113.87, which corresponds to the sequence L^{147}LNHIMAVTTHALDIGAMTPFFWMFEER^{174}, was detected in only the control sample. The modified peptide (m/z 1226.93) was detected in only the alkynylated 49 kDa subunit. Taken together, our results unambiguously reveal that the alkynylation occurred specifically at Asp160. 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 5B), although this peptide bond was reproducibly cleaved in the previous studies.[21,24]

Estimation of the Reaction Yield of the Alkynylation Step. The amount of the alkynylated 49 kDa subunit must be determined to estimate a reaction yield of LDT chemistry (i.e., the alkynylation step) using ligand **1**. The direct detection of the intact alkynylated and nonalkynylated 49 kDa subunit by MS analysis was unsuccessful. Therefore, we estimated the amount of the alkynylated subunit by an indirect method.

We prepared a calibration plot of the fluorescence intensity of TAMRA by directly dropping 2.0 μL of its aqueous solution (0.5−4.0 μM) onto a glass plate (Fla Fluor Stage 4046, Fuji Film) and quantifying the fluorescence by the model FLA-5000 Bio-imaging analyzer (Fuji Film). By interpolating the

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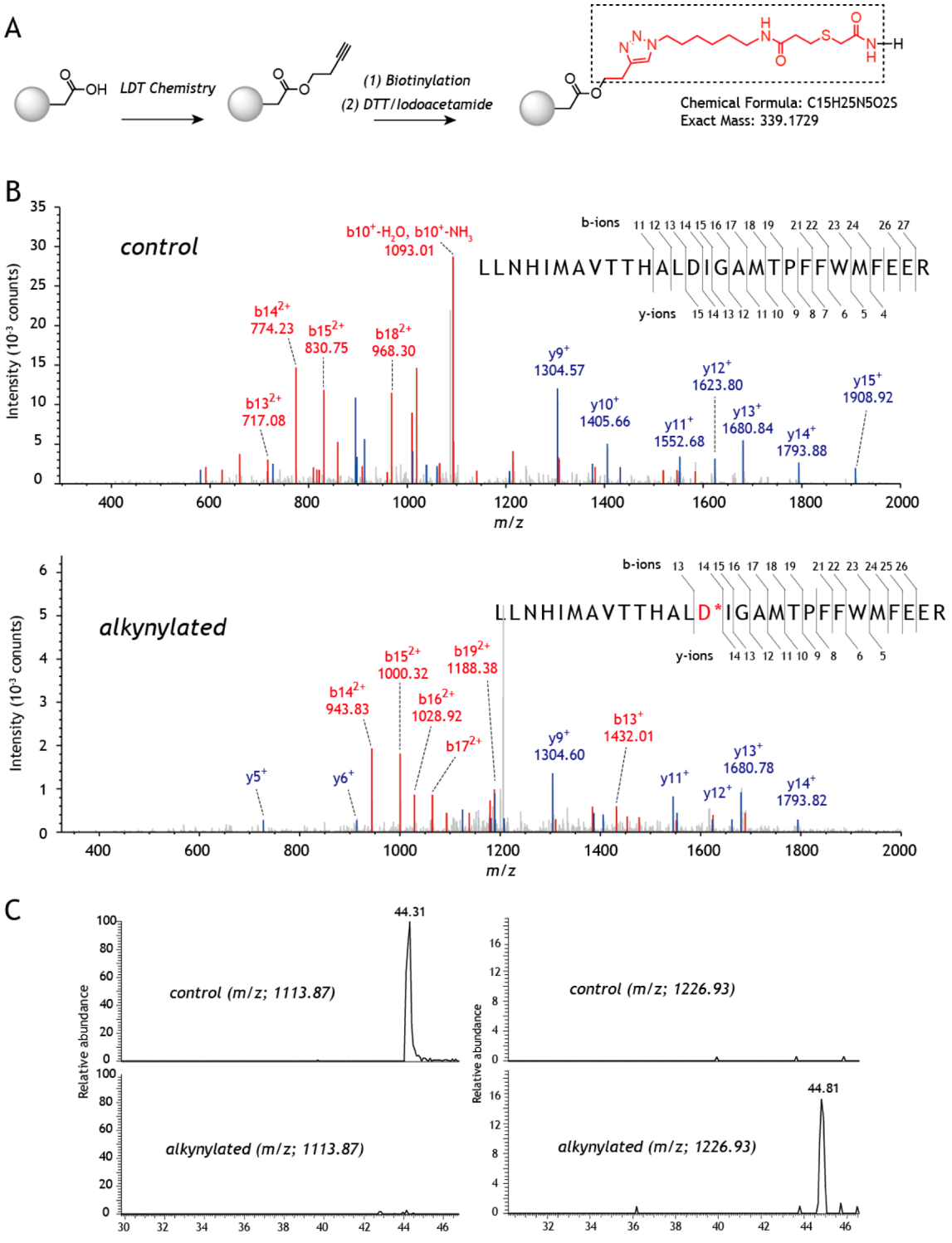

