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The ND2 Subunit Was Labeled by a Photoaffinity Analogue of Asimicin, a Potent Complex I Inhibitor*

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Abstract

NADH:ubiquinone oxidoreductase (complex I) is the entry enzyme of mitochondrial oxidative phosphorylation. To obtain the structural information on inhibitor/quinone binding sites, we synthesized [³H]benzophenone-asimicin ([³H]BPA), a photoaffinity analogue of asimicin, which belongs to the acetogenin family known as the most potent complex I inhibitor. We found that [³H] BPA was photo-crosslinked to ND2, ND1 and ND5 subunits, by the three dimensional separation (blue-native/doubled SDS-PAGE) of [³H]BPA-treated bovine heart submitochondrial particles. The cross-linking was blocked by rotenone. This is the first finding that ND2 was photo-crosslinked with a potent complex I inhibitor, suggesting its involvement in the inhibitor/quinone-binding.

Keywords

complex I; bovine heart mitochondria; asimicin; ND2; photoaffinity labeling; quinone binding

1. Introduction

NADH-ubiquinone oxidoreductase (complex I) is the entry enzyme of the respiratory chain in both mitochondria and bacteria. Complex I is the most elaborate iron-sulfur protein with a total mass close to 1000 kDa, composed of 45 dissimilar protein subunits [1,2]. Seven subunits are encoded by mitochondrial DNA (designated ND1-6 and 4L) and the others by nuclear DNA [1,3]. The recent crystal structure of the hydrophilic domain of complex I from *Thermus thermophilus* HB-8 at 3.3 Å resolution clearly showed that the electron transfer pathway from FMN to iron-sulfur cluster N2, which has the highest midpoint redox potential and is therefore considered to be the site of electron transfer to quinone (Q) molecules [4,5]. Although the detailed mechanism of proton translocation coupled with Q reduction remains unsolved, Ohnishi's group has reported the involvement of two different types of semiquinone species,

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namely, SQ_{NF} (the gate of proton transport) [6,7] and SQ_{NS} (the converter between one-electron and two-electron transfer) [8].

Currently, more than 60 different families of compounds are known to inhibit complex I [9]. Many of these inhibitors are with a clear resemblance to the structure of the natural quinones in terms of positions of hydrogen bonding groups and hydrophobic character. They are generally believed to bind to the site in the membrane, where it is responsible for Q reduction and/or proton transport. Therefore, to identify the inhibitor/Q binding site(s) in complex I, photoaffinity labeling experiments with various inhibitors and/or Q analogs have been conducted. Rotenone-derived photoaffinity probes [10] and an acetogenin analog, [¹²⁵I] (trifluoromethyl)-phenyldiazirinylnacetogenin ([¹²⁵I]TDA) labeled the ND1 subunit [11], a photoaffinity analogue of pyridaben labeled the PSST and ND1 subunits [12]. A classical carboxyl group modifying reagent, [¹⁴C]*N,N'*-dicyclohexylcarbodiimide, also labeled the ND1 subunit in a manner correlated with the inhibition of NADH-Q reductase activity [13]. It suggests that the Q reduction site is coupled to proton translocation. An experiment with an azido-ubiquinone derivative suggested the NuoM subunit (*E. coli* ND4 homolog) was labeled [14], although their mass spectrometry (MS) identification was later questioned [15]. An analogue of fenpyroximate, which is another potent complex I inhibitor and a phenoxypyrazole acaricide, labeled the ND5 subunit [16]. A photoaffinity probe of quinazoline-type inhibitor labeled 49 kDa and ND1 subunits [17].

The question of whether Q binding sites and inhibitor binding sites as determined by labeling studies are identical or not still remains to be answered due to the difficulty of doing competitive experiments with exogenous short-chain ubiquinones [18]. However, information obtained from labeling experiments are important to understand the Q binding site(s) and/or its neighboring site(s) in complex I. These labeling studies certainly indicate that the inhibitor/Q binding pocket must be very large. However, this immediately raises an important question. Is it possible that all the subunits, namely 49 kDa, PSST, ND1 and ND5 construct a single large inhibitor/Q binding pocket in complex I [19]? It is more likely that more than one distinct Q binding sites exist in complex I. Thus, to explore this possibility, we designed and synthesized benzophenone-asimicin (BPA) (Fig. 1). Asimicin, which belongs to a chemical family of acetogenins [20], is one of the most potent inhibitors of complex I [9]. We used a benzophenone photoprobe, because it offers the advantage of being more stable and having a longer lifetime than other photolabeling devices such as diazo and azido groups [21].

