Bioorganic Chemistry

Design and Synthesis of Photochemically Controllable Caspase-3**

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Apoptosis, known as programmed cell death, is an essential biological phenomenon for maintaining a living system by removal of unnecessary cells and unfavorable damaged cells.[1] Caspase-3 is a cysteine protease which exists as an inactive precursor (pro-caspase-3) and is activated through two general signaling pathways for apoptotic cell death.[1,2] In death-receptor-induced apoptosis, activated caspase-8 cleaves pro-caspase-3 at the specific position for activation; then the activated caspase-3 degrades an inhibitor (DFF 45) of DNase (DFF 40) which functions for DNA fragmentation.[1,2] In the mitochondria mediated pathway an apoptosis signal also finally reaches pro-caspase-3 for activation.[2] Thus, caspase-3 is a key player in the apoptosis signaling pathway, and the artificial activation of pro-caspase-3 in a cell can be a powerful tool for induction of apoptotic cell death for removal of tumor cells and virus-infected cells. Photochemically controllable caged proteins have been utilized as initiators for the activation of some specific phenomena in the cell.[3] Incorporation of a photochemical switch into pro-caspase-3 enables photofunctionalized caspase-3 to be utilized as a trigger to induce apoptotic cell death.

Herein, we demonstrate the photochemical activation of a synthetic caspase-3 having a photoreactive amino acid (2-nitrophenylglycine, Npg)[5] at a specific position on the peptide chain. The details of the reaction mechanism of 2-nitrophenylglycine derivatives have been investigated by laser flash photolysis using a Nd:YAG laser (355 nm)[5a] and a XeCl excimer laser (308 nm).[5b,c] Npg groups have been incorporated into an ion channel and a nicotinic acetylcholine receptor in *Xenopus Oocyte* cells for investigation of their biochemical functions to be determined by measuring the cell response.[6,7] To mimic the activation mechanism of pro-caspase-3 which is specifically cleaved by activated caspase-8 through apoptotic signaling, we introduced the Npg residue at the cleavage position (S176) of caspase-3 for control of the caspase-3 activity by cleaving the peptide backbone using photoirradiation (Figure 1). In addition, the peptide sequence of the cleavage site is substituted in an unnatural way by introduction of the Npg residue at position 176; thus, the undesired autocleavage mediated by caspase-3 itself should be prohibited.[2]

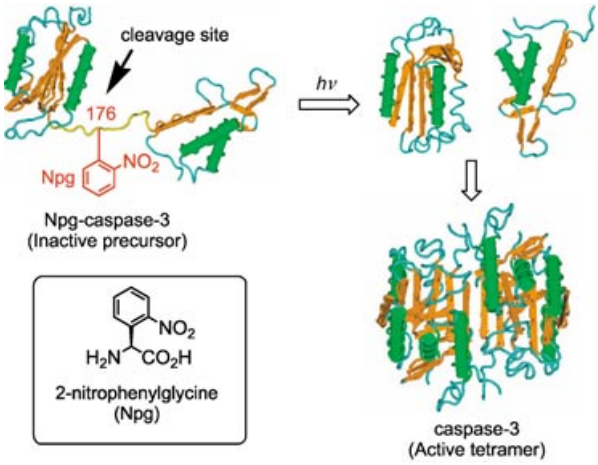

Figure 1. An illustration of the photofunctionalized caspase-3 possessing a photoreactive Npg residue at position 176 and its activation with photoirradiation. The figures of the caspase-3 and the presumed structures are reconstituted from the tetramer structure (PDB, 1CP3).[4]

Site-selective incorporation of Npg was achieved by an in vitro transcription/translation system with a plasmid containing a four-base codon (CGGG) and Npg-$tRNA_{CCCG}$.[6,8] *N*-(4-Pentenoyl)-2-nitrophenylglycine cyanomethyl ester and a nucleotide dimer pdCpA were first coupled and then protected Npg-pdCpA was ligated onto a $tRNA_{CCCG}$(-CA) by RNA ligase. The pentenoyl group was removed with saturated aqueous iodide to give Npg-$tRNA_{CCCG}$.[6] In vitro translation was performed with mutated DNA having a CGGG codon (position 176) and Npg-$tRNA_{CCCG}$ (Figure 2). The full-length Npg-incorporated caspase-3 (Npg-caspase-3) was obtained in the presence of the Npg-$tRNA_{CCCG}$ (compare lanes 2 and 3 in Figure 2), thus indicating that the Npg residue was selectively incorporated into position 176. The efficiency for the incorporation of Npg was 15%. Since the incorpo-

