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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

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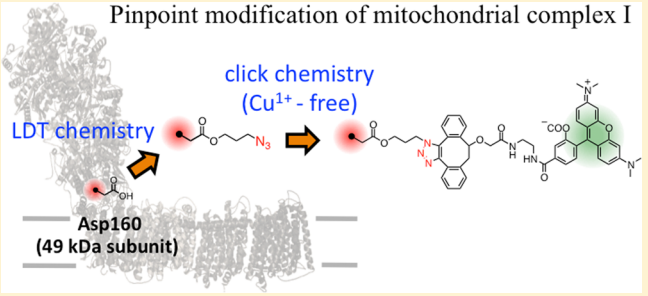
Supporting Information

ABSTRACT: Through a ligand-directed tosyl (LDT) chemistry strategy using the synthetic acetogenin ligand AL1, we succeeded in the pinpoint alkynylation (−C≡CH) of Asp160 in the 49 kDa subunit of bovine complex I, which may be located in the inner part of the putative quinone binding cavity of the enzyme [Masuya, T., et al. (2014) *Biochemistry*, *53*, 2307−2317]. This study provided a promising technique for diverse chemical modifications of complex I. To further improve this technique for its adaptation to intact complex I, we here synthesized the new acetogenin ligand AL2, possessing an azido ($-N_3$) group in place of the terminal alkyne in AL1, and attempted the pinpoint azidation of complex I in bovine heart submitochondrial particles. Careful proteomic analyses revealed that, just as in the case of AL1, azidation occurred at 49 kDa Asp160 with a reaction yield of ∼50%, verifying the high site specificity of our LDT chemistry using acetogenin ligands. This finding prompted us to speculate that a reactivity of the azido group incorporated into Asp160 (Asp160-N_3) against externally added chemicals can be employed to characterize the structural features of the quinone/inhibitor binding cavity. Consequently, we used a ring-strained cycloalkyne possessing a rhodamine fluorophore (TAMRA-DIBO), which can covalently attach to an azido group via so-called click chemistry without Cu^{1+} catalysis, as the reaction partner of Asp160-N_3. We found that bulky TAMRA-DIBO is capable of reacting directly with Asp160-N_3 in intact complex I. Unexpectedly, the presence of an excess amount of short-chain ubiquinones as well as some strong inhibitors (e.g., quinazoline and fenpyroximate) did not interfere with the reaction between TAMRA-DIBO and Asp160-N_3; nevertheless, bullatacin, a member of the natural acetogenins, markedly interfered with this reaction. Taking the marked bulkiness of TAMRA-DIBO into consideration, it appears to be difficult to reconcile these results with the proposal that only a narrow entry point accessing to the quinone/inhibitor binding cavity exists in complex I [Baradaran, R., et al. (2013) *Nature*, *494*, 443−448]; rather, they suggest that there may be another access path for TAMRA-DIBO to the cavity.



NADH-quinone oxidoreductase (respiratory complex I[1]) couples the electron transfer from NADH to quinone with the translocation of protons across the membrane, which drives energy-consuming processes such as ATP synthesis.[1,2] The enzyme from bovine heart mitochondria is composed of 44 different subunits with a total molecular mass of ∼1 MDa.[3] The crystal structures of entire complex I from *Thermus thermophilus*[4] and *Yarrowia lipolytica*[5] were solved at 4.5 and 6.3 Å, respectively. As expected from electron microscopic studies, the enzyme is L-shaped, with membrane and hydrophilic domains. The diffraction of crystals of the entire *T. thermophilus* complex I has been improved to 3.3 Å resolution,[6] providing more useful structural information on the mechanism of coupling between electron transfer and proton translocation in the enzyme. Together with the crystal structure of the six membrane subunits of *Escherichia coli* complex I at 3.0 Å,[7] possible proton translocation pathways were proposed in the subunits Nqo12 (NuoL in *E. coli*, ND5 in bovine), Nqo13 (NuoM, ND4), and Nqo14 (NuoN, ND2).[6] These subunits are homologous to the subunits from the Mrp family of Na^+/H^+ antiporters,[8] suggesting that their mechanisms of ion translocation are related. Vinothkumar et al. recently described a 5 Å structure of bovine heart complex I determined by single-particle electron cryomicroscopy and assigned and modeled 14 supernumerary subunits around the core domains,[9] thereby advancing our knowledge of the structure of mammalian complex I.

Site-specific chemical modifications of complex I by various functional probes, such as fluorophores and spin probes, may provide unique biophysical techniques to study complex I;[10] however, they have not yet been investigated in detail. In a previous study,[11] we succeeded in the pinpoint alkynylation of Asp160 in the 49 kDa subunit of complex I in bovine heart submitochondrial particles (SMP) through a ligand-directed tosyl (LDT) chemistry strategy[12,13] using a synthetic acetogenin ligand (AL1, Figure 1). The 49 kDa Asp160

