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Synthesis of azobenzenealkylmaleimide probes to photocontrol the enzyme activity of a bacterial histone deacetylase-like amidohydrolase

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ABSTRACT

A series of azobenzenealkylmaleimides (AMDs) with different spacer length was synthesized and coupled *via* Michael-Addition to a specific mutant of a bacterial histone deacetylase-like amidohydrolase (HDAH). Michaelis–Menten parameters (V_{max} and K_m) were employed to characterize the effect of both, the spacer length and the configuration (*cis* vs. *trans*) of the attached azobenzene moiety, on the HDAH enzyme activity. The photoswitch behavior of the AMD/enzyme conjugate activity was clearly influenced by the AMD spacer length. This study highlights the importance of steric rearrangement of the photoswitch with respect to the active site and describes a strategy to optimize the photocontrol of HDAH.

1. Introduction

Bioconjugation of light-switchable molecules is a widely used method to introduce stimulus sensitive moieties to biomolecules [1–3]. The isomerization process of light-responsive molecules offers diversified applications like photocontrol of the protein function [4–8] or the conformational change of protein structures [9,10] as well as protein assembly [11]. Azobenzene is the most studied light-switchable molecule. It undergoes a reversible isomerization process from the *E*- to the *Z*-isomer by irradiating with UV-light. The back-isomerization to the lower energy *E*-isomer (*trans*) can be realized by visible light (vis) or by thermal relaxation [12,13]. In addition to the configuration, the polarity of the azobenzene group is also switched. Both factors play an important role if azobenzene containing molecules are covalently bound to a protein surface. Bioconjugation of small molecules requires mild conditions and an orthogonal coupling strategy whereupon the Michael-Addition towards the thiol group of cysteine residues fulfills these conditions [14]. The variation of linker length is a common motif to probe the influence of a specific group to a defined area [15,16]; this elongation can be achieved by the use of different diamines. These advantages were combined by the synthesis of a series of new azobenzenealkylmaleimides (AMDs) **1a–e**. Subsequently, the reactive probes were conjugated to a specific cysteine-variant (M30C) of a photoswitchable bacterial histone deacetylase-like amidohydrolase (HDAH) from *Bordetella/Alcaligenes* strain FB188 [7] to investigate the influence of AMD spacer length variation as well as the *cis*- and *trans*-configuration on the photocontrol behavior of the enzyme activity. Furthermore, spatio-temporal controlled deacetylation reactions could be employed in biotechnological and biomedical applications to regulate the deacetylation of biomolecules or chemical compounds in a precise and defined manner to meet desired needs.

2. Materials and methods

2.1. General methods

All chemical reagents were of analytical grade, obtained from commercial suppliers and used without further purification. THF was freshly distilled under argon from sodium and stored over sodium and under argon atmosphere. TEA was distilled and stored over KOH. Column chromatography was carried out on silica gel 60 (40–60 μm), Macherey-Nagel. Reactions and chromatography

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fractions were analyzed on precoated silica gel plates (silica gel 60, F254, 20 × 20 cm, 0.25 mm thick, Merck thin-layer chromatography). Amino and Boc protected amino groups were stained by dipping the TLC plate in a ninhydrin solution (0.66% m/v in EtOH) following by heating or by absorbance of UV light at 254 nm. NMR-spectra were recorded with an Avance 300 (AC300) (300 MHz ^{1}H, 75 MHz ^{13}C) and a Bruker DRX 500 (500 MHz ^{1}H, 126 MHz ^{13}C)-spectrometer. The chemical shift data for each signal is given in units of δ (ppm) relative to tetramethylsilane (TMS), assigned δ (TMS) = 0. Coupling constants (J) are stated in Hz. HPLC–MS was performed with an Agilent Technologies Series 1200 instrument using an Agilent Eclipse XDB-C18 column. Method: 5–95% B from 2 to 17 min, 95–5% B from 20 to 25 min. Solvent A: water + 0.1% formic acid; solvent B: acetonitrile + 0.1% formic acid. The *cis*- and *trans* photoisomerization of AMDs (**1a–e**) conjugated to HDAH variant M30C was verified by UV/Vis spectroscopy measurements in MC buffer (40 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.2) at 30 °C. A solution of 3 μM of the respective AMD/HDAH M30C conjugate was irradiated by UV light by use of a hand lamp (6 W, Heraeus) at 364 nm for 25 min at a distance of 6 cm (43 mJ cm^{-2}) or blue light (1 W, LED, Osram) at 464 nm for 25 min at a distance of 6 cm (1.5 J cm^{-2}) to achieve the *cis*- or *trans*-configuration. UV/Vis spectra (280–400 nm) of photoisomerized samples were subsequently recorded with a UV/Vis spectrophotometer (Jasco V-630).

2.2. Biochemical methods

2.2.1. Employed HDAH variants

HDAH variant C51S was used to investigate the inhibitory effect of the respective *cis*- and *trans*-configuration of (*E*)-1-(4-(phenyldiazenyl)phenyl)-1*H*-pyrrole-2,5-dione (4-PAM) and AMDs (**1a–e**) by determination of IC_{50} values. HDAH variant M30C was used as conjugate and test model to investigate the AMD dependent influence on the photoswitch capability with respect to the spacer length. Both HDAH variants were generated by site-directed mutagenesis, expressed in *Escherichia coli* strain XL1-blue and purified as previously reported [7].

