Angewandte
International Edition Chemie

DOI: 10.1002/anie.201307207

Light-Regulated Enzyme Activity

Exploiting Protein Symmetry To Design Light-Controllable Enzyme Inhibitors[**]

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Abstract: *The activity of the metabolic branch-point enzyme PriA from Mycobacterium tuberculosis (mtPriA) can be controlled reversibly by light. Two-pronged inhibitors based on the dithienylethene scaffold were designed utilizing mtPriA's natural rotational symmetry. Switching from the flexible, ring-open to the rigid, ring-closed isomer reduces inhibition activity by one order of magnitude.*

The artificial control of biological processes by light is a rapidly emerging area of protein design.[1] Three basic strategies for the light regulation of biomolecules have been reported: key positions have been functionalized with photolabile protecting groups,[2] naturally occurring photoreceptors have been reprogrammed,[3] and designed molecules that can be reversibly switched by light (photoswitches) have been used to direct protein or cellular function.[4] With respect to the latter, substantial progress has been made in the regulation of neuronal activity by designing light-inducible ligands for ion channels and receptors.[5] As the molecular recognition of specific ligand parts leads to a nonlinear signal response in neural systems, even small changes in the binding efficacy upon light irradiation significantly influence the cellular output.[6] In contrast, for the reversible control of enzymatic activities, the switching of a photoresponsive group must substantially affect the enzyme's active site. This task can be fulfilled either by the covalent incorporation of a molecular photoswitch near the catalytic center[4,7] or by the design of a noncovalently bound, light-controlled inhibitor.[4,8]

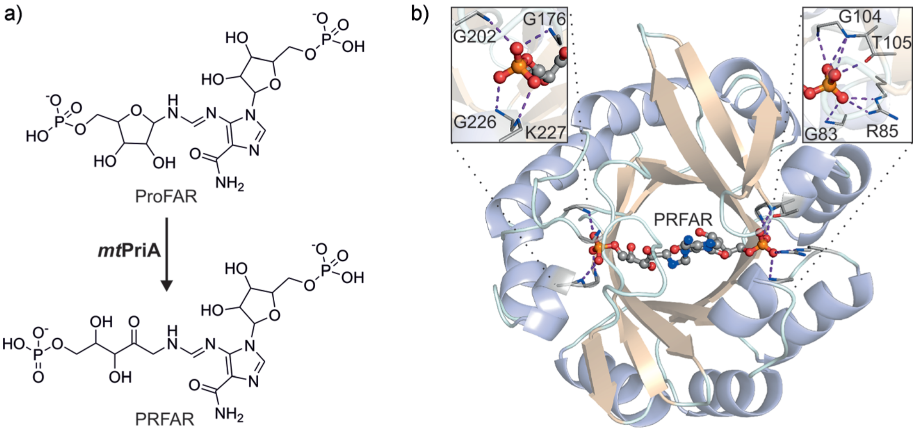

Figure 1. Reaction and structure of *mt*PriA. a) *mt*PriA catalyzes the conversion of ProFAR to PRFAR in the histidine biosynthesis. b) Ribbon representation of the $(\beta\alpha)_8$-barrel structure of *mt*PriA with bound product PRFAR (PDB ID: 3ZS4[12a]). The view is along the twofold symmetry axis of the protein. PRFAR is anchored by two opposite phosphate binding sites, which are enlarged in the insets. Hydrogen bonds are indicated by dashed lines.

