Light Control of Mitochondrial Complex I Activity by a Photoresponsive Inhibitor†

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ABSTRACT: We recently developed a new class of inhibitors of bovine heart mitochondrial NADH−ubiquinone oxidoreductase (complex I), named Δlac-acetogenin [Ichimaru et al. (2005) *Biochemistry 44*, 816−825]. The inhibitory potency of Δlac-acetogenin is remarkably affected by the molecular shape of the alkyl side chains. We speculated that if the shape of the side chains can be changed by the *trans*−*cis* photoisomerization of the azobenzene unit that is introduced into the chain moiety, the inhibitory effect could be switched on and off in a reversible manner. Such a photoresponsive inhibitor may allow rapid, remote, and noninvasive control of complex I activity. Therefore, we here synthesized Δlac-acetogenin (**3**) possessing an azobenzene unit in the side chains. ^{1}H NMR, HPLC, and UV−visible absorption analyses indicated that the azobenzene unit in **3** is rapidly and reversibly *trans*−*cis* isomerized by photoirradiation in chloroform and ethanol. The inhibitory effect of *trans*,*trans*-**3** on complex I activity in submitochondrial particles was more potent than that of *cis*,*cis*-**3**. When **3** was applied at the nanomolar level to complex I, the inhibitory effect was reversibly reduced and enhanced by alternating irradiation by UV and visible light, respectively. The present study gives a positive clue to the light control of complex I activity.

NADH−ubiquinone oxidoreductase (complex I)[1] is the first energy-transducing enzyme of the respiratory chains of most mitochondria and many bacteria. It catalyzes the oxidation of NADH by ubiquinone, coupled to the generation of an electrochemical proton gradient across the membrane that drives energy-consuming processes such as ATP synthesis and flagella movement (*1*). Complex I is the most complicated and least understood multisubunit enzyme in the respiratory chain; for example, the enzyme from bovine heart mitochondria is composed of 46 different subunits with a total molecular mass of about 1 MDa (*2*). Recently, the crystal structure of the hydrophilic domain (peripheral arm) of complex I from *Thermus thermophilus* was solved at 3.3 Å resolution (*3*), whereas our knowledge about the ubiquinone redox reaction (*4*, *5*), proton translocation mechanism (*6*, *7*), and mode of action of numerous specific inhibitors (*8*, *9*) is still highly limited.

We recently developed a new class of inhibitors of the terminal electron transfer step of bovine heart mitochondrial complex I, named Δlac-acetogenin (*10*, *11*). As Δlac-acetogenins are highly unique in structure and have a different mode of inhibition compared to ordinary inhibitors including natural acetogenins (*11*), they are expected to be valuable molecular probes for investigating the mechanism of complex I. The structure−activity study of a series of Δlac-acetogenins indicated that their inhibitory effect is remarkably influenced by the physicochemical properties of the alkyl side chains (*10*, *11*). For instance, the *p*-*n*-butyl derivative (**1**, Figure 1A) has a very potent activity at the nanomolar level, but the *o*-*n*-butyl derivative (**2**) has almost no activity (*11*). This means that the molecular shape of the side chains has a remarkable influence on the inhibitory potency of Δlac-acetogenins. On the basis of this finding, we speculated that if the shape of the side chains can be changed by photoirradiation, the inhibitory effect could be switched on and off in a reversible manner. Accordingly, such a photoresponsive inhibitor may allow rapid, remote, and noninvasive control of complex I activity. The light control of the enzyme activity, in combination with other techniques, might provide a new experimental methodology to the complex I researches.

To this end, the Δlac-acetogenins possessing an azobenzene unit in the side chain moiety are suitable not only because azobenzene has been widely used as an efficient photoresponsive switch (for recent examples, see refs *12*−*16*) but also because the change in molecular shape of the substituted azobenzene due to *trans*−*cis* isomerization seems to be similar to that seen between compounds **1** and **2** (Figure 1B). Therefore, we herein synthesized Δlac-acetogenin possessing an azobenzene unit in both chains (**3**) and examined the reversible regulation of complex I activity by UV and visible light.