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(Asp139 in the Nqo4 subunit of *T. thermophilus*) may be located in the inner part of the putative quinone binding cavity of the enzyme.[6] This study provided a promising technique for diverse chemical modifications of intact complex I because the incorporated terminal alkyne may serve as a footing for subsequent chemical modifications by second molecular probes possessing an azido ($-N_3$) group via so-called click chemistry.[13,14] We attached a TAMRA fluorescent tag possessing an azido group to the incorporated alkyne group (Asp160-alkyne) via Cu^{1+}-catalyzed click chemistry using a commercially available reaction kit (Click-iT reaction buffer kit) to promote the efficient covalent attachment of the TAMRA tag to Asp160-alkyne;[11] however, this reaction was conducted with solubilized complex I by SDS, not intact complex I in SMP. This reaction kit contains high concentrations of Cu^{1+} as a catalyst, a reducing reagent of Cu^{2+} to Cu^{1+}, and a Cu^{1+} stabilizer. Hence, if we want to chemically modify intact complex I in SMP using diverse second tags, then click chemistry must be carried out without solubilization of the enzyme and catalytic reagents.

To overcome these problems, an azide group should ideally be incorporated into complex I (as a first tag) prior to click chemistry using highly reactive ring-strained cycloalkynes (as a second tag) because the ring-strained cycloalkynes do not require the catalytic Cu^{1+} to covalently attach to an azido group (Figure S1 and refs 15 and 16). Therefore, we here synthesized a new acetogenin ligand, AL2, possessing an azido group (Figure 1), in place of the terminal alkyne in AL1, and attempted the pinpoint azidation of complex I in bovine heart SMP. Careful proteomic analyses revealed that azidation occurred at 49 kDa Asp160, confirming the high site specificity of our LDT chemistry using acetogenin ligands. With this result, we investigated the reactivity (susceptibility) of the azido group incorporated into Asp160 (Asp160-N_3) against an externally added ring-strained cycloalkyne (TAMRA-DIBO) via click chemistry under various experimental conditions in order to characterize the structural features of the quinone/inhibitor binding cavity in intact complex I. The results obtained seem to be difficult to reconcile with the idea that only a narrow entry point accessing the quinone/inhibitor binding cavity exists in complex I;[6] rather, they suggest that there may be another access path for TAMRA-DIBO to the cavity.

EXPERIMENTAL PROCEDURES

Materials. Bullatacin and fenpyroximate were kindly provided by J. L. McLaughlin (Purdue University, West Lafayette, IN) and Nihon Nohyaku Co. Ltd. (Tokyo, Japan), respectively. Aminoquinazoline (AQ) was synthesized according to previously described procedures.[17] 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-alkyne (Figure S2), and TAMRA-DIBO alkyne (Figure 1) were purchased from Life Technologies (Carlsbad, CA). Two cleavable biotin tags (biotin-SS-alkyne and biotin-SS-ADIBO; Figure S2) were synthesized in our laboratory (see the Supporting Information). Other reagents were all of analytical grade.

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[18] and stored in 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 previously described procedures.[19,20] 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;[21] the content of complex I in 1.0 mg of SMP protein was estimated to be 0.11 nmol. The IC_{50} value, which is the molar concentration needed to reduce the control NADH oxidase activity by one-half, was estimated using Prism (GraphPad, La Jolla, CA).

LDT chemistry was conducted by the incubation of SMP (2.0 mg of protein/mL, 100−200 μL) with AL2 in a buffer containing 250 mM sucrose, 1 mM $MgCl_2$, and 50 mM KP_i (pH 7.4) for 24 h at 37 °C. Anionized and nonreacted AL2 residing in SMP were removed by washing SMP with buffer containing 1% (w/v) bovine serum albumin (BSA) several times according to previously described procedures.[11] The reaction yield of azidation by AL2 was also estimated according to previously described procedures.[11]

Click Chemistry between Azidated Proteins and Alkyne Probe. Regarding conventional Cu^{1+}-catalyzed click chemistry, SMP treated with AL2 were collected by ultracentrifugation (200 000*g* for 20 min at 4 °C), solubilized in buffer containing 50 mM Tris/HCl, 1% (w/v) SDS (pH 8.0, 25−50 μL), and conjugated with terminal alkyne derivatives such as TAMRA-alkyne and biotin-SS-alkyne tags using the Click-iT reaction buffer kit (Life Technologies) according to previously described procedures.[11]

For Cu^{1+}-free click chemistry, SMP azidated by AL2 were resuspended in buffer containing 250 mM sucrose, 1 mM $MgCl_2$, and 50 mM KP_i (pH 7.4) and then incubated with 30 μM TAMRA-DIBO or biotin-SS-ADIBO at 35 °C for 1 h without Click-iT reaction buffer and SDS. SMP were collected by ultracentrifugation (200 000*g* for 20 min at 4 °C), and the proteins were subjected to further analyses.

Electrophoresis. Mitochondrial proteins were resolved by Laemmli[22] or Schägger[23]-type SDS-PAGE. Clear native (CN)-PAGE was conducted using a native PAGE Novex Bis-Tris gel system with a 4−16% precast gel (Life Technologies) without Serva Blue G dye (CBB-G250). To improve the resolution of oxidative phosphorylation enzyme complexes, 0.02% DDM and 0.05% DOC were added to the cathode buffer according to the conditions reported by Wittig et al.[24] To isolate complex I, SMP were separated by blue native (BN)-PAGE followed by electroelution and covalent conjugation with TAMRA tag via click chemistry as described previously.[11] The migration pattern of the fluorescent proteins was visualized by the model FLA-5100 (Fuji Film, Tokyo, Japan), using a 532 nm light source and LPG (575 nm) filter. Data processing and the quantification of fluorescence were conducted using Multi-Gauge software (Fuji Film).