Figure 6. Identification of the alkynylated site in the 49 kDa subunit. The alkynylated 49 kDa subunit was enriched by the same procedure described in the legend of Figure 3. The modified 49 kDa subunit was digested in gel with trypsin, and the peptide mixture was extensively characterized using an Orbitrap mass spectrometer. (A) Predictable formation of a stable adduct by site-specific alkynylation via LDT chemistry, biotinylation via click chemistry, and carbamidomethylation. (B) Fragment ion spectrum of the control and modified peptide L^{147}LNHIMAVTTHALD*IGAMTPF-FWMFEER^{174}, where D* corresponds to an *O*-alkynylated aspartic acid with a mass shift of +339.458. The identified b- and y-fragment ions were mapped onto the amino acid sequence shown in the inset. (C) Extracted ion chromatograms of the peptide L^{147}LNHIMAVTTHALDIGAM-TPFFWMFEER^{174} (m/z 1113.87; left) and its theoretical modified product (m/z 1226.93, right) from the control and modified 49 kDa subunits, respectively, during the reverse-phase separation of the tryptic digests.

fluorescence intensity of the TAMRA-attached 49 kDa subunit in the SDS−PAGE gel, which was alkynylated by 1.0 μM ligand **1** using 2.0 mg of SMP proteins/mL (equivalent to 0.20 μM complex I), to the calibration plot and presuming that the attachment of the TAMRA tag to the alkyne incorporated into the 49 kDa subunit via click chemistry was achieved

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quantitatively (~100%), we estimated the reaction yield of the alkynylation step to be >100%. This overestimation presumably occurs because the amount of the TAMRA-attached 49 kDa subunit in the gel cannot be exactly estimated using the calibration plot, which was prepared by directly coating the glass plate with aqueous solution of TAMRA.

To accurately estimate the amount of the TAMRA-attached 49 kDa subunit in the SDS−PAGE gel, we chose carbonic anhydrase II (CAII, human isozyme II, 260 amino acids, 29246 Da) as a model protein and covalently attached the TAMRA tag (Figure 1) by two reaction steps using *N*-(3-butynyl)-maleimide, as illustrated in Figure S4A of the Supporting Information. CA II has only one cysteine (Cys205) reactive to SH reagents. The amount of TAMRA attached to CAII was estimated to be 14 nmol/mg of CAII based on the fluorescence calibration of definite concentrations of TAMRA. We then prepared a calibration plot of the fluorescence intensity of TAMRA-attached CA II in the SDS−PAGE gel versus the molar quantity of TAMRA attached to CA II (Figure S4B of the Supporting Information). By interpolating the fluorescence intensity of the TAMRA-attached 49 kDa subunit in the SDS−PAGE gel (prepared under the same experimental conditions described above) to the plot, we showed that the fluorescent band of the 49 kDa subunit (Figure S4C of the Supporting Information), in which 2.0 pmol of complex I was charged, corresponds to 0.91 pmol of TAMRA. Because the reaction yield of the covalent attachment of the TAMRA tag to the alkyne via click chemistry was presumed to be 100%, we concluded that at least 45% of the 49 kDa subunit was alkynylated under the experimental conditions in which the extent of the alkynylation reaction is maximal (see Figure 2).