2.2.2. Modification of HDAH variant M30C

The single solvent accessible cysteine of HDAH variant M30C was chemically modified by 4-PAM or AMDs (**1a–e**) by a 4-fold molar excess in MC buffer (supplemented with 8% DMSO) and the modification procedure continued as described previously [7].

2.2.3. Determination of Michaelis–Menten kinetics

The determination and analysis of Michaelis–Menten kinetics of unmodified and AMD (**1a–e**) modified HDAH M30C variants, photoisomerized to their *cis*- (UV light, 364 nm, 43 mJ cm^{-2}) and *trans*-configuration (blue light, 464 nm, 1.5 J cm^{-2}), was performed as reported previously [7]. Pluronic F 68 was purchased from BASF and bovine serum albumin from Sigma–Aldrich. Measurements were performed as independent triple measurements and Michaelis–Menten parameters (K_m and V_{max}) represented as means ± standard deviation (SD), n = 3. The photoswitch efficiency of V_{max}- and K_m-values, between the *cis*- and *trans*-configuration, was calculated as percentages by definition of the more active isomer.

2.2.4. DOL value determination

The DOL value (average number of conjugated AMD molecules per each HDAH variant M30C molecule) calculation of AMD/HDAH M30C variant conjugates was performed as described previously [7] *via* Lambert–Beer equation by use of the determined molar extinction coefficient (ε) of *trans* 4-PAM at its absorbance maximum after conjugation to HDAH variant M30C (15,700 l mol^{-1} cm^{-1}; 330 nm) and the predicted ε of HDAH wild type (wt) (43,890 l mol^{-1} cm^{-1}; 280 nm).

2.2.5. Determination of IC_{50} values

To determine the inhibitory effect of the *cis*- and *trans*-configuration of 4-PAM and AMDs (**1a–e**) on HDAH variant C51S activity, dose–response curves were performed and IC_{50} values analyzed. The HDAH C51S activity was determined by employing a fluorescence-based activity assay in a similar way as described previously [17,18]. 5 mM 4-PAM or AMD (**1a–e**) DMSO solutions were isomerized to their *cis*-configuration by UV light irradiation (364 nm, 43 mJ cm^{-2}) using a hand lamp as described before and serially diluted in FB188 buffer (15 mM Tris, 50 mM KH_2PO_4/K_2HPO_4, 250 mM NaCl, 250 μM EDTA, 0.001% Pluronic F 68 and 0.005% bovine serum albumin; pH 8.0). The *trans*-configuration of mentioned compounds was achieved by thermal relaxation of the serially diluted samples due to incubation at 30 °C for 5 h in the dark. The HDAH catalyzed deacetylation reaction, in dependence on increasing 4-PAM or AMD concentrations, was performed as a one-step assay in the dark in black 96-well half-area microplates (flat bottom, polystyrene, medium binding; Greiner Bio-One) providing final concentrations of 100 nM HDAH variant C51S, 0.5 mg/mL Trypsin from bovine pancreas (Serva) and 50 μM BocLys(Ac)AMC (Bachem) as substrate. The fluorescence intensity of 7-amino-4-methylcoumarin (AMC) was detected at 450 nm (350 nm excitation) over a period of 2 h at 30 °C and the HDAH C51S activity determined from the slope of the linear initial phase of measured kinetics and expressed as relative fluorescence units per second (RFU s^{-1}). The latter was correlated to the activity of a sample of HDAH C51S where no compound was added (stated as 100%), the activity expressed as percentages and plotted against the log of applied compound concentration. Dose–response curves were analyzed by the program GraphPad Prism (GraphPad Software) using a three-parameter dose–response model and IC_{50}-values determined in independent triplicates and specified as means ± SD, n = 3. Dose–response curves of DMSO were equally determined as described before using HDAH-wt to verify the real inhibitory effect, induced by 4-PAM and AMDs (**1a–e**) on HDAH activity. Additionally, a dose–response curve of a known histone deacetylase inhibitor like suberoylanilide hydroxamic acid (SAHA) was tested on HDAH-wt activity to compare the inhibitory potency with 4-PAM and AMDs. The dose–response curve of SAHA was performed as a single determination by use of the above described assay conditions and data points comparably analyzed by a three-parameter dose–response model.

3. Experimental procedure

3.1. Synthesis of AMD 1b–e

The procedure of the synthesis of compounds **3c–e, 4c–e, 5c–e** and **7** as well as the ^{1}H NMR and ^{13}C NMR spectra of AMDs **1a–e** can be found in supplementary material.

3.1.1. (E)-N-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)-4-(phenyldiazenyl)benzamide (**1b**)

To a solution of 1-(2-aminoethyl)-1*H*-pyrrol-2,5-dione 2,2,2-hydrochloride: (150 mg, 0.849 mmol) and TEA (350 μL, 2.50 mmol) in THF (4 mL) was added (*E*)-perfluorophenyl 4-(phenyldiazenyl)benzoat **7** (666 mg, 1.70 mmol) in THF (4 mL) dropwise. After 16 h at rt, the solvent was evaporated, the resulting residue was dissolved in $CHCl_3$ and washed with H_2O (30 mL), 1 M HCl (30 mL) and brine (30 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by silica column (eluent EtOAc:hexane: 1:4 → 1:1; R_f: 0.10 → 0.50)