EXPERIMENTAL PROCEDURES

Synthesis of Compound ***3.*** The synthetic procedures and spectral data of compound **3** are described in the Supporting Information.

Photoirradiation Conditions. The photoirradiation was carried out using a LAX-102 illuminator containing a 100

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[1] Abbreviations: complex I, mitochondrial proton-pumping NADH−ubiquinone oxidoreductase; SMP, submitochondrial particles; **3** (t, t), compound **3** in which both azobenzenes adopt *trans* form; **3** (t, c), compound **3** in which one azobenzene adopts *trans* form and the other adopts *cis* form; **3** (c, c), compound **3** in which both azobenzenes adopt *cis* form.

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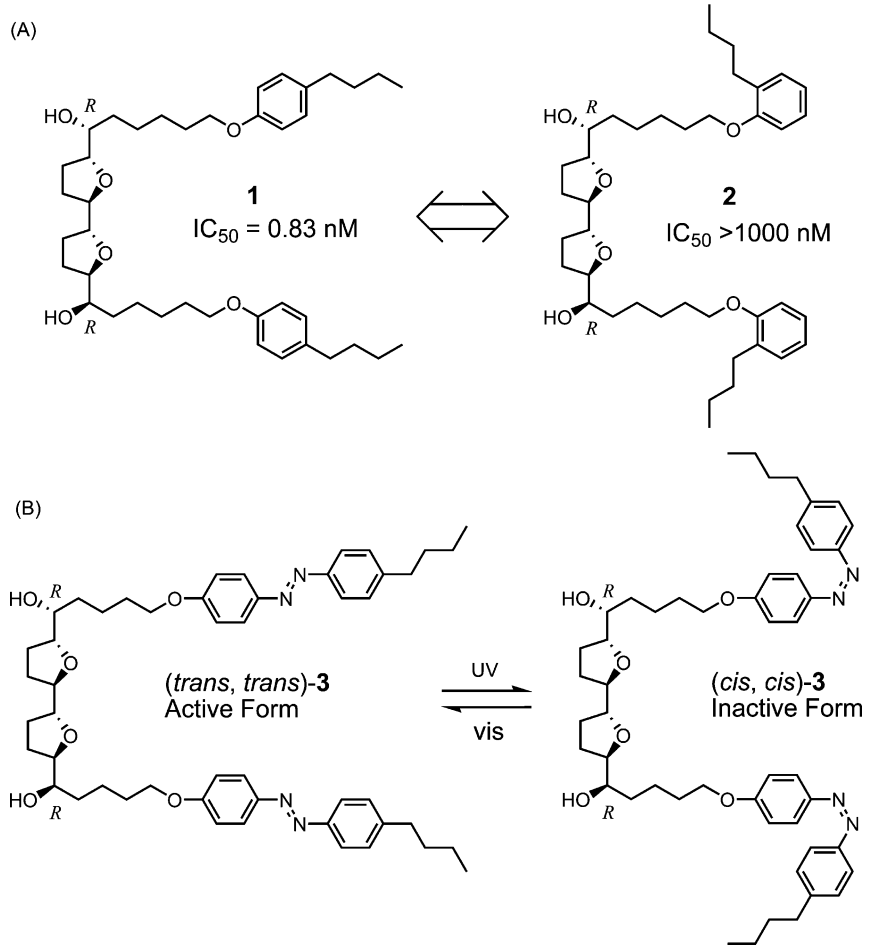

FIGURE 1: Structures of Δlac-acetogenin derivatives (**1**, **2**, and **3**) examined in this study.

W xenon arc lamp (Asahi Spectra, Tokyo, Japan) equipped with a mirror module and narrow band-pass UV−vis filters (±10 nm bandwidth) to select the required wavelength. UV or visible light was focused on sample using an appurtenant fiber cable at a distance of 3 cm.