To analyze the TAMRA-attached 49 kDa subunit of complex I, the subunit was partially purified by SDS-PAGE and electroelution.[25,26] The purified subunit was digested with lysylendopeptidase (Lys-C), endoprotease Asp-N, or trypsin in 50 mM Tris/HCl buffer (containing 0.1% SDS), 50 mM NaP_i buffer (containing 0.01% SDS), or 50 mM NH_4HCO_3, respectively.[26] The digests were separated on a Schägger-type SDS gel (16.5% T and 6% C containing 6.0 M urea[23]).

Enrichment of Biotinylated Proteins. Biotin-attached proteins, via click chemistry, were captured by immobilized

streptavidin on agarose.[11] Briefly, azidated proteins covalently conjugated with a cleavable biotin-SS-alkyne or biotin-SS-ADIBO tag were recovered by methanol/chloroform precipitation or collected by ultracentrifugation (200 000*g* for 20 min at 4 °C), respectively. The biotin-attached proteins were 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 twice with TBS buffer without the detergent. The biotinylated proteins were eluted in Laemmli's sample buffer containing 2.5% mercaptethanol, separated on a Laemmli-type SDS-PAGE gel, and visualized by Coomassie brilliant blue R250 (CBB) staining.

Mass Spectrometry. Identification and characterization of the 49 kDa subunit were conducted using Bruker Autoflex III Smartbeam (MALDI-TOF MS, Bluker Daltonics) and LTQ Velos Orbitrap mass spectrometers equipped with Ultimate 3000 nano-LC (LC-MS, Thermo Scientific), respectively, under the same experimental and data-processing parameters as those previously reported.[11]

RESULTS

Synthesis of Acetogenin Ligand AL2. As described in the opening section, because an azide group must be incorporated into the quinone/inhibitor binding cavity in complex I as a first tag prior to Cu^{1+}-free click chemistry, we synthesized a new acetogenin ligand, AL2, possessing an azido group, in place of the terminal alkyne in AL1 (Figure 1). The synthetic procedure is described in the Supporting Information. In our acetogenin ligands (AL1 and AL2), the adjacent bis-THF ring skeleton, which is a common structural motif in many natural acetogenins, was replaced with the enantiometric 1,2-cyclopentandiol bis-ether motif because the latter can substitute for the former while maintaining very potent inhibitory activity.[27]

The inhibitory effects of AL2 and bullatacin, a member of the natural acetogenins, were examined against the NADH oxidase activity in SMP (30 μg of protein/mL). The IC_{50} values of AL2 and bullatacin were 1.8 (±0.20) and 0.90 (±0.15) nM, respectively, which indicated that AL2 is slightly less active than bullatacin but still retains a potent inhibitory effect at the nanomolar level, as was also the case with AL1 [IC_{50} = 3.2 (±0.20) nM, ref 11].

LDT Chemistry with Intact Complex I and Identification of the Azidation Site. Bovine SMP (2.0 mg/mL) were incubated with 1.0 μM AL2 at 37 °C for 24 h followed by conjugation with TAMRA-alkyne via Cu^{1+}-catalyzed click chemistry using the Click-iT reaction buffer kit in the presence of 1% SDS. As shown in Figure 2, a strong fluorescent band was detected at ~50 kDa, showing that the azide group of AL2 was specifically incorporated into the ~50 kDa protein in a concentration-dependent manner. Azidation of the ~50 kDa protein was completely suppressed in the presence of a 10-fold molar excess of bullatacin or aminoquinazoline (Figure 2A). These results strongly suggest that the azide group was incorporated into the 49 kDa subunit, which comprises a part of the putative inhibitor/ubiquinone binding pocket in complex I, as observed with AL1.[11]

To identify the azidated protein and amino acid residue, the protein azidated by AL2 was conjugated with a cleavable biotin-SS-alkyne tag, enriched using immobilized streptavidin, and subjected to in-gel tryptic digestion.[11] The tryptic digests were extensively characterized by LC-MS/MS, and the protein was identified as the 49 kDa subunit (27 sequences, 82.3% coverage, Figure S3). A triply charged ion with an m/z value of 1197.58 (z = 3) was detected in the tryptic digests, which corresponds to the calculated mass of the azidated peptide Lys147−Arg174 (1103.21 + 94.37 = 1197.58, z = 3). The fragment spectra of the peptide confirmed the sequence L^{147}LNHIMAVTTHAL-DIGATPFFWMFEER^{174} with azidation at Asp160 (Figure 3). It is therefore concluded that azidation by AL2 and alkynylation by AL1 both occur at 49 kDa Asp160.