We aimed to design a light-controlled inhibitor for phosphoribosyl isomerase A from *Mycobacterium tuberculosis* (*mt*PriA). *mt*PriA is a branch-point enzyme in amino acid biosynthesis as it catalyzes two chemically equivalent sugar isomerization reactions in tryptophan and histidine biosynthesis.[9] In the latter, the aminoaldose *N*′-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (ProFAR) is converted to the corresponding aminoketose *N*′-[(5′-phosphoribulosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (PRFAR) (Figure 1 a). Since humans can synthesize neither histidine nor tryptophan, *mt*PriA is a potential target for anti-tuberculosis drugs.[10] Structurally, *mt*PriA belongs to the class of $(\beta\alpha)_8$-barrels, which is a frequently encountered and highly versatile fold among enzymes.[11] The protein exhibits a clear twofold symmetry (Figure 1 b),[12] which indicates its evolution from a $(\beta\alpha)_4$-half-barrel precursor.[13] Consequently, two phosphate binding sites are found opposite each other to fix the substrate ProFAR and the product PRFAR (Figure 1 b). We thus reasoned that a C_2-symmetric photoswitch with terminal phosphate anchors would be an excellent foundation for building a light-controllable inhibitor of *mt*PriA.

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[**] B.R. was supported by a PhD fellowship from the Cusanuswerk. Financial support by the Deutsche Forschungsgemeinschaft (GRK 1910) is gratefully acknowledged.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201307207.

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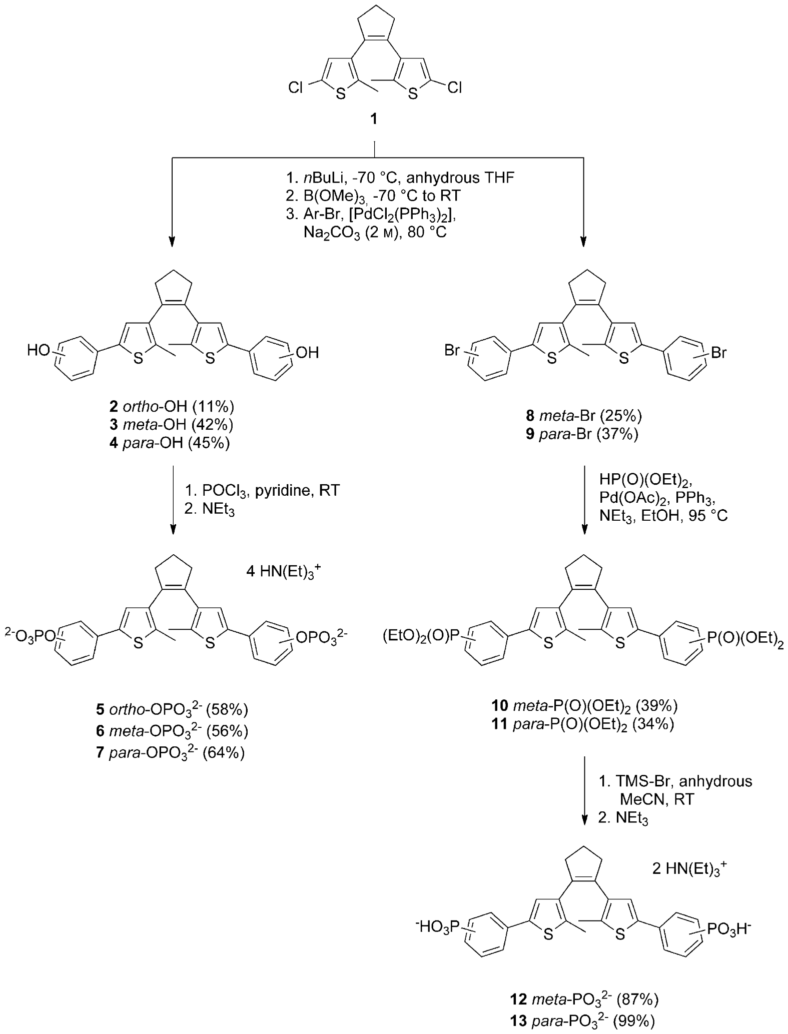
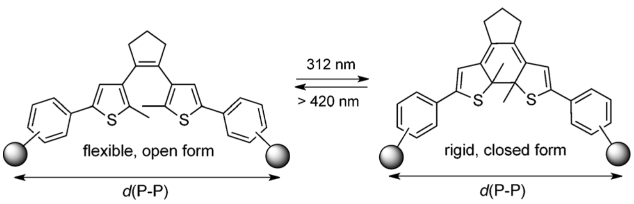
Two types of organic photochromic systems possess the desired twofold rotational symmetry: stilbene[14] and azobenzene switches[15] and the diarylethene scaffold.[16] Although azobenzene derivatives have been widely used in biological systems, they suffer from incomplete photoconversion and thermal reversibility.[1] In contrast, photoresponsive compounds based on 1,2-dithienylethene (DTE) generally feature photoconversions of over 90 % with both photoisomers being thermally stable.[8b,16] Hence, we opted for DTE as a core and provided it with different phosphate and phosphonate anchors (Scheme 1). Starting with the bis-chlorodithienylethene **1**, Suzuki coupling yielded either the aromatic hydroxides **2**–**4** or the aromatic bromides **8** and **9**. The former were subsequently converted to *ortho*-, *meta*- and *para*-phosphates **5**–**7**, while the latter were used to synthesize the phosphonic acid esters **10** and **11**, which were finally hydrolyzed to afford *meta*- and *para*-phosphonates **12** and **13**.