2. Materials and Methods

Materials

Bovine heart mitochondria were kindly provided by Dr. Chang-An Yu (Oklahoma State University, OK). Bovine heart submitochondrial particles (SMP) were prepared as described in ref. [22]. NaB[³H]₄ was purchased from American Radiolabeled Chemicals (St. Louis, MO). Asimicin was chemically synthesized as described in ref [23]. Anti-ND2 used here had been raised by Dr. Yagi.

Synthetic Procedures of [³H]BPA

[³H]-labeled BPA was prepared using partially protected [³H]-labeled 34-hydroxyasimicin according to ref. [23]. Briefly, NaB[³H]₄ (6.25 μmol, 500 mCi/18.5 GBq, 80 Ci/mmol) was added to a solution of the corresponding aldehyde (21 mg, 24 μmol) in ethanol (0.3 ml) at -10 °C. After 2 hrs, the solution was poured into iced water and extracted with ethyl-acetate. The combined organic layer was dried on anhydrous MgSO₄. The resulting residue was dissolved in CH₂Cl₂ (0.6 ml), 3-(4-benzoylphenyl)-propionic acid and N,N-dimethyl-4-aminopyridine (1 mg) and then 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (5 mg, 27

μmol) were added at 0° C. The mixture was stirred overnight at room temperature. Then the solvent was passed through a short bed of silica gel to give the corresponding ester (17 mg, 70% yield). BF₃•Et₂O was added to the ester solution in dimethylsulfide (0.9 ml) at 0 °C. The mixture was stirred at room temperature for 2 hrs. Solvents were removed, and the residue was purified by silica gel chromatography to obtain the final product [³H]BPA (10 mg, 73% yield). The purity was >95 % based on ¹H-NMR (300 MHz) analysis. The specific activity was 17.82 Ci/mmol.

Enzyme Activity

The NADH oxidase activity was spectrophotometrically measured using 340 nm at 30 °C and the effect of inhibitors was determined. The standard assay mixture contained 150 μM NADH, 0.25M sucrose, 50mM sodium phosphate (pH 7.5), 1 mM EDTA, and SMP (10-30 μg/ml).

Photoaffinity Labeling

SMP (0.2 mg/ml) in 50 mM sodium phosphate at pH 7.5 containing 250 mM sucrose was treated with [³H]BPA (50 nM) for 10 min at room temperature. Competitive inhibitors were added before incubation with [³H]BPA and left on ice for 10 min. Samples were then irradiated for 10 min at 4 °C under 12W UV lamp (365 nm) at a distance of 2.5 cm. The proteins were precipitated (256,600 × g for 15 min at 4 °C) for analyses by electrophoresis. For a localization analysis of labeled protein bands, the gels were cut into 1-2.5 mm slices, individually incubated with 0.5-0.75 ml of 30% hydrogen peroxide overnight at 75 °C. Radioactivity was measured by liquid scintillation counter as previously described [16].

Electrophoresis

SDS-PAGE was performed according to Laemmli [24] or Schagger and von Jagow [25]. Blue native (BN)-PAGE was carried out on a 5-12% or 4-18% linear acrylamide gradient using a full sized gel format [26]. The position of complex I band was confirmed by activity staining with nitroblue tetrazolium. These BN-PAGE gels were subjected to subsequent SDS-PAGE. To isolate hydrophobic subunits in complex I, we performed doubled SDS-PAGE (dSDS-PAGE) [27].

Immunoblot analyses

Protein bands in SDS gels were transferred onto 0.2 mm nitrocellulose membranes. Immunoblot patterns were visualized with enhanced chemiluminescence, Supersignal kits (Pierce, Rockford, IL).

Mass spectrometry

MALDI-TOF-MS experiments were performed on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) according to ref. [27].

