Published in final edited form as:

J Am Chem Soc. 2018 January 10; 140(1): 14–17. doi:10.1021/jacs.7b08709.

Computational Design of a Photo-controlled Cytosine Deaminase

Kristin M. Blacklock, Brahm J. Yachnin, G. Andrew Woolley, Sagar D. Khare*

Department of Chemistry and Chemical Biology, Center for Integrative Proteomics Research,
Rutgers University Department of Chemistry, University of Toronto

Abstract

There is growing interest in designing spatiotemporal control over enzyme activities using noninvasive stimuli such as light. Here, we describe a structure-based, computation-guided predictive method for reversibly controlling enzyme activity using covalently attached photo-responsive azobenzene groups. Applying the method to the therapeutically useful enzyme yeast cytosine deaminase, we obtained a ~3-fold change in enzyme activity by the photo-controlled modulation of the enzyme's active site lid structure, while fully maintaining thermostability. Multiple cycles of switching, controllable in real time, are possible. The predictiveness of the method is demonstrated by the construction of a variant that does not photoswitch as expected from computational modeling. Our design approach opens new avenues for optically controlling enzyme function. The designed photo-controlled cytosine deaminases may also aid in improving chemotherapy approaches that utilize this enzyme.

Graphical Abstract

*Corresponding Author: sagar.khare@rutgers.edu.

Supporting Information. Table S1 and S2. Figures S1–S5. Computational and Experimental Methods.

Externally modulating the activity of an enzyme allows for the precise control over the location, timing, and amplitude of its activity¹. Many successful advances in photo-control over enzymes have arisen through the use of photocaged unnatural amino acids², or fusing split enzymes to naturally occurring photoswitchable protein-protein interactions^{3,4}. In many biological systems, however, it is beneficial to design regulatory mechanisms with reversible, photoswitching properties without splitting proteins, and to this end, labeling enzymes with azobenzene derivatives is an attractive option. Azobenzene is a photochromic molecule capable of undergoing *trans*-to-*cis* isomerization upon illumination with UV light and *cis*-to-*trans* isomerization via thermal relaxation or illumination with visible (VIS) light, which causes reversible end-to-end length changes^{5,6}. Coupling these changes to the conformational modulation of the attached protein has enabled the design of a switchable cadherin⁷, restriction enzyme⁸, leucine zipper⁹, and an allosterically modulated ionotropic glutamate receptor¹⁰. However, azobenzene attachment is generally performed using trial-and-error as a generalizable, predictive design protocol for the crosslinking of a protein structure with an azobenzene derivative is not available.

Directed enzyme prodrug therapy (DEPT) is a strategy that aims to reduce undesirable side effects of chemotherapy by selectively activating prodrugs at tumor sites while sparing normal tissue from damage¹¹. The zinc-containing dimeric metalloenzyme cytosine deaminase from yeast (yCD) is a prototypical DEPT enzyme, that catalyzes the conversion of cytosine to uracil, and is used with the prodrug 5-fluorocytosine (5FC) to generate the toxin 5-fluorouracil (5FU). A reversibly photoactivatable yCD variant would be desirable in

DEPT to site-specifically induce toxicity with an external, noninvasive optical signal. Previous structural and mechanistic studies have shown that yCD activity involves a dynamic active site lid region comprising an amphipathic helical segment at its C-terminal^{12–15}. The lid helix covers the active site, and is spatially proximal to the dimer interface of the enzyme.

We developed a generalizable computational protocol for the design of a photo-controlled yCD, a prototypical DEPT enzyme, *via* the crosslinking of two suitably placed cysteine residues with a chosen azobenzene derivative. In a first step, we used the RosettaMatch algorithm¹⁶ to query all pairs of residue positions in a thermostabilized variant of yCD (called yCD-Triple¹⁷) crystal structure (PDB:1YSB) to find suitable cysteine substitutions that could sterically accommodate the attachment of an azobenzene derivative in the UV-light induced *cis*-conformation. We chose the bifunctional azobenzene derivative 4,4'-bis(maleimido)azobenzene (ABDM) (Fig. 1A) that can be attached with high efficiency to designed cysteines. The Rosetta Match algorithm requires the generation of a conformational ensemble of the azobenzene derivative and a set of defined parameters for the maleimide-cysteine attachment geometry, (Fig. 1B), which were derived from a search of the Cambridge Crystallographic Database (CCDC)¹⁸. Once geometrically compatible locations were obtained (Fig. 1C), further sequence optimizations were performed using RosettaDesign (Fig. 1D) and five designs were chosen for further characterization, based on (1) distance of the designed cysteine pairs from the active site, (2) Rosetta energies, and (3) the conformational strain at the azobenzene-cysteine connection (Table S1), and (4) the predicted ability of *cis-trans* isomerization to induce distortions in the active site structures, as determined by Rosetta-based simulations of modified proteins. We illustrate the ABDM cross-linked models for two designs, called yCD-Opt1 and yCD-Opt2 (Fig. 1E).