Scheme 1. Synthesis of DTE phosphates and DTE phosphonates.

The DTE molecular structure can be toggled reversibly between a ring-open and a ring-closed photoisomer (Table 1), which significantly alters its overall conformational flexibility.[8b] Energy minimizations of the open and closed forms of all potential inhibitors confirmed distances between the phosphorus atoms of 15.8 to 19.6 Å (Table 1), which is in good accordance with the 16.9 Å observed for the corresponding atoms in the *mt*PriA structure with co-crystallized PRFAR (PDB ID: 3ZS4[12a]; see the Supporting Information for details). Only the open and closed isomers of *ortho*-phosphate **5** exhibit rather short P–P distances of 12.3 Å and 11.3 Å, respectively, in their energetically most favorable geometries; in addition, less populated, more extended conformers can be observed.

Table 1: Photochemical switching and corresponding calculated P–P distances in DTE phosphates and DTE phosphonates **5**–**7**, **12**, and **13**.



Compound	d(P–P) [Å] open	d(P–P) [Å] closed
5	12.3	11.3
6	16.7	16.2
7	19.6	18.4
12	16.9	15.8
13	18.9	18.1

When ring-open forms of compounds **5**–**7**, **12**, and **13** are irradiated with UV light (312 nm), the absorption band at 280 nm immediately decreases. Simultaneously, new absorption maxima at 350 nm and 525 nm formed, turning the initially colorless solutions pink (Figure S1). In each case, the spectral changes are complete after 30 s of irradiation and the corresponding photostationary states consist of between 93 % and 97 % of the closed isomers, as judged by HPLC analyses (Figure S2). The open forms can be recovered by irradiation with visible light (> 420 nm) and all switches are robust over several ring-closing/ring-opening cycles (Figure S3).

The activity of *mt*PriA can be measured spectrophotometrically at 300 nm in a coupled enzyme assay (Scheme S1).[17] As all synthesized compounds were stable under the assay conditions, their inhibitory effect could be investigated in steady-state enzyme kinetics. For this purpose, substrate saturation curves were monitored in the presence of different concentrations of compounds **5**–**7**, **12**, and **13** in their open and closed forms (curves are shown for compound **6** in Figure S4). Indeed, all investigated DTE phosphates and DTE phosphonates are able to inhibit the *mt*PriA reaction in both isomeric forms, thus proving the viability of the design concept. As expected for competitive inhibition, the turnover numbers k_{cat} were identical in the presence and absence of inhibitor (Table S1). The observed increase of the Michaelis constants caused by the inhibitors (Table S1) was used to calculate the inhibition constants K_i (Equation S1), which are given in Table 2.