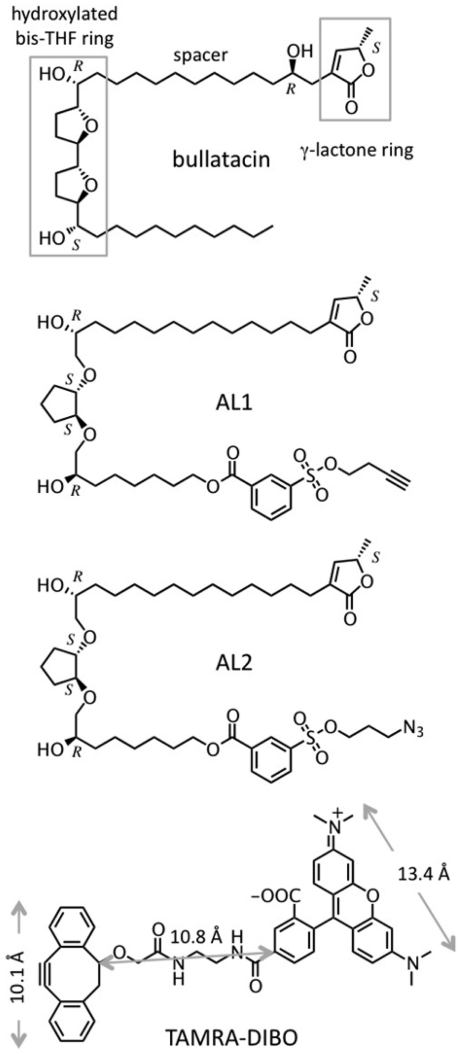

Figure 1. Structures of compounds used in this study. The length of the TAMRA and DIBO moieties, indicated by an arrow, means the distance between the corresponding two (C)−H atoms.

We also carried out exhaustive digestion of the azidated 49 kDa subunit, which was attached by the TAMRA-alkyne tag via Cu^{1+}-catalyzed click chemistry, by Lys-C, Asp-N, or trypsin. The digestion afforded a single fluorescent digest at ~8 kDa (for Lys-C), ~12 kDa (for Asp-N), and ~4 kDa (for trypsin) (Figure S4). The digestion patterns were also identical to those observed with AL1.[11] These results indicate that azidation exclusively occurred at Asp160.

Moreover, according to previously described procedures,[11] we investigated the reaction yield of the azidation step and the

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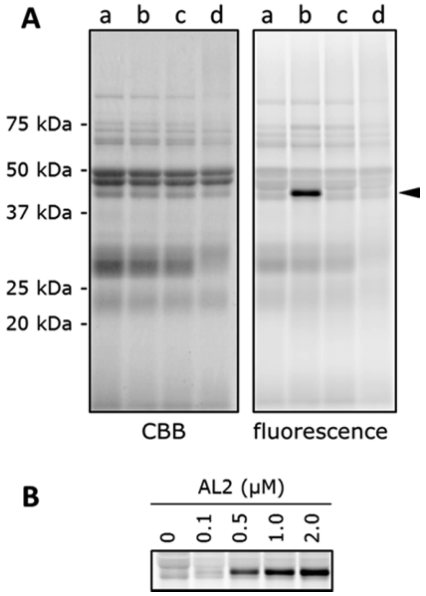

Figure 2. Azidation of complex I in SMP via LDT chemistry using AL2. (A) Bovine heart SMP (2.0 mg of protein/mL) were incubated in the absence (a) or presence (b) of 1.0 μM AL2. Alternatively, SMP were incubated with 1.0 μM AL2 in the presence of 10 μM bullatacin (c) or 10 μM aminoquinazoline (d) to examine the effect of these inhibitors on LDT chemistry. The incorporated azido group(s) was conjugated with fluorescent TAMRA-alkyne via click chemistry using the Click-iT reaction buffer kit in the presence of 1% SDS and analyzed on a 12.5% Laemmli-type SDS gel. (B) Concentration dependence of azidation against the ~50 kDa protein. A fluorescent image of the ~50 kDa band at the given concentration is shown. Approximately 50 μg of SMP proteins was loaded in each well. Data are representative of three independent experiments.

effect of azidation on complex I activity. The yield was estimated to be 53%, on average, which is comparable to that of alkynylation by AL1 (~50%). We also found that azidated complex I almost completely loses its activity, as was observed for AL1. Thus, the azidation and alkynylation of 49 kDa Asp160 occur and affect the enzyme's activity in a similar manner.

Click Chemistry between Asp160-N_3 and a Ring-Strained Cycloalkyne (TAMRA-DIBO). We succeeded in incorporating an azido group into 49 kDa Asp160 of intact complex I in SMP. This finding prompted us to speculate that the reactivity of this azido group (Asp160-N_3) against externally added ring-strained cycloalkyne derivatives can be employed to characterize the structural features of the quinone/inhibitor binding cavity in intact complex I. Ring-strained cycloalkynes are highly reactive and can covalently attach to an azido group via click chemistry without Cu^{1+} catalysis, leading to a triazole product (ref 16 and Figure S1). Here, we used a cycloalkyne possessing a rhodamine fluorophore (TAMRA-DIBO, Figure 1) as the reaction partner of Asp160-N_3 and examined whether TAMRA-DIBO can directly react with Asp160-N_3 of intact complex I in SMP.