Effect of Alkynylation on Complex I Activity. Ligand **1** becomes a sulfonate anion after the nucleophilic substitution of the terminal alkyne (Figure 1 and Figure S1 of the Supporting Information), which results in a marked increase in its hydrophilicity. As the large hydrophobicity of complex I inhibitors is favorable for their inhibitory action, the anionized ligand may lose its binding affinity for complex I; therefore, the enzyme activity may gradually recover. To confirm this, we assessed the changes in NADH oxidase activity in SMP (30 μg of protein/mL) after the addition of ligand **1**. The concentration of ligand **1** was set to 2, 8, and 20 nM, which elicited ~40, ~80, and ~90% inhibition, respectively. Unexpectedly, the enzyme activity did not recover over a period of 24 h (data not shown). This may be because anionized (and also nonreacted) ligand **1** could still reside in the membrane environment and/or enzyme. As ligand **1** is inherently very hydrophobic, the anionic form may be able to reside in hydrophobic environments. In addition, because the alkyl tail moiety of acetogenins is not an essential structural factor for their inhibitory action,[30,31] the formation of a negative charge in this moiety may not drastically affect its inhibitory activity.

In light of the purpose of this study, it is critical to verify whether complex I maintains the intact activity even after the alkynylation of the 49 kDa subunit. Therefore, we attempted to remove the residual anionized and nonreacted ligand **1** in SMP using bovine serum albumin (BSA). BSA has been widely used to remove hydrophobic chemicals from the mitochondrial membrane.[35] SMP (2.0 mg of protein/mL), which were incubated with 0.5 or 1.0 μM ligand **1** at 35 °C for different periods of time, were washed several times with a buffer containing 1% (w/v) BSA. Ligand **1** exhibited ~70 and ~90% inhibition of the NADH oxidase activity in SMP at 0.5 and 1.0 μM, respectively.

When SMP were incubated with ligand **1** for 5 min, the NADH oxidase activity was almost completely recovered by the BSA washing, irrespective of the concentration of ligand **1** (Figure 7, squares). In contrast, after the 24 h incubation, the activity recovered to maximal levels of ~70 and ~50% at 0.5 and 1.0 μM ligand **1**, respectively (Figure 7, circles). Because the maximal recovery was achieved with once or two washes with SMP, most residual anionized and nonreacted ligands were removed by the BSA washing. These results, along with the fact that the reaction yield of the alkynylation step under the same experimental conditions was ~50%, strongly suggest that the alkynylated complex I almost completely loses its activity. This result was disappointing for us but will be discussed later.

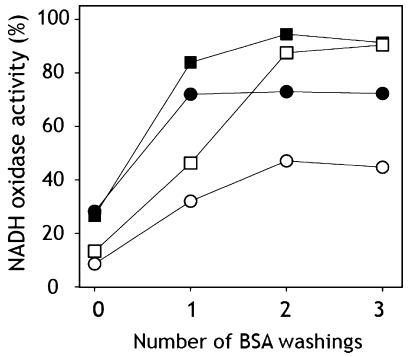

Figure 7. Recovery of the NADH oxidase activity in SMP by BSA washing. SMP (2.0 mg of protein/mL) were incubated with 0.5 μM (filled symbols) or 1.0 μM (empty symbols) ligand **1** for 5 min (squares) or 24 h (circles) at 35 °C. The protein concentration of SMP was set to be 30 μg of protein/mL for the measurement of the NADH oxidase activity. The 100% value is the NADH oxidase activity determined with SMP treated with 1% (w/v) BSA without ligand **1**. Data are representative of two independent experiments.