Table 2: Inhibition constants K_i for compounds **5–7**, **12**, and **13** in their ring-open and ring-closed forms.

Inhibitor	K_i [μM][a] ring-open	ring-closed
5	8.1 ± 2.1	7.0 ± 1.2
6	0.55 ± 0.12	4.4 ± 0.3
7	3.5 ± 0.3	3.7 ± 0.4
12	1.6 ± 0.1	4.9 ± 0.3
13	6.8 ± 0.6	22.7 ± 4.7

[a] The values of K_i were obtained from the data shown in Table S1 using Equation S1.

The determined inhibition constants are in the low micromolar range (Table 2) and therefore comparable or even better than the enzyme's K_M value for the natural substrate ProFAR ($K_M^{ProFAR} = 8.6$ μM; Table S1). In agreement with the appropriate distance between its phosphorus atoms (Table 1), the open isomer of phosphate **6** exhibits the highest binding affinity ($K_i = 0.55$ μM). However, when **6** is switched to its rigid, closed form, the inhibition activity is lowered by roughly one order of magnitude ($K_i = 4.4$ μM). A similar trend is observed for the corresponding phosphonate **12**, whose binding affinity in the open form ($K_i = 1.6$ μM) decreases about threefold upon ring-closure ($K_i = 4.9$ μM). In contrast, the inhibitory effects of phosphates **5** and **7** are nearly identical in their ring-open and ring-closed forms (Table 2). Interestingly, the introduction of a phosphonate moiety in *para* position (compound **13**) causes a threefold difference in the inhibition activity of the open ($K_i = 6.8$ μM) and closed photoisomer ($K_i = 22.7$ μM). The removal of the oxygen bridge between the terminal anchor and the switchable core further reduces the overall flexibility, which seems to predominantly affect the already rigid, closed isomer.

The performance of *mt*PriA can also be controlled by irradiation with light during catalysis. When the reaction was started with compound **6** in its strongly inhibiting, open form, the reaction rate could be enhanced by about threefold upon switching to the less active, ring-closed isomer (Figure 2).

As pointed out before, the mechanistic principle behind the different binding affinities of the photoisomers is based on a change in conformational flexibility.[8b] Due to free rotation around the C−C bonds joining the thiophene heterocycles to the central cyclopentene ring and the terminal phenyl groups, DTE phosphate **6** is able to adopt various geometries in its ring-open form. On the other hand, the closed isomer is completely conjugated and thus far more restricted in its mobility. To gain insight into the binding modes of the inhibitors, we performed molecular dynamics (MD) simulations of the open and closed isomers of compound **6** bound to *mt*PriA. Although both forms are clearly fixed at the phosphate binding sites (Figure 3 a,c), obvious differences can be observed in their structural cores. While the open isomer converges to similar, C_2-symmetric conformers in

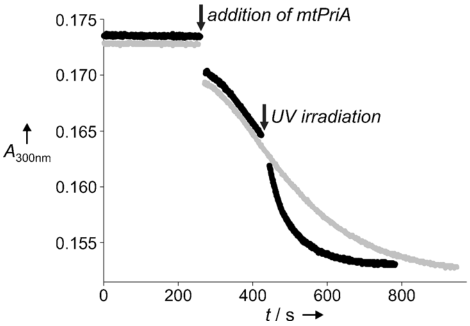


Figure 2. Change in *mt*PriA activity upon ring-closure of compound **6**. The turnover of 5 μM ProFAR was followed photometrically at 300 nm in the presence of 4 μM **6** in its open form under typical assay conditions (25 °C, 50 mM Tris/acetate pH 8.5, 100 mM ammonium acetate, 0.18 μM HisF, and 0.15 μM *mt*PriA). After having reached its maximum rate, the reaction mixture was either left in the spectrophotometer (gray) or removed and irradiated with 312 nm light for 10 s (black). A reference solution without enzymes was used to correct the baseline shift resulting from different absorption values of the open and closed isomer at 300 nm (see Figure S1).