SMP (2.0 mg of protein/mL), which had been subjected to LDT chemistry with different concentrations of AL2 (0.1−2.0 μM), were incubated with 30 μM TAMRA-DIBO at 35 °C for 1 h followed by solubilization with 1% DDM or 1% SDS for CN- or SDS-PAGE, respectively. Complex I in the SMP samples is a mixture harboring Asp160-N_3 and an unlabeled group. If Cu^{1+}-free click chemistry occurs between Asp160-N_3 and TAMRA-DIBO, then the reaction product can be visualized as a fluorescent band on the gel, the fluorescent intensity of which would increase with an increase in the concentration of AL2. As seen in Figure 4A, the CN-PAGE analysis of SMP treated with TAMRA-DIBO revealed that TAMRA-DIBO was predominantly incorporated into complex I, albeit slightly into complex V (F_oF_1-ATPase). SDS-PAGE showed that the fluorescent intensity of the 49 kDa subunit increased with an increase in the concentration of AL2 (Figure 4B). Although the fluorescent intensity of the ~75 kDa band appear to increase with an increase of the concentration of AL2, the reproducibility of this result was poor in every experiment. Through Cu^{1+}-catalized click chemistry using TAMRA-alkyne in the presence of 1% SDS, as conducted in Figure 2, we confirmed that azidation of the ~75 kDa protein was negligible (Figure S5).

The fluorescent bands in other regions in Figure 4B, the intensities of which were not dependent on the concentration of AL2, may be attributable to the reaction between highly reactive TAMRA-DIBO and sulfenic acids (−SOH), which are formed by the reaction of cysteine thiols with reactive oxygen species.[28] The reaction mechanism of TAMRA-DIBO and sulfenic acid is illustrated in Figure S6. We note that the protein corresponding to the strong fluorescent band in the ~30 kDa region was identified as an ADP/ATP carrier by MALDI-TOF MS analysis of its tryptic digests (43% coverage). The proteins corresponding to the two fluorescent bands at ~75 kDa could not be identified.

Effects of Quinones or Inhibitors on the Reaction between TAMRA-DIBO and Asp160-N_3. Baradaran et al. suggested that the quinone/inhibitor binding cavity is completely enclosed from the solvent and is a ~30 Å long cavity with only a narrow entry point for quinone/inhibitor.[6] If the entry point of quinone, inhibitors, and TAMRA-DIBO into the putative cavity is identical, then the presence of an excess amount of quinones or inhibitors may interfere with the click reaction between TAMRA-DIBO and Asp160-N_3 by occupying the cavity. We examined this using SMP, which were previously subjected to the azidation of 49 kDa Asp160 by AL2 and subsequently washed with BSA to remove residual AL2. The SMP samples were reacted with 30 μM TAMRA-DIBO in the absence or presence of inhibitor (or quinone) and subjected to BN-PAGE to separate complex I. The excised gel slice containing the complex I band was then subjected to SDS-PAGE (Figure 5). Unexpectedly, ubiquinone-2 and -6 as well as a large excess of strong inhibitors (quinazoline and fenpyroximate) did not interfere with the reaction; nevertheless, bullatacin, a member of the natural acetogenins, significantly interfered with this reaction. We note that the concentration (10 μM) of the three inhibitors was a large excess to give a complete inhibition of complex I activity and also that their binding positions in the quinone/inhibitor binding cavity slightly differ.[19,20,26,29] It appears to be difficult to reconcile these results with the idea that only a narrow entry point accessing the quinone/inhibitor binding cavity exists,[6] as discussed later.

■ DISCUSSION

A novel protein labeling methodology termed LDT chemistry, which is based on the principle of affinity labeling, has been applied to various proteins.[12,13] We previously demonstrated that intact complex I in bovine heart SMP can be site-specifically alkynylated at 49 kDa Asp160 via LDT chemistry