Determination of the Trans:Cis Ratio by HPLC. The *trans*:*cis* ratio of the azobenzene unit of **3** was analyzed by a reverse-phase HPLC (Shimadzu LC-10ATvp) using COSMOSIL 5C_{18}-MS-II (4.6 × 150 mm) and methanol as an eluent. The detector wavelength was set to 310 nm, which is an isosbestic wavelength of *trans*- and *cis*-**3** (see Figure 3).

Spectroscopy. UV−visible absorption spectra of **3** were recorded using a MultiSpec-1500 photodiode-array spectrophotometer (Shimadzu) at 25 °C. ^{1}H NMR spectra were measured at 400 MHz with a Bruker AVANCE400 spectrometer using tetramethylsilane as the internal standard. When the spectra of *trans*,*trans*-**3**, in which both azobenzenes adopt *trans* form, were measured, the sample solution was kept in the dark at 25 °C for 4 days.

Measurement of Complex I Activity. Bovine heart submitochondrial particles (SMP) were prepared by the method of Matsuno-Yagi and Hatefi (*17*) using a sonication medium containing 0.25 M sucrose, 1 mM succinate, 1.5 mM ATP, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 10 mM Tris-HCl (pH 7.4) and stored in a buffer containing 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4) at −84 °C.

The NADH oxidase activity in SMP was followed spectrometrically with a Shimadzu UV-3000 (340 nm, $\epsilon = 6.2$ mM^{-1} cm^{-1}) at 25 °C. The reaction medium (2.5 mL) contained 0.25 M sucrose, 1 mM $MgCl_2$, and 50 mM phosphate buffer (pH 7.4). The final mitochondrial protein concentration was 30 μg of protein/mL. The reaction was started by adding 250 μM NADH after the equilibration of SMP with inhibitor for 5 min. When a change in complex I activity due to photoirradiation was monitored, UV or visible light was focused on a quartz reaction cuvette containing a reaction mixture using an appurtenant fiber cable at a distance of 3 cm.

RESULTS AND DISCUSSION

*Photochemical Tran−Cis Isomerization of **3** in Organic Solvents.* Compound **3** has two azobenzene units and, hence, adopts three different configurations: *trans*,*trans*, **3** (t, t); *trans*,*cis*, **3** (t, c), and *cis*,*cis*, **3** (c, c) forms. ^{1}H NMR spectra clearly indicate a difference in chemical shift between the aromatic protons of *trans*- and *cis*-azobenzenes (*18*). As judged by integration of the ^{1}H NMR signals assigned to the four sets of four aromatic protons of *trans*- and *cis*-azobenzenes, almost 100% of the azobenzene groups in **3** have the *trans* form in chloroform after the sample was kept in the dark at 25 °C for 4 days (i.e., at the thermal equilibrium state, Figure 2A). At the photostationary state achieved by irradiation at 370 ± 10 nm for 1 h, the four

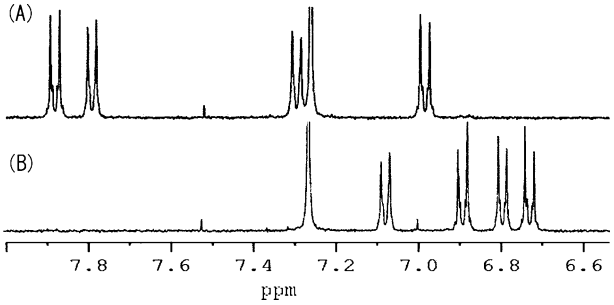

FIGURE 2: ^{1}H NMR spectra of **3** in chloroform-d_1 before (A) and after (B) irradiation at 370 (±10) nm for 1 h. The sample was kept in the dark at 25 °C for 4 days before ^{1}H NMR measurements.