To computationally predict the impact of light-induced *cis-trans* isomerization on the active site structure, we simulated the *cis*-to-*trans* isomerization of the designed ABDM-crosslinked enzymes by “mutating” the azobenzene derivatives to their *trans* isomers and performing Rosetta FastRelax^{19,20} simulations. We allowed for the regions flanking the secondary structure elements that contained the cysteine mutations to sample alternative backbone and sidechain movements in order to conservatively sample structural changes during the conformational switching (Fig. 2A, 2B). The degree of active site disruption and reconstitution was measured by C α -RMSDs with respect to the native state.

The lowest scoring *trans* conformations of the yCD-Opt1 (Fig. 2C) and yCD-Opt2 (Fig. 2D) designs show widely different C α -RMSDs in each of their respective subsets of moveable residues, indicating the yCD-Opt1 cross-linking position requires a wider range of motion to accommodate the *trans* ABDM conformation as compared to yCD-Opt2. All of the movement exhibited by yCD-Opt1 in the *trans* state was shown to be in the swinging of active site lid helix $\alpha 6$ away from the active site pocket (Fig. 2E). In stark contrast, the design yCD-Opt2 shows little movement from the native conformation in the *trans*-ABDM state, demonstrating an ability to absorb the motion of the ABDM conformational switch using local flexibility in glycine residues adjacent to the helix (Fig. 2F).

For experimental testing, first, we reduced the number of non-designed crosslinkable residues by substituting three of the four non-catalytic native cysteine residues in yCD-Triple. The resulting variant, yCD-Triple- C3, featured three mutations, C36A, C71S, and C137A, and this template was used as the backbone on which yCD-Opt1 (E75C/F153C) and yCD-Opt2 (K117C/T124C) substitutions were made. Both designed proteins were expressed in the *E. coli* strain BL21(DE3)pLysS using the pET15b expression vector, and purified with standard Ni²⁺-affinity chromatography followed by thrombin cleavage to remove the His₆-tag (Supplementary Information). The cysteine pair in yCD-Opt1 is adjacent to one another on different monomers of the dimer, and is expected to covalently link the dimer post-labeling (Fig. 1E). In yCD-Opt2, the pair of cysteines is located on the same interior alpha-helix, with the cysteines flanking the α helical segment (Fig. 1E).

We first investigated the labeling efficiency of yCD-Opt1 and yCD-Opt2 with ABDM, as well as the ability of the post-labeled ABDM molecule to reversibly isomerize under UV and blue light signals. Denaturing and reducing SDS-PAGE results show an increased intensity of the dimer-sized molecular weight band in the labeled yCD-Opt1 sample versus the unlabeled yCD-Opt1 sample, indicating the existence of a majority population of yCD-Opt1 species with at least one bridging ABDM molecule between the two monomers (Fig. 3A). ESI-MS of yCD-Opt1 variants (Fig. 3B) revealed a mass change of 820 Da upon labeling, which was close to the expected change of 816 Da for two ABDM moieties per dimer, assuming the maleimide moieties were hydrolyzed during workup⁸. Electrophoretic patterns of yCD-Opt2, on the other hand, show a majority of the monomer species in both labeled and unlabeled samples although some labeling induced multimerization was observed indicating a more heterogeneous labeling pattern (Fig. S1B). We further tested the completeness of ABDM crosslinking by confirming the lack of reactivity of cysteine residues to a PEG-maleimide derivative post-crosslinking (Fig. S2).

To investigate the conformational changes upon labeling in yCD-Opt1, we performed ¹⁹F-NMR spectroscopy with protein samples containing genetically incorporated 5-fluorotryptophan (5-FW) (Fig. 3C). yCD-Triple and yCD-Opt1 contain two tryptophan residues: W10 (distal from active site) and W152 (in the active site lid). Comparisons of the spectra of yCD-Triple and yCD-Opt1 (before and after labeling) indicate that one tryptophan residue (likely W152) experiences an altered environment due to cysteine mutations and labeling by ABDM (detected in the *trans* state), in accord with our simulations (Figs. 2 and 3C). Intrinsic tryptophan fluorescence experiments also showed clear differences in UV-irradiated and blue-irradiated ABDM-labeled yCD-Opt1 spectra further supporting the change in the environment of W152 in the active site lid upon irradiation (Fig 3D).