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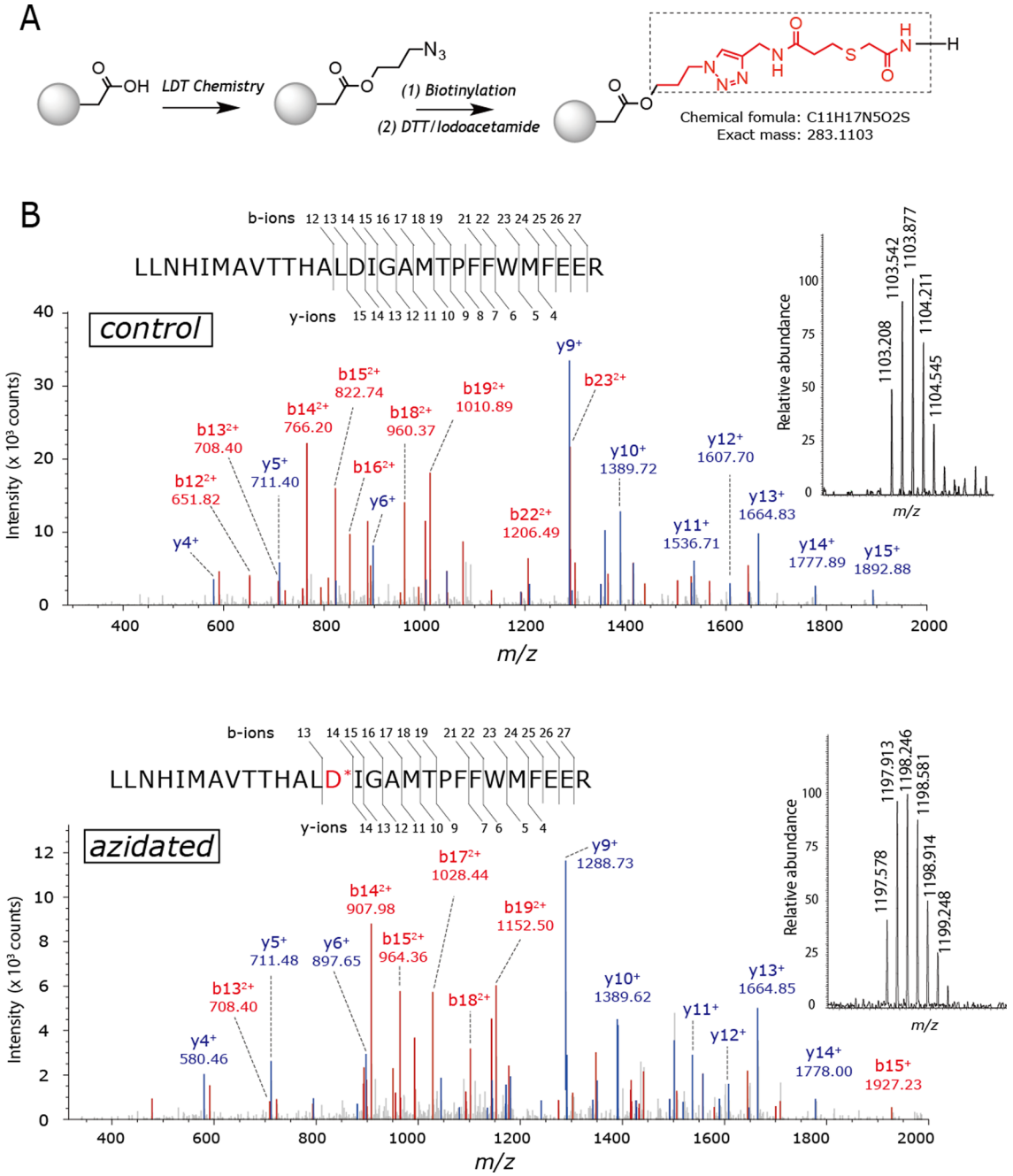

Figure 3. Identification of the azidated position in the 49 kDa subunit. The azidated 49 kDa subunit was enriched using immobilized streptavidin as described in the Experimental Procedures. The modified 49 kDa subunit was digested in-gel with trypsin, and the peptide mixture was extensively characterized using an Orbitrap mass spectrometer. (A) Predictable formation of a stable adduct by azidation via LDT chemistry, biotinylation via click chemistry, and carbamidomethylation. (B) Fragment ion spectra of the control and azidated peptides L^{147}LNHIMAVTTHALDIGAMTPFFWMFEER^{174}, in which D* corresponds to an *O*-azidated aspartic acid with a mass shift of +94.37. The identified b- and y-fragment ions were mapped onto the amino acid sequences shown in the inset. Insets are the respective precursor ion spectra. The residue numbers refer to the mature sequence of the bovine 49 kDa subunit (Swiss-Prot entry: P17694), and oxidation (+15.9994) occurred at Met168.

using the acetogenin ligand AL1.[11] However, the incorporation of a terminal alkyne (−C≡CH) as the first tag was not necessarily appropriate for subsequent diverse chemical modifications via click chemistry, as described in the opening section. To further improve the chemical modifications of complex I by a combination of LDT chemistry and click chemistry, in the present study, we performed the pinpoint azidation of intact complex I via LDT chemistry using the newly synthesized acetogenin ligand AL2. Careful proteomic analyses revealed that, just as in the case of AL1, azidation occurred at 49 kDa Asp160 with a reaction efficiency of ∼50%, confirming the high site specificity of our LDT chemistry using acetogenin ligands. According to a sequence alignment for the bovine 49 kDa subunit and *T. thermophilus* Nqo4 subunit, Asp160 (Asp139 in Nqo4) is located in the inner part of the putative quinone/inhibitor binding cavity.[6]

The azidation of 49 kDa Asp160 resulted in an almost complete loss of enzyme activity, which was disappointing for us; nevertheless, we speculated that the reactivity of the azido group incorporated into 49 kDa Asp160 (Asp160-N_3) against