3. Results

Photoaffinity Labeling of SMP with [³H]BPA

We investigated the inhibitory effect of the photoreactive analogue, BPA on complex I activity in SMP. Although BPA gained a considerable bulkiness (Fig. 1A), it remarkably retained the original strong inhibitor potency (IC₅₀ = ~1 nM) in SMP (Fig. 1B). Photoaffinity labeling of SMP with 50 nM [³H]BPA resulted in one major and two minor labeled regions, with an apparent *M_r* 85 kDa, and ~33 kDa and 23 kDa on a 12.5% Laemmli's SDS gel (Fig. S1 (a)). But, with Tricine-SDS-PAGE system, the radioactivities were detected at one major ~85 kDa and one minor 50 kDa bands on a 10% gel, and much less aggregation at the entry of the gel was observed (Fig. S1 (b)). Labeling was time dependent, and about 90% of labeling seemed

to be completed within 10 min irradiation (data not shown). Higher concentrations (> 80 nM) significantly increased background on the SDS gels caused by the highly lipophilic nature of BPA, while the labeling of 23 kDa and 33 kDa bands correlated with the inhibition of NADH oxidase activity (data not shown). The labeling of the 85 kDa band did not clearly saturate even at 160 nM, but this band is not a complex I component, because it is larger than the largest subunit (75 kDa) of complex I.

Labeling of Complex I with [³H]BPA

We examined how specific is the labeling of [³H]BPA to complex I. We analyzed [³H]BPA-treated SMP by BN-PAGE. Without covalent binding initiated by UV irradiation, [³H]BPA was still strongly associated with complex I on a BN gel (Fig. 2A). However, we also found a substantially high background radioactivity in other mitochondrial complexes isolated by BN-PAGE. Non-specific [³H]BPA binding is likely caused by its lipophilic interaction with membrane phospholipids, which account for ~30% of the total mass [28]. In fact, when each band containing complex II, III, IV and V was excised from a BN gel and directly loaded onto a SDS gel, no radioactivity band was detected on a SDS-gel (data not shown) because non-covalently bound BPA was removed from the protein by SDS. The UV irradiation enhanced the binding of [³H]BPA only to complex I by ~30%, while there was no effect on other respiratory complexes (Fig. 2A). In contrast, in the presence of cold BPA, the non-covalent binding of [³H]BPA to complex I was significantly suppressed to the background levels regardless of the UV irradiation (Fig. 2B). Since no radioactivity band was detected on a SDS-gel from the band containing complex I and II excised from a BN gel of Fig. 2B regardless of UV irradiation (data not shown), we concluded that the radioactivities in Fig. 2B were all non-covalently bound BPA via membrane lipids. The incorporation of radioactivity into complex I was completely saturated at 80 nM (Fig. S2), and the binding of [³H]BPA to complex I basically paralleled to the inhibition of NADH oxidase activity (Fig. S2). The small dissociation in the relationship between the labeling and inhibition seemed to be caused by poor efficiency of photoactivation of [³H]BPA inside SMP. Since it was known that NADH increases the labeling efficiency for the cases such as pyridaben [12] or fenpyroximate [16], we also tried the labeling by incubating SMP with [³H]BPA in the presence of NADH (400 μM) for 10 min at room temperature. Then sodium pyruvate (1 mM) and L-Lactate dehydrogenase (3 units) was added to destroy the remaining NADH before UV-irradiation. The labeling efficiency and patterns did not change by the addition of NADH.

Two- and three-dimensional separation of the [³H]BPA-labeled complex I subunits

To identify which subunits were labeled with [³H]BPA, the band containing complex I was excised from a BN gel and directly loaded onto a 10% Tricine-SDS gel containing 6M urea. As shown in Fig. 3A(a), we detected the radioactivity peaks at 85 kDa, ~33 kDa, 23 kDa, and at 50 kDa as already observed in Fig. S1. Using 6 M urea for SDS-PAGE separation is known to have some dispersing effect and alter SDS-binding to highly hydrophobic proteins in gels. In fact, in the absence of 6 M urea, the radioactivity peaks around 50 kDa became much smaller (Fig. 3A(b)). Furthermore, in the presence of rotenone, the labeling of ~33 kDa band was almost completely blocked, while the labeling of 85 kDa was only partially decreased to ~70% of the control (Fig. 3A(c)). Basically similar results were obtained with piericidin A or methyl-N-isobutyl amiloride, a specific inhibitor for Na⁺/H⁺ transporters (data not shown). Then, we applied a novel technique of dSDS-PAGE. This new method has been very effective to separate highly hydrophobic membrane proteins from water soluble proteins, and it has been successfully employed to analyze integral membrane proteins such as mitochondrial respiratory chain complexes [27] or those found in synaptic vesicles [29]. Especially, hydrophobic proteins in SMP are readily identified by their position on the gel and do not require identification by sequencing or peptide fingerprinting [27]. The vertical alignment of the spots in the second dimension yields familiar spot patterns that agree with many published