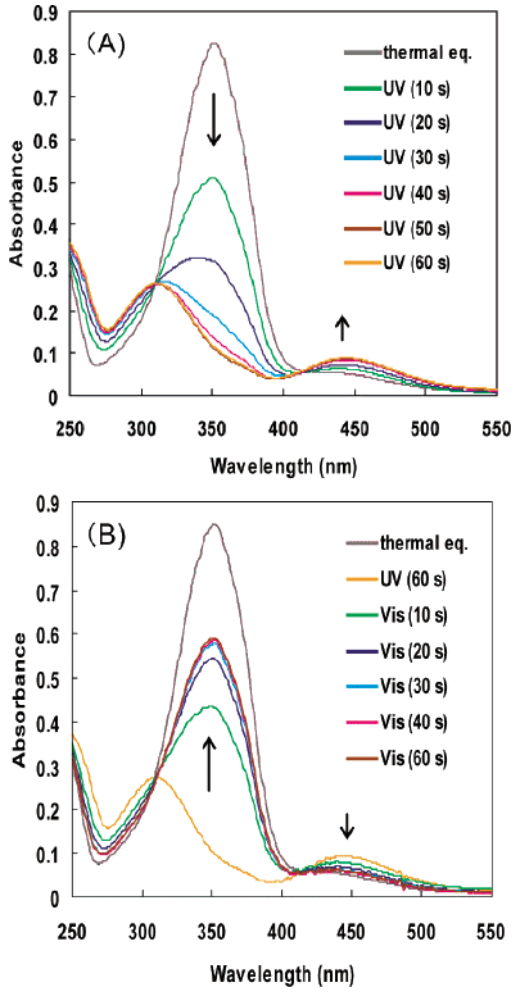

FIGURE 3: UV−visible absorption spectra of **3**. (A) The spectral changes of **3** (t, t) (1.5×10^{-5} M) upon irradiation at 370 (±10) nm in chloroform at 25 °C. (B) After reaching the photostationary state by UV irradiation, the sample was irradiated at 470 (±10) nm so the reverse isomerization could take place.

doublets showed upfield shifts, giving almost 100% *cis* form (Figure 2B).

The *trans*−*cis* photoisomerization was also confirmed from the UV−visible absorption spectra of **3**. When **3** (t, t) was irradiated at 370 ± 10 nm in chloroform, the intensity of the absorption band centered at 350 nm decreased, while that of the absorption band centered at 445 nm associated with an increase of the *cis* form increased (Figure 3A). These spectral changes are typical for the *trans*-to-*cis* isomerization of azobenzene derivatives (*19*). On the other hand, a reverse isomerization took place upon irradiation at 470 ± 10 nm

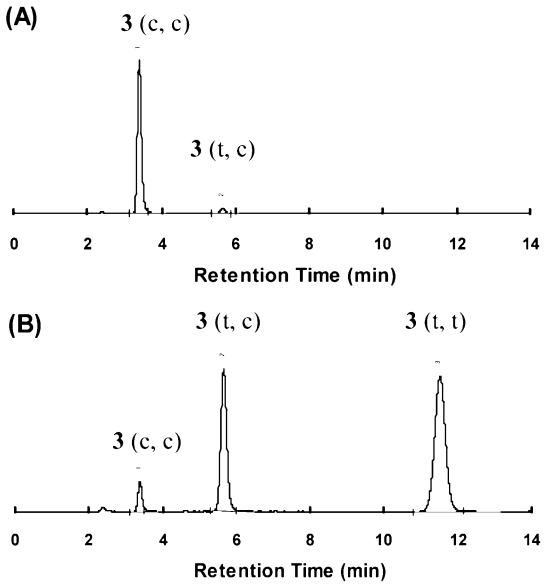

FIGURE 4: Chromatogram of HPLC analysis of **3**. (A) The ratio of **3** (t, t):**3** (t, c):**3** (c, c) after irradiation at 370 (±10) nm for 1 min was 0:5:95. (B) The ratio of **3** (t, t):**3** (t, c):**3** (c, c) after irradiation at 470 (±10) nm for 1 min was 60:35:5. The photoirradiation was carried out in ethanol (1.5×10^{-5} M).