DISCUSSION

We performed the site-specific alkynylation of complex I in bovine SMP via the LDT chemistry strategy using the newly synthesized acetogenin ligand **1**. Detailed proteomic analyses revealed that the alkynylation occurred at Asp160 in the 49 kDa subunit with a reaction efficiency of ~50%. According to a sequence alignment for the bovine 49 kDa subunit and the *T. thermophilus* Nqo4 subunit, Asp160 (Asp139 in Nqo4) is located in the inner part of the putative quinone-binding cavity.[6] This is implicated as a cause of the severe inhibition of the enzyme activity by the alkynylation. To the best of our knowledge, this and its corresponding residues (e.g., Asp136 in *E. coli* and Asp196 in *Y. lipolytica*)[36] have never been functionally characterized in previous mutagenesis studies. On the other hand, *O*-alkylation of Asp and Glu residues to form an ester product is generally impossible in an aqueous solution because of the weak nucleophilicity of the carboxy group. However, the esterification reaction may be able to proceed between the LDT reagents and target proteins because of the so-called proximity effect.[37]

Various methods of chemical labeling of complex I have been investigated. For instance, SH reagents reacting to the cysteine thiol have been used to examine the location and/or function of certain cysteine residues.[38,39] "Zero-length" bifunctional cross-

linkers {e.g., 1-ethyl-3-[3-(dimethylamine)propyl]-carbodiimide} have been employed to investigate near-neighbor relationship between the subunits under different experimental conditions.[40,41] However, the site specificity of labeling cannot be expected with conventional chemical labeling but is with our labeling strategy. Additionally, the alkynylation itself is not the final goal of our strategy; in other words, the alkynylation is conducted to prepare a footing for subsequent diverse modifications by second tags via click chemistry. Furthermore, our procedure could change not only the labeling position finely by modifying the length of the linker between the 1,2-cyclopentanediol bis-ether moiety and the terminal alkyne but also the labeling position drastically by using different inhibitors as the ligand. We note that amiloride derivatives synthesized in our laboratory bind to the ND3 or ND6 subunit, but not to the quinone cavity, of bovine complex I (unpublished data); hence, these may be promising candidates for different ligands.

Baradaran et al. proposed that a 30 Å long cavity, which is completely shielded from the solvent, with a narrow apparent entry point in the membrane may be the quinone access route toward the cluster N2 and may also accommodate the hydrophobic isoprene tail.[6] As this notion was based on a snapshot structure of the oxidized form of *T. thermophilus* complex I, it remains unclear whether the same holds true for transitional forms during enzyme turnover and for the enzymes of different species. According to a sequence alignment for the bovine 49 kDa and ND1 subunits and *T. thermophilus* Nqo4 and Nqo8, respectively, the position alkynylated by ligand **1** in the 49 kDa subunit (Asp160) is a part of the quinone-binding cavity, as mentioned above. The third matrix loop of ND1, to which the bis-THF ring moiety of acetogenin binds,[33] also constitutes the quinone cavity. These findings mean that all of ligand **1** entered into the quinone-binding cavity. Considering the putative active conformation of acetogenins[30] as well as the steric bulkiness of ligand **1**, this seems to be difficult to reconcile with the idea of a narrow cavity for quinone−inhibitor binding.[6] Therefore, the quinone cavity in bovine complex I may be fairly sufficiently spacious, at least under some transitional states, to accommodate bulky ligand **1**. We also cannot exclude the possibility that the structure around the interface between the hydrophilic domain and membrane domain markedly changes: for instance, the transmembrane helices of the ND1 subunit might move and change their spacing with respect to each other, thereby allowing the long and flexible ligand **1** to enter into the binding site. The following finding may support the possibility of fairly large structural changes in the interface region: Cys39 in bovine ND3 (equivalent to Ser46 in *T. thermophilus* Nqo7) is accessible to the SH reagent, *N*-fluoresceinyl maleimide, only in the "deactive" form of the enzyme;[38] however, Ser46 is completely shielded from the solvent in the loop that provides a part of the seal of the quinone cavity in the crystal structure.[6]