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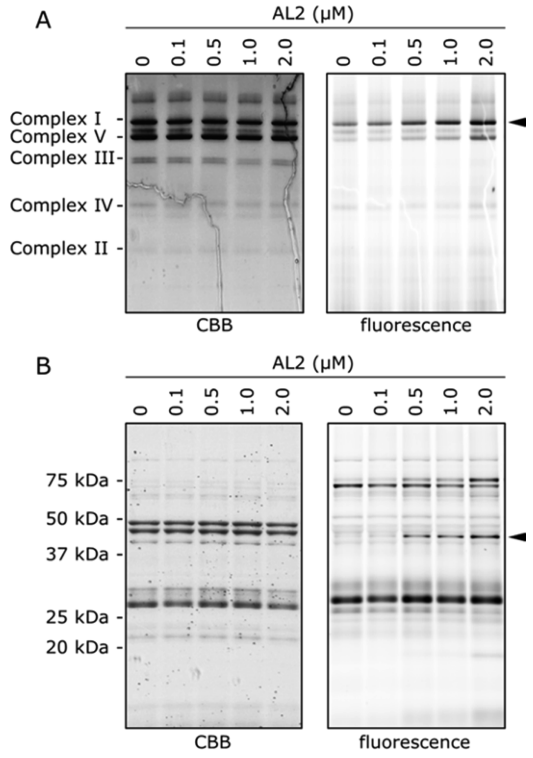

Figure 4. Cu^{1+}-free click chemistry against Asp160-N_3 using the ring-strained cycloalkyne TAMRA-DIBO. Bovine heart SMP (2.0 mg of protein/mL) were azidated via LDT chemistry using AL2 (0−2.0 μM) at 37 °C for 24 h followed by the direct attachment of fluorescent TAMRA-DIBO via Cu^{1+}-free click chemistry. (A) Treated SMP were resolved by CN-PAGE[24] using a 4−16% precast gel and subjected to fluorescent gel imaging and CBB staining. (B) Treated SMP were also separated on a 12.5% Laemmli-type SDS gel and subjected to fluorescent gel imaging and CBB staining. Approximately 20 and 10 μg of SMP proteins was loaded on CN and SDS gels, respectively. Data are representative of four independent experiments.

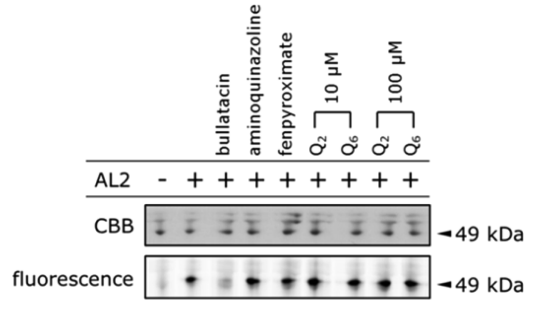

Figure 5. Effects of quinones or inhibitors on the reaction between Asp160-N_3 and TAMRA-DIBO via Cu^{1+}-free click chemistry. SMP (2.0 mg of protein/mL) were incubated with 1.0 μM AL2 at 37 °C for 24 h and washed three times with buffer containing 1% BSA to remove nonreacted AL2. SMP were then incubated with 30 μM TAMRA-DIBO at 35 °C for 1 h in the presence of bullatacin (10 μM), aminoquinazoline (10 μM), fenpyroximate (10 μM), Q_2 (10 and 100 μM), or Q_6 (10 and 100 μM). The proteins (120 μg of SMP proteins/well) were separated by BN-PAGE using a 4−16% precast gel, and the complex I band was then excised and subjected to the Laemmli-type SDS-PAGE using a 12.5% gel.[20] The separated proteins were analyzed by fluorescent gel imaging and stained by CBB. Data are representative of three independent experiments.

some externally added chemicals can be employed to characterize the structural features of the quinone/inhibitor binding cavity of intact complex I in SMP. Therefore, we investigated the reactivity between Asp160-N_3 and the ring-strained cycloalkyne TAMRA-DIBO, which can covalently attach to an azido group via click chemistry without Cu^{1+} catalysis. Baradaran et al. suggested that the quinone/inhibitor binding cavity leading from the membrane interior to the Fe−S cluster N2 is completely enclosed from the solvent and is a ∼30 Å long cavity with only a narrow entry point for quinone/inhibitor.[6] This entry point is framed by the helices TM1 and TM6 and amphipathic helix AH1 of the Nqo8 subunit (ND1) as well as TM1 of the Nqo7 subunit (ND3). If there is only an entry point for quinone/inhibitor, as suggested, then TAMRA-DIBO would have to enter through the narrow entry point and pass along the cavity to react with Asp160-N_3. Then, if an excess amount of quinone or strong inhibitor occupies the cavity, TAMRA-DIBO may be unable to come close to Asp160-N_3; however, this was not the case. Namely, Q_2 and Q_6 as well as an excess amount of strong inhibitors (quinazoline and fenpyroximate) did not interfere with the reaction between TAMRA-DIBO and Asp160-N_3 (Figure 5). In contrast, bullatacin significantly interfered with the reaction. Thus, some differences were identified among the inhibitors concerning their effects on the behavior of TAMRA-DIBO. Since this difference may be attributable to the unique characteristics of lipid-like acetogenins as complex I inhibitors, we briefly summarize their modes of action elucidated from structure−activity relationship (SAR) studies and photoaffinity labeling as follows.