subunits compositions of purified complex I [30]. In our typical dSDS-PAGE patterns in the presence or absence of 6M urea (Fig. 3B and 3C), highly hydrophobic subunits were found above the diagonal axis and they were clearly separated from water-soluble hydrophobic proteins as reported. This pattern was exactly the same as the previously published data of bovine heart mitochondria, in which each protein spot has already been identified by MS [27,30]. By comparing to the known patterns, we clearly assigned four hydrophobic spots, ND5, ND4, ND2 and ND1 on our 3-D gels. The small spot for COX1 (complex IV) and the faint spot for Cyt.b (complex III) were also observed (Fig. 3B and 3C). To confirm our spot assignment, we also carried out MALDI-TOF-MS analyses. We succeeded in identifying two peptides, ⁵²²FSTLLGYFPTDMHR⁵³⁵ and ⁴²⁶IIFALLGQPR⁴³⁶ from the ND5 spot on our gels. We also tried to confirm ND1 and ND2 spots and used the corresponding spots from five 3-D gels together for MS analysis, but we failed to detect any peptides. The ND2 spot was confirmed by Western blot (Fig. S3). We measured the radioactivity of these ND spots on the 3-D gels (Table I). The size of the gels (18 × 14 cm) used in Fig. 3B and 3C was large enough to separate ND1 and ND2 spots. These small spots were obtained with a puncher made of a stainless tube. Consistent with 2-D patterns (Fig. 3A(a) and (b)), twofold higher radioactivity for the ND5 spot was detected in the presence of 6M urea (Table I). In contrast, 6M urea did not affect the radioactivities of ND1, ND2, and ND4 spots. As expected from the molecular size (Fig. 1B and 3A), we finally identified that the radioactivity peaks at 50 kDa and ~33 kDa are corresponding to the spots of ND5, and ND1&ND2, respectively. The radioactivity of COX I was 27 at the background level. The radioactivities of well-isolated hydrophilic spots of 51 kDa and 49 kDa were ~35 cpm, close to the background level. When ND1 and ND2 bands were separated on a 1-D 10% SDS, we found two but fused radioactivity peaks around 30 and 33 kDa, which were reacted with specific antibodies against ND1 and ND2 respectively with no overlapping (data not shown). Taken together, it was concluded that ND2 was labeled as well as ND1.

We detected high radioactivities (~400 cpm) for the 85 kDa spot. However, we concluded that the 85 kDa protein does not belong to complex I by the following reasons: (i) this 85 kDa spot was very faint: (ii) no subunit larger than 75 kDa exists in complex I; (iii) it did not react to any complex I subunit antibodies against ND1, ND2, ND5, 51 kDa, 49 kDa, and PSST. In fact, although more detailed study must be done, we recently found that the 85 kDa could be long-chain 3-hydroxyacyl-CoA dehydrogenase, which is an important enzyme for fatty acid oxidation in mitochondria and reduces NAD to NADH.

4. Discussion

In the present study, we demonstrated that [³H]BPA was covalently incorporated into ND2, ND1 and ND5 subunits. We also observed that the labeling was blocked by rotenone. This is the first report that ND2 was photoaffinity-labeled with a complex I inhibitor, and that this subunit is involved in the inhibitor/Q binding sites. Unfortunately, we could not identify labeled amino acid residue(s) due to a low labeling efficiency of [³H]BPA. This is probably caused by the conformational flexibility of benzophenone [31]. In general, labeling experiments of complex I have been extremely challenging for the last two decades. Only recently, Miyoshi's group has succeeded in locating the [¹²⁵I]TDA-labeled region within the 4th and 5th transmembrane helices of the ND1 subunit [11]. Using photoreactive azidoquinazoline, another cross-linked domain was identified in the membrane interface region, at a sub-subunit level (within 23 amino acid polypeptide) in the 49 kDa subunit [17].