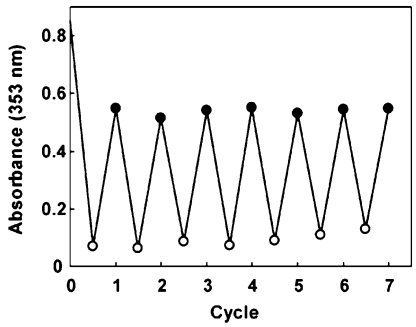

FIGURE 5: Changes in absorption of **3** observed at 353 nm after alternating photoirradiation with UV and visible light. The sample was irradiated at 370 (±10) nm (open circles) and 470 (±10) nm (closed circles) for 1 min in chloroform (1.5×10^{-5} M) at 25 °C. Data shown are representative of three independent experiments.

(Figure 3B). For both processes, 1 min irradiation was enough to attain the maximal photoisomerization under the experimental conditions.

We also carried out reverse-phase HPLC analysis to determine the molar ratio of **3** (t, t):**3** (t, c):**3** (c, c) under the adopted conditions. In general, *cis*-azobenzene is less hydrophobic than the *trans* form (*19*). The analysis indicates that the molar ratio of **3** (t, t):**3** (t, c):**3** (c, c) determined after irradiation at 370 ± 10 nm for 1 min in ethanol was 0:5:95 (Figure 4A), indicating that almost all azobenzene adopts a *cis* form. The molar ratio of **3** (t, t):**3** (t, c):**3** (c, c) determined after irradiation at 470 ± 10 nm for 1 min was 60:35:5 (Figure 4B). We confirmed that by alternating irradiation at 370 and 470 nm for 1 min, maximal photoisomerization is reproducible for at least seven cycles (Figure 5). Thus, the azobenzene unit can reversibly, but not completely (*19*), *trans*−*cis* isomerize in response to photoirradiation.

*Inhibitory Effect and Photoisomerization of **3** in Submitochondrial Particles.* We examined the inhibitory potency of **3** (t, t) and **3** (c, c) to know whether the potency of the latter is actually weaker than that of the former. The

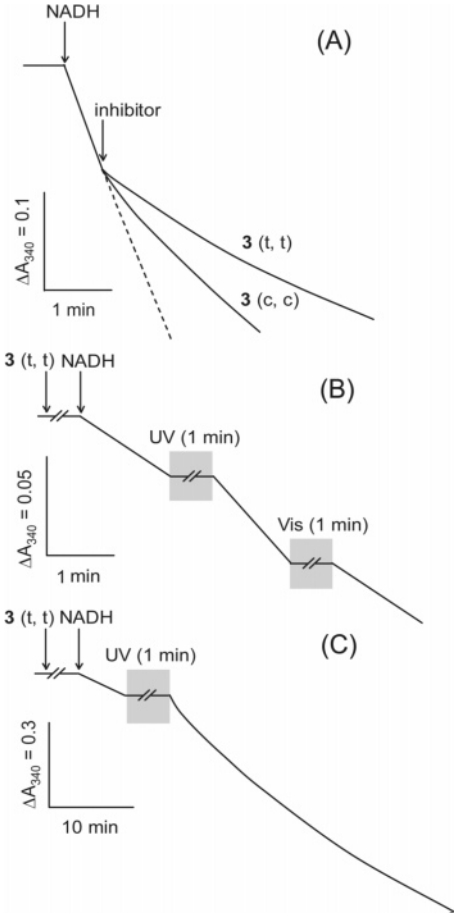

FIGURE 6: Changes in NADH oxidase activity in the presence of **3**. NADH oxidation was monitored by following the absorption at 340 nm. (A) Comparison of the inhibitory effect between **3** (t, t) and **3** (c, c). Where indicated, an ethanol solution of **3** (t, t) or **3** (c, c) was added to give a final concentration of 48 nM. (B) The inhibitory effect of **3** (t, t) on NADH oxidase activity after irradiation at 370 and 470 (±10) nm. The concentration of **3** is 48 nM. (C) NADH oxidase activity in dark after irradiation at 370 (±10) nm for 1 min at 25 °C. The concentration of **3** is 48 nM. In (B) and (C), but not (A), SMP were preincubated with **3** for 5 min before the enzyme reaction starts. Final mitochondrial protein concentration is 30 μg of protein/mL.