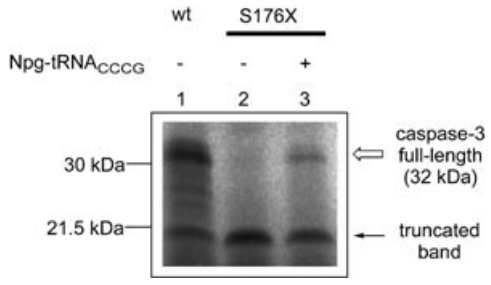

Figure 2. In vitro transcription/translation of the Npg-caspase-3 labeled with [^{35}S]methionine. Lane 1: wild-type caspase-3; lane 2: translation with mutant DNA containing a four-base codon; lane 3: translation with mutant DNA containing a four-base codon in the presence of Npg-$tRNA_{CCCG}$. S176X represents the DNA mutated by CGGG at position 176.

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ration efficiency depends on the shape of the amino acid side chains,[8b] the lack of a β-methylene group may reduce the incorporation efficiency.

The photoreactivity of the Npg-caspase-3 was investigated by photoirradiation at 0 °C by using a high-pressure mercury lamp (500 W) equipped with a monochrometer for extraction of 366 nm light. Degradation of the Npg-caspase-3 was observed on photoirradiation, and the protein almost disappeared in 5 min (see Supporting Information). No degradation of the wild-type caspase-3 was observed under these photoirradiation conditions.

To examine the photoinduced activation of Npg-caspase-3 the activity was quantified with DEVD peptide attached to rhodamine 110 (Z-DEVD-rhodamine 110) as a substrate. The activity of the Npg-caspase-3 with different photoirradiation times is shown in Figure 3. The Npg-caspase-3 showed no

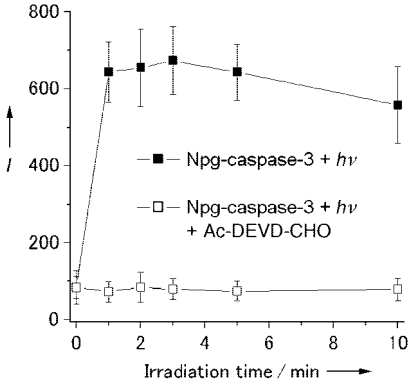

Figure 3. Expression of the caspase-3 activity using Npg-caspase-3 with photoirradiation. Photoirradiation at 366 nm was carried out for 0, 1, 2, 3, 5, and 10 min at 0 °C. The enzymatic activity after photoirradiation was quantified with Z-DEVD-rhodamine 110 as a substrate (λ_{ex} = 485 nm; detection at 538 nm). Inhibition experiments were carried out using Ac-DEVD-CHO as an inhibitor. Error bars indicate standard deviations for three independent experiments. *I* = fluorescence intensity.

activity without photoirradiation (0 min). The activity was clearly expressed after irradiation for one minute, and almost no change was observed during five minutes of irradiation. Addition of a caspase-3 inhibitor Ac-DEVD-CHO suppressed the activity of the photoirradiated Npg-caspase-3, thus indicating that the photoreacted Npg-caspase-3 specifically cleaves the substrate Z-DEVD-rhodamine 110. These results show that the photoirradiated Npg-caspase-3 can recover the intrinsic activity and function of caspase-3 and that the Npg residue can effectively function as a photochemical switch to control the activity of caspase-3.

The regulation of undesired autocleavage mediated by caspase-3 was investigated by comparison of the activities of wild-type and Npg-caspase-3 without and with photoirradiation (Figure 4). The translated wild-type caspase-3 showed activity on incubation at 25 °C. In contrast, the Npg-caspase-3 exhibited significantly lower activity compared to the wild-type caspase-3, which suggests that the substitution to the Npg residue effectively suppresses the intrinsic autocleavage of the native caspase-3. In addition, Npg-caspase-3 can clearly

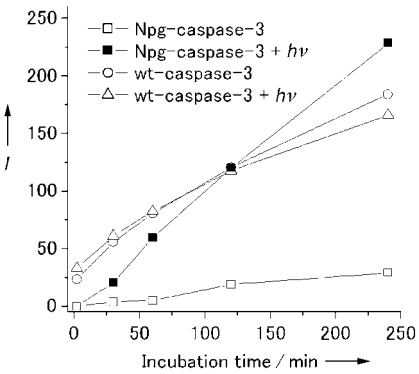

Figure 4. Suppression of autocleavage of caspase-3 by introduction of the Npg residue into the cleavage site of caspase-3. Photoirradiation at 366 nm was carried out at 0 °C for 5 min. The enzymatic activity was quantified with incubation at 25 °C for 2, 30, 60, 120, 240 min with Z-DEVD-rhodamine 110. Wt = wild type.

recover its activity after photoirradiation, thus indicating that the activity of the caspase-3 can be controlled by introduction of the Npg residue at position 176 and subsequent cleavage by photoirradiation.