SAR studies of a number of acetogenins for their inhibitory effects on bovine complex I provided interesting information regarding their modes of action.[30,31] In particular, SAR studies concerning the flexible spacer region linking the two critical toxophoric moieties (i.e., the γ-lactone ring and hydroxylated THF ring; Figure 1) provided hints for predicting an active conformation of acetogenins in their bound state. For example, acetogenin derivatives, in which a spacer covering 10 carbon atoms was hardened into a rodlike shape by introducing a tetrayne skeleton (Figure S7), maintained very potent inhibitory activities at the nanomolar level. It is difficult to imagine that a whole acetogenin molecule having such a rodlike spacer could enter into the quinone/inhibitor binding cavity through the putative narrow entry point. This finding, along with those of another SAR study in which the spacer region was markedly modified by the introduction of an azobenzene unit (Figure S7), strongly suggested that this region is free from steric restriction arising from the binding site. Therefore, it is likely that the spacer region may somewhat protrude from the enzyme into the lipid phase.[31] On the other hand, photoaffinity labeling studies with bovine complex I showed that acetogenins reside in the ND1 subunit: the hydroxylated THF ring moiety (Figure 1) occupies the lower part of the quinone binding cavity by binding to the third matrix-side loop connecting the fifth and sixth transmembrane helices in the ND1 subunit,[29] whereas the γ-lactone ring moiety binds to the fourth or fifth transmembrane helix in the ND1.[32] Contrarily, quinazoline and fenpyroximate occupy the upper part of the cavity, being close to the Fe−S cluster N2 (PSST and/or 49 kDa subunits).[25,26] Taken together, it is reasonable to speculate that acetogenins reside in the ND1 subunit by passing through some opening (or gap) between the transmembrane helices of ND1 rather than through the narrow entry point.

With the modes of action of acetogenins in mind, we again consider the reaction between TAMRA-DIBO and Asp160-N_3,

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based on the X-ray crystallographic structure of *T. thermophilus* complex I.[6] The distance between the DIBO ring and TAMRA fluorophore in its extended conformation (~11 Å; Figure 1) is much shorter than that between the putative entry point and Asp160-N_3 (~30 Å; Figure S8). Therefore, if there is only an entry point accessing the quinone/inhibitor binding cavity, then a whole TAMRA-DIBO molecule must enter the cavity through the entry point to react with Asp160-N_3. However, considering the diameter of the narrowest part of entrance to the cavity of ~8 Å (Figure S8), this may be impractical because of steric hindrance arising from bulky DIBO and TAMRA moieties. Furthermore, it is important to note that short-chain quinones and an excess amount of inhibitors, which occupy the cavity,[25,26,32] could not interfere with the reaction between TAMRA-DIBO and Asp160-N_3, as discussed above. With all things taken together, it is difficult to reconcile the present results with the idea of there being only a narrow entry point accessing the cavity. Rather, it is likely that there is another access path to the cavity; TAMRA-DIBO may enter the cavity by passing through some opening (or gap) between the transmembrane helices of the ND1 subunit in a similar manner to that of acetogenins. Although bullatacin significantly interfered with the passage of TAMRA-DIBO into the cavity, it is unclear whether bullatacin directly competes with TAMRA-DIBO or induces some structural changes of the ND1 subunit, thereby hampering the passage of TAMRA-DIBO.

We found that Asp160-N_3 in intact complex I directly reacts with the externally added ring-strained alkyne TAMRA-DIBO. However, during the course of the present study, we came to realize that TAMRA-DIBO reacts not only with Asp160-N_3 but also with sulfenic acids (−SOH) in mitochondrial proteins,[28] which arose from the reaction of reactive oxygen species with protein thiols. This undesired side reaction could be detected as background fluorescence, as seen in Figure 4B, and is a common property of ring-strained alkynes.[33] To overcome this problem and in order to achieve the pinpoint chemical modifications of complex I in SMP by a combination of the LDT strategy and click chemistry, a ring-strained alkyne must be incorporated into the 49 kDa Asp160 as the first tag prior to Cu^{1+}-free click chemistry using azido-containing probes as second tags. For this procedure, the synthesis of new acetogenin ligands possessing a ring-strained alkyne in the tosyl moiety is currently under way in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures for AL2, biotin-SS-alkyne, and biotin-SS-ADIBO. Figure S1: Schematic representation of the Cu^{+1}-free click chemistry between an azido group and a ring-strained cycloalkyne. Figure S2: Structures of TAMRA-alkyne, cleavable biotin-SS-alkyne, and biotin-SS-ADIBO. Figure S3: Characterization of the ~50 kDa protein of bovine complex I by LC-MS. Figure S4: Exhaustive digestion of the azidated 49 kDa subunit. Figure S5: Bovine SMP azidated via LDT chemistry using different concentrations of AL2 followed by conjugation with 30 μM TAMRA-alkyne via Cu^{+1}-catalyzed click chemistry in the presence of 1% SDS at 35 °C for 1 h and subjected to SDS-PAGE. Figure S6: Sulfenic acid can react readily to ring-strained cycloalkynes like TAMRA-DIBO. Figure S7: Inhibitory activities of rigid acetogenin derivatives. Figure S8: Structure of the quinone/inhibitor binding cavity in *T. thermophilus* complex I. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

BSA, bovine serum albumin; CBB, Coomassie brilliant blue R250; complex I, proton-translocating NADH-quinone oxidoreductase; DDM, *n*-dodecyl-β-maltoside; DOC, deoxycholic acid sodium salt; LDT chemistry, ligand-directed tosylate chemistry; 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

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