The binding site of two pharmacophoric components of acetogenin (i.e., γ-lactone and THF ring moieties) was shown to be located in hydrophobic membrane subunit ND1.[32,33] Therefore, we presumed that anionized ligand **1**, which is formed after nucleophilic substitution (Figure S1 of the Supporting Information), dissociates from complex I because of a remarkable increase in hydrophilicity. Contrary to our presumption, anionized ligand **1** resided in the membrane environment and/or enzyme (Figure 7). Nevertheless, we found that externally applied BSA can efficiently remove both anionized and nonreacted ligand **1** from SMP; therefore, the residual ligands themselves are not a significant obstacle for chemical labeling via LDT chemistry. Instead, the fact that alkynylation of the 49 kDa subunit (Asp160) severely inhibited the enzyme activity is the problem to be solved, although it is not clear at present whether Asp160 is actually a part of the seal of the quinone cavity in bovine complex I, as in *T. thermophilus* complex I. To find the best position for the alkynylation, labeling conditions must be exhaustively examined by modifying the length of the linker between the 1,2-cyclopentanediol bis-ether moiety and terminal alkyne in ligand **1**. To do so might require synthesizing a series of ligand **1** analogues and then characterizing both their labeling sites and effects on enzyme activity. However, these tasks are too time-consuming, and we came to realize through this study that ligand **1** is not necessarily an ideal ligand for our purpose (as discussed below); therefore, the optimization of labeling conditions was not investigated further.

This study demonstrates an example of the site-specific alkynylation of complex I via LDT chemistry. Therefore, the formation of a footing for subsequent chemical modifications through click chemistry may be in sight. Our next task is to investigate whether click chemistry can be performed with intact complex I in SMP using the incorporated alkyne as the footing, in other words, whether a second tag possessing an azido group can be attached directly to the incorporated alkyne. With regard to this point, it should be mentioned that we performed click chemistry with solubilized complex I, not intact complex I in SMP, using a commercially available reaction kit (Click-iT reaction buffer kit) to promote the efficient covalent attachment of the TAMRA tag to the incorporated alkyne. This reaction kit contains high concentrations of Cu(I) as a catalyst, a reagent for reducing Cu(II) to Cu(I), and a Cu(I) stabilizer. Hence, if we wish to achieve the chemical modification of intact complex I in SMP by a second tag, click chemistry must be conducted without solubilization of the enzyme and catalytic copper. Copper-free click chemistry has been exhaustively investigated[42] and is actually possible between azide and ring-strained cycloalkyne [e.g., 3,3-difluorocycloactyne (Figure S1 of the Supporting Information)] instead of the terminal alkyne used in this study. However, compared to the small terminal alkyne, the bulky ring-strained cycloalkyne may be unfavorable both for transfer into the binding site by the ligand and for the substitution reaction by nucleophilic amino acids. It is therefore ideal that, as opposed to the procedure conducted in this study, azide must be incorporated into complex I as a first tag prior to click chemistry using the ring-strained cycloalkyne as a second tag. Consequently, acetogenin ligand **1** can no longer be used for this procedure. The syntheses of new acetogenin ligands possessing an azido group and ring-strained cycloalkynes having a proper tag such as fluorophore are currently underway in our laboratory.

In conclusion, we demonstrated that complex I in SMP can be site-specifically alkynylated through LDT chemistry. Although the alkynylation conditions, including the ligand structure, must be further improved to fully retain the enzyme active, this study provides a clue about diverse chemical modifications of the enzyme in combination with click chemistry.