Repeated irradiation cycles of UV and blue light demonstrated the reversible photoisomerization of ABDM post-crosslinking with no loss of intensity over 15 cycles for yCD-Opt1 (Fig. 4A). In addition, labeled yCD-Opt1 is able to produce similar and right-shifted characteristic absorbance spectra changes when illuminated with blue light (455 nm) followed by UV light (375 nm) compared to the uncrosslinked azobenzene derivative, in which the *trans* isomer has a local maximum absorbance at 363 nm that decreases upon illumination (Fig. 4B). The half-life for thermal relaxation of the azobenzene moiety in the context of bound ABDM to yCD-Opt1 is approximately 90 minutes at 25°C (Fig. 4B).

These experiments were not performed with yCD-Opt2 as it did not show activity changes upon irradiation (*vide infra*). The longer half-life of the ABDM upon attachment to the yCD-Opt1 protein compared to its reported half-life in the unattached state (~30 minutes²¹) indicates that the protein structure stabilizes the otherwise higher-energy *cis* state, and indicates that further modulation of the half-life of the more active state, an important parameter for chemotherapeutic utilization of the enzyme, may be feasible by altering the local stability of the protein environment.

Next, we tested the activity of the cross-linked yCD variants using a previously-described cytosine consumption assay²². The cross-linked yCD variant, yCD-Opt1, showed increased activity under UV light when the azobenzene adopted predominantly the *cis* conformation compared to the *trans* (Fig. 4C, S3A). The second yCD variant, yCD-Opt2, showed no difference in activity toward cytosine under both UV and blue light after labeling, and the activities of both were higher than the unlabeled yCD-Opt2. Gratifyingly, this behavior of yCD-Opt1 and yCD-Opt2 is in agreement with our computational prediction (Fig. 2D,2F).

Further characterization of yCD-Opt1 specific activity and Michaelis-Menten kinetics under UV and blue light indicated that UV-light irradiation leads to an approximately 2.5-fold increase in the specific activity of the enzyme compared to the dark state (Fig. 4C), and that the increase appears to primarily affect the k_{cat} value ($2.2 \pm 0.10 \text{ s}^{-1}$ and $1.4 \pm 0.07 \text{ s}^{-1}$ under UV and blue light, respectively; Table S2). These results are consistent with previous mechanistic studies which have found that product release is rate-limiting in the overall mechanism of the enzyme^{13,15}, and Trp152 plays a key role in this process. Our NMR and fluorescence experiments suggest that the environment of Trp152 is altered in yCD-Opt1 by labeling and irradiation, potentially leading to the observed changes in k_{cat} .

Optical control over the activity of yCD-Opt1 could be observed in real time by switching the external signal from UV light to blue light over several five-minute iterations during the course of the assay (Fig. 4D). The more negative slopes of each UV-light interval compared to each blue-light interval after normalizing the slopes to the starting concentrations of cytosine at the beginning of each interval indicates that the photomodulation of yCD-Opt1 is nearly fully reversible.

We next tested the photosensitized activity of yCD-Opt1 against its DEPT prodrug, 5FC, and found a smaller (~1.5-fold) difference in activity between UV and blue-irradiated samples compared to the cytosine assays (Fig. S3B). The *cis*-conformation azobenzene derivative has a higher activity toward 5FC than the *trans* version, but both are closer in activity to the unlabeled yCD-Opt1 than in the cytosine case.

Having demonstrated the photosensitization of yCD-Opt1 (Fig. 4), we investigated whether the light-induced change in activity was due to global destabilization of the enzyme. We used CD thermal melt experiments to probe protein stability under increasing temperatures, and found similar melting curves and temperatures for the design starting point variant (yCD-Triple) and yCD-Opt1 labeled with ABDM in the *trans* conformation (Fig. S4), which further suggest that a local change in structure as detected by ¹⁹F-NMR and intrinsic

fluorescence measurements is likely the cause of the photosensitization rather than an overall global instability imparted to the enzyme by the ABDM cross-link in the *trans* state.