A difference between our results and those by Miyoshi's group, which also used acetogenin derivatives, is attributed to the different locations of photo-affinity groups between [³H]BPA and [¹²⁵I]TDA. In other words, the cytotoxic γ -lactone ring was replaced with the photo-affinity aryl diazirine group in [¹²⁵I]TDA, while the photo-affinity benzophenone group was attached

to the opposite end of the alkyl chain end of asimicin in [³H]BPA (Fig. 1A). According to the detailed study on how acetogenins interact with the membrane bilayers by ¹H NMR spectroscopy, two tetrahydrofuran rings act as a hydrophilic anchor in the center of lipid bilayers and the γ -lactone ring and the other end are stretched out into the membrane in U-shaped conformations [32]. Thus the [³H]BPA-labeled site could be far from the primary inhibitor/Q-binding site labeled with [¹²⁵I]TDA. Amarneh et al. previously reported that mutants K158C and H224K in the NuoN subunit (homologous to ND2) of *E. coli* complex I showed enhanced inhibition by decylubiquinone (DB) along with decreased deamino-NADH oxidase and proton pumping activities. These findings indicate that NuoN interacts with quinones [33]. It is interesting to note that these residues are facing the cytoplasm surface and located very close or in the predicted Q motif (LxxxHxxT) [34].

Two distinct SQ signals in complex I were detected in bovine heart SMP by EPR: fast-relaxing uncoupler sensitive SQ_{Nf} and slow-relaxing uncoupler insensitive SQ_{Ns} species. Based on their spin-spin interaction analyses, the distance between SQ_{Nf} and cluster N2 was calculated as 12 Å [6], while SQ_{Ns} is >30 Å away from cluster N2 [35]. Both SQ species showed the same IC₅₀ to piericidinA while SQ_{Ns} showed much lower sensitivity to rotenone than that to SQ_{Nf} [35]. Thus, it has been suggested that these two quinone species have different functions and occupy different locations [35]. Based on a relatively high stability constant ($K_{\text{stability}}$) of the SQ_{Ns}, its function was suggested to be a converter between n=1 (Q_{Nf}) and n=2 (Q pool) electron transfer processes [8]. These previous findings and our current data strongly suggest that ND2 is involved in the secondary Q-binding site where the SQ_{Ns} signals arise.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations

BN-PAGE	blue native-PAGE
BPA	benzophenone-asimicin
1-D	one-dimensional
2-D	two-dimensional
3-D	three-dimensional
IC ₅₀	the molar concentration of substance that provides 50% inhibition of the control NADH oxidase activity
Q	quinone
SMP	submitochondrial particles
SQ	semiquinone
[¹²⁵ I]TDA	[¹²⁵ I](trifluoromethyl)phenyldiazirinyacetogenin

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Fig. 1.

A, Structures of asimicin, its photoaffinity probes, benzophenone-asimicin (BPA). B, Inhibition of NADH-oxidase activity in bovine heart SMP by BPA (closed circles) or asimicin (open circles).

Fig. 2. Localization of radioactivities in SMP (A) and the effect of cold BPA on the labeling (B). ~50 μ g of [3 H]BPA-labeled SMP was loaded onto a BN gel (4-13%) to separate five respiratory enzyme complexes. In (B), SMP were treated with cold BPA (2 μ M) for 10 min on ice before incubation with [3 H]BPA (80 nM). The columns represent the radioactivity of each enzyme complex without (open bars) or with UV-irradiation (solid bars). This is a representative data from three different sets of experiments.

Fig. 3.

Identification of the photoaffinity-labeled proteins in complex I. (A), 2-D gel analyses (BN- and 10% tricine-SDS PAGE) in the presence (a) and absence (b and c) of 6M urea. In (c), SMP was incubated with rotenone (10 μ M) before irradiation. The arrows indicate the major radioactivity peaks, 85 kDa, 50kDa, and 33kDa. (B) and (C), a typical gel pattern of 3-D gel analyses (BN-, 10% tricine- with or without 6M urea-, and 16% tricine SDS-PAGE). The gel was stained with silver. The spots 1, 2, 3, and 4 were identified as subunits ND5, ND4, ND2, and ND1 respectively.

Table 1Radioactivity of the Labeled ND Subunits after 3-D separation of complex I ^a

Spot on 3-D gels	+6M urea	-6M urea
ND1	107	114
ND2	131	130
ND4	47	44
ND5	245	157

The values represent the averages of three different samples. Background counts were around 25.

^a Bovine heart SMP (50 µg) labeled with [³H]BPA were first subjected to BN-PAGE, and followed by 2-D (dSDS-PAGE) analysis.