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■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures for ligand **1**, TAMRA tag, biotin tag, *N*-(3-butynyl)maleimide, Figures S1−S4, and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

BSA, bovine serum albumin; CAII, carbonic anhydrase II; CBB, Coomassie brilliant blue R250; complex I, proton-translocating NADH-quinone oxidoreductase; LDT, ligand-directed tosylate; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; PVDF, polyvinylidene fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SMP, submitochondrial particles.

■ 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. 81*, 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) Efremov, R. G., Baradaran, R., and Sazanov, L. A. (2010) The architecture of respiratory complex I. *Nature 465*, 441−445.

(5) Hunte, C., Zickermann, V., and Brandt, U. (2010) Functional modules and structural basis of conformational coupling in mitochondrial complex I. *Science 329*, 448−451.

(6) Baradaran, R., Berrisford, J. M., Minhas, G. H., and Sazanov, L. A. (2013) Crystal structure of the entire respiratory complex I. *Nature 494*, 443−448.

(7) Efremov, R. G., and Sazanov, L. A. (2011) Structure of the membrane domain of respiratory complex I. *Nature 476*, 414−420.

(8) Morino, M., Natsui, S., Ono, T., Swartz, T. H., Krulwich, T. A., and Ito, M. (2010) Single site mutations in the hetero-oligomeric Mrp antiporter from alkaliphilic *Bacillus pseudofirmus* OF4 that affect Na^+/H^+ antiport activity, sodium exclusion, individual Mrp protein levels, or Mrp complex formation. *J. Biol. Chem. 285*, 30942−30950.

(9) Roberts, P. G., and Hirst, J. (2012) The deactive form of respiratory complex I from mammalian mitochondria is a Na^+/H^+ antiporter. *J. Biol. Chem. 287*, 34743−34751.

(10) Yasuda, R., Masaike, T., Adachi, K., Noji, H., Itoh, H., and Kinosita, K., Jr. (2003) The ATP-waiting conformation of rotating F_1-ATPase revealed by single-pair fluorescence resonance energy transfer. *Proc. Natl. Acad. Sci. U.S.A. 100*, 9314−9318.

(11) Watanabe, R., Tabata, K. V., Iino, R., Ueno, H., Iwamoto, M., Oiki, S., and Noji, H. (2013) Biased Brownian stepping rotation of F_oF_1-ATP synthase driven by proton motive force. *Nat. Commun. 4*, 1631.

(12) Sletten, E. M., and Bertozzi, C. R. (2009) Bioorthogonal chemistry: Fishing for selectivity in a sea of functionality. *Angew. Chem., Int. Ed. 48*, 6974−6998.

(13) Stephanopoulos, N., and Francis, M. B. (2011) Choosing an effective protein bioconjugation strategy. *Nat. Chem. Biol. 7*, 876−884.

(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) 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.

(16) 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.

(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) Heal, W. P., Jovanovic, B., Bessin, S., Wright, M. H., Magee, A. I., and Tate, E. W. (2011) Bioorthogonal chemical tagging of protein cholesterylation in living cells. *Chem. Commun. 47*, 4081−4083.

(19) Matsuno-Yagi, A., and Hatefi, Y. (1985) Studies on the mechanism of oxidative phosphorylation. *J. Biol. Chem. 260*, 14424−14427.

(20) 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.

(21) 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.

(22) 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.

(23) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680−685.

(24) Murai, M., Mashimo, Y., Hirst, J., and Miyoshi, H. (2011) Exploring interactions between the 49 kDa and ND1 subunits in mitochondrial NADH-ubiquinone oxidoreductase (complex I) by photoaffinity labeling. *Biochemistry 50*, 6901−6908.

(25) 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.

(26) Schägger, H. (2006) Tricine-SDS-PAGE. *Nat. Protoc. 1*, 16−21.

(27) Cleveland, D. W., Fishcher, M. W., Kirschner, M. W., and Laemmli, U. K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem. 252*, 1102−1106.