In summary, we have developed a predictive computational approach for designing photo-control over enzyme activity, and validated our method using the therapeutically relevant enzyme yCD. Designs that were predicted to show (yCD-Opt1) and to not show (yCD-Opt2) photoswitchability behaved in accord with the computational models. Our results provide some insights into design rules enzyme photoswitchability using azobenzene crosslinking: labeling sites must be placed close enough to the active site to affect substrate binding or catalytic turnover, but not so close as to disrupt catalytic activity in a non-switchable manner. In comparing the two yCD variants, it appears that placing the azobenzene cross-linker in a position that reversibly disrupts local dynamics that are required for catalysis (lid-opening and closing) is likely to be a good strategy for selection of azobenzene attachment sites. It is also likely that greater conformational effects due to photoswitching are inducible at protein termini as opposed to internal positions. While further improvements in switching efficacy, especially with 5-FC, are being pursued in our laboratory, we expect that the described computational method should assist with the predictive design of azobenzene attachment-mediated photo-control over a variety of other enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

SDK acknowledges support from the Cancer Institute of New Jersey (grant P30CA072720–18) and NSF (grant MCB1330760). BJY is supported by a Canadian Institutes of Health Research Postdoctoral Fellowship. We thank B. L. Stoddard for a gift of the pET15b plasmid harboring the yCD-Triple gene, and S. Gurla and G. Montelione for help with NMR experiments.

Funding Sources

No competing financial interests have been declared.

REFERENCES

- (1). Beierle JM; Kistemaker HAV; Velema WA; Feringa BL Chem. Rev 2013, 113, 6114. [PubMed: 23614556]
- (2). Riggsbee CW; Deiters A Trends Biotechnol 2010, 28 (9), 468. [PubMed: 20667607]
- (3). Brechun KE; Arndt KM; Woolley GA Curr. Opin. Struct. Biol 2017, 45, 53. [PubMed: 27907886]
- (4). Guntas G; Hallett RA; Zimmerman SP; Williams T; Yumerefendi H; Bear JE; Kuhlman B Proc. Natl. Acad. Sci. U. S. A 2015, 112 (1), 112. [PubMed: 25535392]
- (5). Beharry AA; Woolley GA Chem. Soc. Rev 2011, 40 (8), 4422. [PubMed: 21483974]
- (6). Pozhidaeva N; Cormier ME; Chaudhari A; Woolley GA Bioconjug. Chem 2004, 15 (6), 1297. [PubMed: 15546196]
- (7). Ritterson RS; Kuchenbecker KM; Michalik M; Kortemme TJ Am. Chem. Soc 2013, 135 (34), 12516.
- (8). Schierling B; Noël A-J; Wende W; Hien LT; Volkov E; Kubareva E; Oretskaya T; Kokkinidis M; Römpf A; Spengler B; Pingoud A Proc. Natl. Acad. Sci. U. S. A 2010, 107 (4), 1361. [PubMed: 20080559]

- (9). Kumita JR; Flint DG; Woolley GA; Smart OS Faraday Discuss 2003, 122, 89. [PubMed: 12555851]
- (10). Volgraf M; Gorostiza P; Numano R; Kramer RH; Isacoff EY; Trauner D Nat. Chem. Biol 2006, 2 (1), 47. [PubMed: 16408092]
- (11). Karjoo Z; Chen X; Hatefi A Adv. Drug Deliv. Rev 2016, 99, 113. [PubMed: 26004498]
- (12). Ko T-P; Lin J-J; Hu C-Y; Hsu Y-H; Wang AH-J; Liaw S-HJ Biol. Chem 2003, 278 (21), 19111.
- (13). Zhao Y; She N; Zhang X; Wang C; Mo Y Biochim. Biophys. Acta - Proteins Proteomics 2017, 1865 (8), 1020. [PubMed: 28478051]
- (14). Ireton GC; Black ME; Stoddard BL Structure 2003, 11 (8), 961. [PubMed: 12906827]
- (15). Yao L; Li Y; Wu Y; Liu A; Yan H Biochemistry 2005, 44 (15), 5940. [PubMed: 15823054]
- (16). Zanghellini A; Jiang L; Wollacott AM; Cheng G; Meiler J; Althoff EA; Röthlisberger D; Baker D Protein Sci 2006, 15 (12), 2785. [PubMed: 17132862]
- (17). Korkegian A; Black ME; Baker D; Stoddard BL Science 2005, 308 (5723), 857. [PubMed: 15879217]
- (18). Groom CR; Bruno IJ; Lightfoot MP; Ward SC Acta Crystallogr. Sect. B Struct. Sci. Cryst. Eng. Mater 2016, 72 (2), 171.
- (19). Fleishman SJ; Leaver-Fay A; Corn JE; Strauch E-M; Khare SD; Koga N; Ashworth J; Murphy P; Richter F; Lemmon G; Meiler J; Baker D PLoS One 2011, 6 (6), e20161. [PubMed: 21731610]
- (20). Misura KMS; Baker D Proteins Struct. Funct. Genet 2005, 59 (1), 15. [PubMed: 15690346]
- (21). Wu D; Dong M; Collins CV; Babalhavaeji A; Woolley GA Adv. Opt. Mater 2016, 4 (9), 1402.
- (22). Mahan SD; Ireton GC; Knoeber C; Stoddard BL; Black ME Protein Eng. Des. Sel 2004, 17 (8), 625. [PubMed: 15381761]

Figure 1. Computational design of ABDM-labeled yCD-dC3 variants.