inhibition of complex I activity was determined by NADH oxidase assay using SMP. Compound **3** (t, t) was obtained by keeping the ethanol solution of **3** in the dark for 4 days, and **3** (c, c) was prepared by irradiating the ethanol solution of **3** at 370 ± 10 nm for 1 min. As shown in Figure 6A, the inhibitory effect of **3** (c, c) was weaker than that of **3** (t, t) at the same concentration (48 nM). However, a difference of the inhibitory potency between the two forms seems to be less than that expected from the activities of compounds **1** and **2**. In this experiment, we omitted the incubation of SMP with **3** (t, t) or **3** (c, c) before the enzyme reaction starts to avoid the thermal *cis*-to-*trans* isomerization during the incubation in SMP. Therefore, the inhibitory effect of both forms gradually increased with time, as generally observed with the assay of ordinary complex I inhibitors. We did not quantitatively evaluate the inhibitory potency of both forms (e.g., in terms of IC_{50} value) since it was impractical to determine the actual *trans*:*cis* ratio of **3** at the equilibrium state immediately after partitioning into SMP.[2] Accordingly, we cannot exclude the possibility that the apparent inhibitory effect of **3** (c, c) is, at least partly, attributable to that of **3** (t, t) [or **3** (t, c)] which was transformed from **3** (c, c) immediately after partitioning into SMP.

Next we studied the changes in the inhibitory effects of **3** due to photoisomerization in SMP. The concentration of **3** (t, t) was set at 48 nM, which exhibited about 70% inhibition of complex I activity before UV irradiation (Figure 6B). In this and the following experiment (Figure 6C), SMP were preincubated with **3** for 5 min before the enzyme reaction starts. After 1 min exposure to UV light (370 ± 10 nm) in a quartz reaction cuvette containing the SMP suspension, the enzyme activity recovered to ~60% due to the *trans*-to-*cis* isomerization (Figure 6B). Sequential irradiation by visible light (470 ± 10 nm) for 1 min reduced the enzyme activity to ~30% reversibly. This result indicates that complex I activity changes reversibly by the photoisomerization of **3**. On the other hand, by keeping the cuvette in the dark after 1 min exposure to UV light, the inhibitory effect was slowly enhanced (Figure 6C), consistent with thermal relaxation of the azobenzene moiety to the more stable *trans* configuration in the absence of light (*19*).

To find the most effective wavelength for the *trans*-to-*cis* isomerization, the reaction mixture was irradiated at 330, 350, or 370 (±10) nm for 1 min. The resulting assay spectrum shows that 370 nm is most effective for the *trans*-to-*cis* isomerization (i.e., recovering the enzyme activity). Similarly, the most effective wavelength for enhancing the inhibitory effect was examined at 430, 450, or 470 (±10) nm for 1 min irradiation, but no significant difference was observed among these wavelengths. Further, to achieve maximal *trans*−*cis* isomerization while minimizing the period of irradiation in order to avoid damage to the enzyme, we also examined the effect of the duration of irradiation on the *trans*−*cis* isomerization. As a result, 1 min irradiation at 370 and 470 nm was found to be enough to lead to the maximal *trans*−*cis* isomerization.