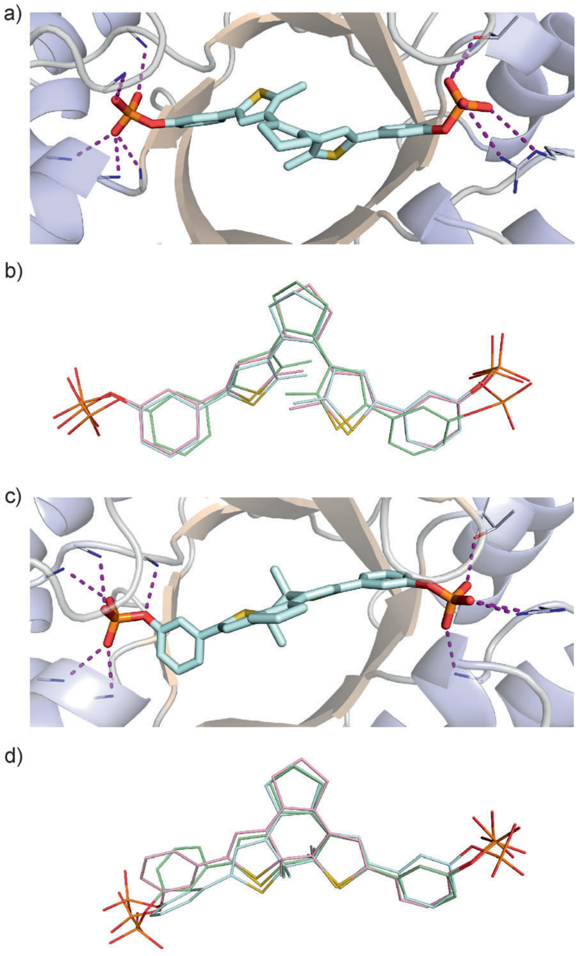


Figure 3. MD simulations of *mt*PriA and bound *meta*-phosphate **6**. For each isomer, three independent calculations were performed and representative enzyme structures for the open (a) and closed (c) form are shown. A superposition of the energetically most favorable conformer of each simulation is depicted in for the open conformation (b) and the closed conformation (d).

three independent calculations (Figure 3b), more diverse binding modes are found for the ring-closed isomer (Figure 3d). Here, one terminal phenyl ring is twisted and adopts various geometries to facilitate proper coordination of the terminal phosphate group. The measured difference in inhibition activity is also reflected in the binding energies determined during the simulations, which consistently show that interaction with the open form is energetically more favorable (Table S2). Taken together, the higher flexibility of the open isomer allows for better adaptation to the enzyme's active site and apparently overcompensates for the loss in entropy upon binding.

In summary, we have demonstrated that natural protein symmetry can be advantageously utilized to design light-controllable enzyme inhibitors. The two-pronged DTE switches can be toggled reversibly between a high- and low-affinity form, where both photoisomers are nearly quantitatively formed and thermally stable. Hence, the enzyme's performance can alternately be enhanced and reduced by irradiation with UV and visible light, respectively. The viability of a dual-anchored DTE inhibitor has been shown before[8b] and the design concept can in principle be transferred to functionally quite different enzyme systems. Phosphate is a frequently encountered element of metabolic substrates, and various other $(\beta\alpha)_8$-barrel enzymes such as pyridoxine 5′-phosphate synthase[18] and aldolases[19] possess two phosphate binding sites. Thus, for these enzymes, inhibitors may be designed by a similar approach, which would allow for the photocontrol of several metabolic processes independently in a spatiotemporal fashion.

Received: August 16, 2013
Published online: November 25, 2013

Keywords: biosynthesis · enzyme catalysis · enzyme inhibitors · molecular switches · photochromism

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