(A) Schematic of the *cis* (purple) and *trans* (cyan) states of ABDM. Isomerization from *cis* to *trans* is induced by UV light, while *trans* to *cis* is induced by blue light or thermal relaxation. The distance between maleimide groups in the *cis* state is shorter than in the *trans* state. (B) The rotatable degrees of freedom within the *cis*-ABDM ligand (purple) and between the maleimide moieties and cysteine (grey) sidechains are shown with circular arrows. (C) The inverse rotamer ensemble (grey lines) generated by the specified degrees of freedom in (B) is shown surrounding *cis*-ABDM (purple). A pair of successful rotamers (green sticks) have been matched with a protein backbone (grey tube, Ca atoms shown as spheres). (D) Flowchart of the RosettaMatch-based design approach for *cis*-ABDM labeled proteins. (E) Designed yCD-Opt1 and yCD-Opt2 ABDM attachments to the yCD dimer.

Figure 2. *Cis-to-trans* simulations of yCD-Opt1 and yCD-Opt2.

Purple and cyan are used to denote the *cis* and *trans* states, respectively, throughout. The Rosetta FoldTree used during *cis-trans* isomerization simulations for (A) yCD-Opt1 and (B) yCD-Opt2. Cysteine substitutions are denoted with yellow stars. (C) Total Score (R.E.U.) vs Cα-RMSD of the terminal helix residues in the *cis*-Designed (black), *cis-to-trans*, and *trans-to-cis* states. The cyan points show that the *trans* state disrupts yCD-Opt1, while the purple points demonstrate that the *cis* state can retain the native structure. (D) Total Score (R.E.U.) vs Cα-RMSD of the residues within and flanking the helix in the *cis*-Designed (black), *cis-to-trans*, and *trans-to-cis* states of yCD-Opt2. Both cyan and purple points have low Cα-RMSDs, indicating that neither conformation severely disrupts yCD-Opt2. (E, F) Lowest-energy decoys from the simulations in (C) and (D) (structures denoted with arrows). In (E), there is considerable displacement of two of three lid-region residues (yellow-bordered sticks), while (F) shows minor displacement of secondary structure near the active site (catalytic zinc is shown as grey sphere).

Figure 3. yCD-Opt1 Labeling with ABDM.

(A) yCD-Opt1 SDS-PAGE Gel showing unlabeled (Lane 1) and labeled (Lane 2) yCD-Opt1. (B) ESI mass spectra of unlabeled (green) and *cis*-ABDM labeled (black) yCD-Opt1. (C) ^{19}F NMR spectra of 5-F-Trp-labeled yCD Triple, yCD-Opt1, and Labeled yCD-Opt1. Resonances of W10 and W152 (denoted with an asterisk) have been inferred from the proximity of W152 to the yCD-Opt1 mutations and the ABDM molecule in the labeled sample. (D) Fluorescence emission spectra of yCD-Opt1 labeled with free-maleimide (FM) or ABDM under blue and UV light. The inset highlights the differences between the spectra at an emission wavelength of 350 nm, where error bars are +1 SD from three independent experiments.

Figure 4: Bound ABDM characterization and optical control of cross-linked yCD-Opt1 cytosine deaminase activity.

(A) The photoisomerizability of bound ABDM under UV (purple squares) and blue (cyan circles) light is reversible. (B) Thermal relaxation of bound ABDM from the *cis*-state (purple) toward unilluminated, *trans*-state (black) monitored for three hours at 25°C. (C) Cytosine concentration vs. time with FM-labeled (short-dashed lines) and cross-linked (solid lines) yCD-Opt1 under UV and blue light, and cytosine-only controls under UV and blue light (dotted lines). Error bars are +1 s.d. from four independent experiments. (D) Slopes of cytosine consumption assays with labeled yCD-Opt1 as a function of repeating five-minute intervals of UV light (purple bars) followed by blue light (cyan bars), normalized to the starting cytosine concentration for that illumination cycle.