In conclusion, we have designed and synthesized Npg-caspase-3, and photochemical regulation of the caspase-3 activity has been achieved. By using this strategy, caspase-3 can be inactivated by introduction of the Npg residue and the intrinsic activity recovered on photoirradiation. This type of photoinduced activation of enzymes may be available for other proteins and enzymes which are activated by site-selective proteolysis. These photofunctionalized synthetic proteins may be introduced into a cell by attaching a protein-transduction domain such as an HIV-1 TAT peptide sequence.[9]

Experimental Section

In vitro translation of wild-type and Npg-caspase-3: An EcoPro T7 System (Novagen, Darmstadt, Germany) was employed for in vitro protein synthesis following the manufacturer's protocol. The in vitro translation reaction was carried out in 10 μL of a reaction mixture containing a plasmid DNA (2–3 μg), Npg-tRNA $_{CCCG}$ (1 μg), 7 μL of EcoPro T7 extract, and L-[^{35}S] methionine (3 μCi) at 30 °C for 3 h. Wild-type caspase-3 was prepared by the same method without Npg-tRNA. The mixtures of proteins were denatured in a solution containing 50 mM Tris-HCl (pH 6.8; tris = tris(hydroxymethyl)aminomethane), 0.1 M 1,4-dithiothreitol (DTT), and 2% sodium dodecylsulfate (SDS) and were loaded onto an 18% SDS-polyacrylamide gel for electrophoresis. The SDS-PAGE gels were visualized and quantified by an imaging analyzer (Fujix BAS1000 analyzer). The wild-type caspase-3 and Npg-caspase-3 were simply purified by a gel-filtration spin column (BioRad Macro Bio-Spin 6 column, exclusion limit 6 kDa) for photoirradiation and subsequent caspase-3 assay. The concentration was quantified by Western blotting using a hexahistidine antibody as a primary antibody. Generation of the full-length proteins was about 10 ng from a 10 μL scale synthesis of the in vitro translation system.

Photoreaction of wild-type caspase-3 and Npg-caspase-3: The Npg-caspase-3 was placed in a Pyrex tube on ice. Photoirradiation was carried out by using a 500 W high-pressure mercury lamp (Ushio USH-500D) equipped with a monochrometor (Ritsu-Oyokogaku MC-10N) which can control the specific wavelength within 4 nm full-width at half-maximum.

Measurements of the enzymatic activity of the photoirradiated Npg-caspase-3 and the inhibition assay: Npg-caspase-3 was prepared at 30 °C for 3 h under the same conditions as noted above. The enzymatic activity was measured with Z-DEVD-rhodamine 110 (Promega, Madison, WI) as a substrate for caspase-3 and quantified by the fluorescence intensity of rhodamine 110 after degradation of the substrate. The photoirradiated samples were diluted with 50 mM Tris-HCl (pH 7.5), and the Z-DEVD-rhodamine 110 solution was added. After incubation at 25 °C for 4 h, the reaction mixtures were placed on a 96-well plate and quantified by a plate reader (λ_{ex} = 485 nm; detection at 538 nm). For the inhibition assay 1 μL of caspase-3 inhibitor (Ac-DEVD-CHO, 10 mM, DMSO solution) was added to the photoirradiated Npg-caspase-3 samples (20 μL). After incubation of the samples at 4 °C for 1 h, the Z-DEVD-rhodamine 110 solution was added, and the mixtures were incubated at 25 °C for 4 h. The activities were measured in the same fashion as noted above.

Measurements of autocleavage of wild-type caspase-3 and Npg-caspase-3: Npg-caspase-3 was prepared at 30 °C for 3 h under the same conditions as noted above. After translation, the reaction mixture (10 μL) was irradiated at 0 °C for 5 min under the same conditions. The samples were incubated at 25 °C for 2 h without or with an inhibitor Ac-DEVD-CHO. The enzymatic activity was measured at 25 °C for 2, 30, 60, 120, and 240 min with Z-DEVD-rhodamine 110. The reaction mixtures were placed on a 96-well plate and quantified by a plate reader (λ_{ex} = 485 nm; detection at 538 nm).

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