(28) Murai, M., Yamashita, T., Senoh, M., Mashimo, Y., Kataoka, M., Kosaka, H., Matsuno-Yagi, A., Yagi, T., and Miyoshi, H. (2010) Characterization of the ubiquinone binding site in the alternative NADH-quinone oxidoreductase of *Saccharomyces cerevisiae* by photoaffinity labeling. *Biochemistry 49*, 2973−2980.

(29) Morisaka, H., Matsui, K., Tatsukami, Y., Kuroda, K., Miyake, H., Tamaru, Y., and Ueda, M. (2012) Profile of native cellulosomal proteins of *Clostridium cellulovorans* adapted to various carbon sources. *AMB Express 43*, 3750−3755.

(30) Abe, M., Kubo, A., Yamamoto, S., Hatoh, Y., Murai, M., Hattori, Y., Makabe, H., Nishioka, T., and Miyoshi, H. (2008) Dynamic function of the spacer region of acetogenins in the inhibition of bovine mitochondrial NADH-ubiquinone oxidoreductase (complex I). *Biochemistry 47*, 6260−6266.

(31) Kojima, N., Abe, M., Suga, Y., Ohtsuki, K., Tanaka, T., Iwasaki, H., Yamashita, M., and Miyoshi, H. (2013) Critical role of a methyl group on the γ-lactone ring of annonaceous acetogenins in the potent inhibition of mitochondrial complex I. *Bioorg. Med. Chem. Lett. 23*, 1217−1219.

(32) 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.

(33) 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.
(34) Fujita, D., Ichimaru, N., Abe, M., Murai, M., Hamada, T., Nishioka, T., and Miyoshi, H. (2005) Synthesis of non-THF analogs of acetogenin toward simplified mimics. *Tetrahedron Lett. 46*, 5775−5779.
(35) Hogan, D. J., and Singer, T. P. (1968) Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. *J. Biol. Chem. 243*, 834−843.
(36) Tocilescu, M. A., Fendel, U., Zwicker, K., Kerscher, S., and Brandt, U. (2007) Exploring the ubiquinone binding cavity of respiratory complex I. *J. Biol. Chem. 282*, 29514−29520.
(37) Tamura, T., Tsukiji, S., and Hamachi, I. (2012) Native FKBP12 engineering by ligand-directed tosyl chemistry: Labeling properties and application to photo-cross-linking of protein complexes in vitro and in living cells. *J. Am. Chem. Soc. 134*, 2216−2226.
(38) Galkin, A., Meyer, B., Wittig, I., Karas, M., Schägger, H., Vinogradov, A., and Brandt, U. (2008) Identification of the mitochondrial ND3 subunit as a structural component involved in the active/deactive enzyme transition of respiratory complex I. *J. Biol. Chem. 283*, 20907−20913.
(39) Gostimskaya, I. S., Cecchini, G., and Vinogradov, A. D. (2006) Topography and chemical reactivity of the active−inactive transition-sensitive SH-group in the mitochondrial NADH:ubiquinone oxidoreductase (Complex I). *Biochim. Biophys. Acta 1757*, 1155−1161.
(40) Kao, M.-C., Matsuno-Yagi, A., and Yagi, T. (2004) Subunit proximity in the H^+-translocating NADH-quinone oxidoreductase probed by zero-length cross-linking. *Biochemistry 43*, 3750−3755.
(41) Berrisford, J. M., Thompson, C. J., and Sazanov, L. A. (2008) Chemical and NADH-induced, ROS-dependent, cross-linking between subunits of complex I from *Escherichia coli* and *Thermus thermophilus*. *Biochemistry 47*, 10262−10270.
(42) Jewett, J. C., and Bertozzi, C. R. (2010) Cu-free click cycloaddition reactions in chemical biology. *Chem. Soc. Rev. 39*, 1272−1279.