Reproducible Light Control of Complex I Activity. Under the best conditions examined above, alternating irradiation in the presence of 96 nM **3** reproducibly enhanced and reduced the enzyme activity between ~20% and ~50% without damage to the enzyme (Figure 7). The range of change in the enzyme activity can be varied by changing the concentration of inhibitor used (Figure 7). In these experiments, NADH oxidase activity was monitored for 1 min immediately after 1 min exposure to UV light in a quartz reaction cuvette containing SMP and **3**; consequently, the reaction cuvette was exposed to visible light for 1 min, and then the enzyme activity was monitored again. We repeated these operations continuously without additional NADH and/or **3**. Under the experimental conditions, 250 μM NADH added initially was enough to continuously monitor the enzyme activity throughout the repeated operation. Since thermal *cis*-to-*trans* relaxation of the azobenzene in SMP is markedly slow (Figure 6C), the effect of the activity changes due to the thermal isomerization on the 1 min measurement of the enzyme activity is negligibly small.

[2] To determine the *trans*:*cis* ratio in SMP, we have to measure UV−visible absorption spectra of **3** in SMP by increasing the concentrations of both **3** and SMP at least by 2 orders. However, under such experimental conditions, precise UV−visible measurement of **3** was impractical because of high turbidity of the SMP suspension and intense absorption by the hemes in the respiratory chain.

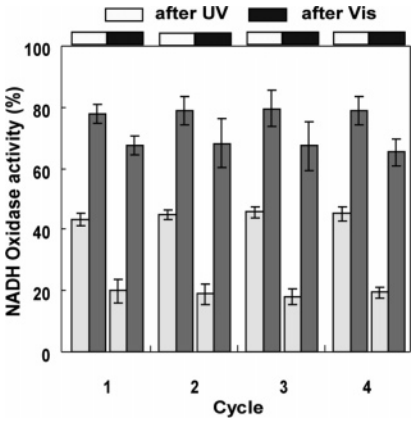

FIGURE 7: Changes in NADH oxidase activity after alternating photoirradiation with UV and visible light. The reaction mixture was irradiated at 370 and 470 (±10) nm for 1 min at 25 °C. The concentrations of **3** are 9.6 nM (dark gray bars) and 96 nM (light gray bars). The final mitochondrial protein concentration is 30 μg of protein/mL. Data are means ± SD ($n = 3$).

Although the reproducible changes in complex I activity due to photoisomerization of **3** were observed, complete recovery of the enzyme activity (i.e., entire loss of the inhibitory effect) was not achieved (Figure 7). A similar tendency due to incomplete *trans*−*cis* isomerization was observed for other photoresponsive reagents containing an azobenzene (for recent examples, see refs *16*, *20*, and *21*). This result seems to be inconsistent with the above spectroscopic analyses, which indicate that the amount of **3** (t, t) is negligibly small after UV irradiation in organic solvents (Figures 2−4). Although the *trans*:*cis* ratio of the azobenzene group in SMP cannot be predicted,[2] this is probably because the *trans*-to-*cis* isomerization process is much less efficient in SMP than in organic solvents since the bent *cis* form is energetically unfavorable due to severe steric congestion in the enzyme and/or membrane environment. Naturally, the fact that **3** (c, c) does not completely lose the inhibitory effect is also the cause of the incomplete recovery of the enzyme activity.

While complete on−off switching of complex I activity was not achieved in the present work, even a partial switching of the enzyme activity is useful to investigate the functional and structural features of complex I since some extent of changes in the redox status of the cofactors and in the enzyme structure might occur. Additionally, concerning the application of the photoresponsive inhibitor to the experiments of cellular level, even a partial change in complex I activity largely influences the physiological events which are closely associated with the activity of a mitochondrial respiratory system such as superoxide production and transport of various substances across the inner mitochondrial membrane (*22*−*25*).

In conclusion, while complete on−off switching of the inhibitory effect was impossible, the concept that complex I activity can be controlled by a photoresponsive inhibitor was in principle verified. The present study gives a positive clue to the light control of complex I activity. The photochemical technique in combination with other techniques, for example, reaction-induced FT-IR spectroscopy (*26*), would provide a new experimental methodology in the research of this highly complicated enzyme.

SUPPORTING INFORMATION AVAILABLE

Synthetic procedures of compound **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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