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A photoregulated racemase mimic

Monochura Saha, Munshi Sahid Hossain, Subhajit Bandyopadhyay*

Abstract: The racemase enzymes convert L-amino acids to their D-isomer. The reaction proceeds through a stepwise deprotonation-reprotonation mechanism that is assisted by a pyridoxalphosphate (PLP) coenzyme. This work reports a PLP-photo switch-imidazole triad where the racemization reaction can be controlled by light by tweaking the distance between the basic residue and the reaction centre.

The active form of vitamin B6, pyridoxal-5-phosphate (PLP), is one of the most versatile coenzymes used by nature in a plethora of biosynthetic transformations.[1] The diversity of reactions promoted by the coenzyme includes transamination, [2-3] decarboxylation, racemization of amino acids,[4-5] catabolism of neurotransmitters, and β-elimination of amino acids such as serine. The structural features of the PLP consist of a central pyridinium moiety that acts as an electron sink functionalized with a formyl unit, a phenolic OH, a phosphorylated primary alcohol, and a methyl substitution (Figure 2A). In terms of the reactivity, both the pyridinium unit and the formyl units are crucial, whereas the role of the –OH/O^- and the $-OPO_3^{2-}$ are primarily for the stabilization of the intermediates. The PLP-aldehyde group hooks up as a Schiff's base with an active site lysine residue of the enzymes.[6] The α-amino group of the amino acid substrates displace the lysine and form an aldimine Schiff's base with the amino acid.[7-9]

The aldimine intermediate is crucial and is common for all PLP-mediated reactions.[10-11] Peptidoglycans on the bacterial cell surface contain the unusual D-alanine amino acid residues. Biosynthetically, the D-alanine isomers are formed upon racemization of the naturally abundant L-alanine using the bacterial cellular machinery using alanine racemase, a PLP-dependent enzyme. In terms of the mechanism (Figure 2), the formation of the alanine-PLP Schiff's base causes a lowering of pK_a of the chiral α-proton, which is subsequently abstracted by a basic amino acid residue at the racemase active site.[12] Reprotonation from the opposite face and subsequent hydrolysis generates the D-isomer.[13]

In his Nobel lecture, Feringa highlighted on the fundamental challenges of controlling molecular functions using dynamic molecular systems.[14] Photochromic systems are excellent examples of dynamic molecular systems.[15]

Upon photoisomerization, they generate light-induced molecular motions which can be exploited to accomplish targeted functions.[16-21] Photoregulation of biological functions is an emerging area of immense importance.[22] Use of bistable photochromic systems for such external regulation offers the additional advantage of reversibility with the non-invasive light as stimulus which additionally offers its precise temporal and spatial control of the activity. Either a photochromic unit attached to a protein can be used to regulate its activity[23-26], or the control can be achieved by using photochromic inhibitors.[27] The mechanism of racemization involves the PLP covalently attaching itself to the L-alanine as a Schiff's base (Figure S16) forming the L-Ala-aldimine intermediate (Figure 6B). The C_α–H of the aldimine is then deprotonated by a basic residue present at the active site (Figure 1A) leading to the destruction of the chirality of the entity and thereby forming a highly delocalized prochiral carbanionic quinonoid intermediate.[28] Reprotonation of this achiral quinoidal carbanion from the other face of the C_α yields the racemic amino acid.[32] A "two-base" mechanism involves a basic residue (Tyr)

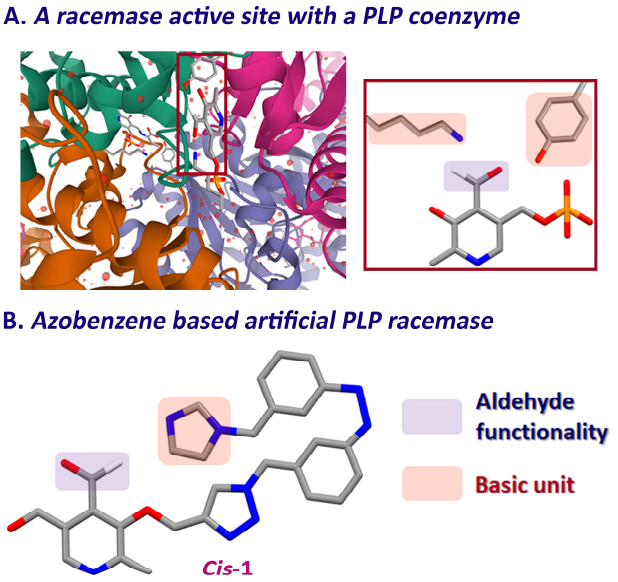

Figure 1. (A) Active site structure of a racemase having a PLP coenzyme (box) (PDB ID 4ysn) [43] *inset:* proximity of two bases Lys and Tyr of the enzyme to the PLP. (B) Our artificial photoregulated PLP racemase.

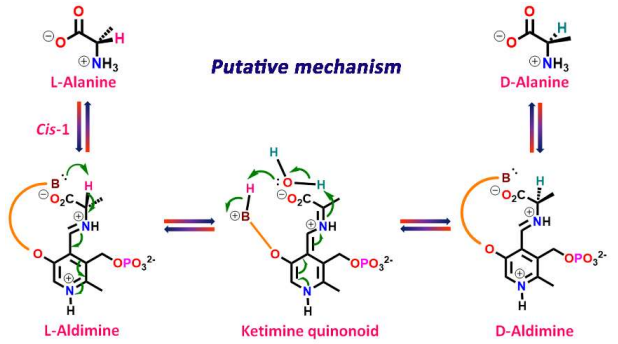

Figure 2. A putative mechanism of L-alanine racemization.

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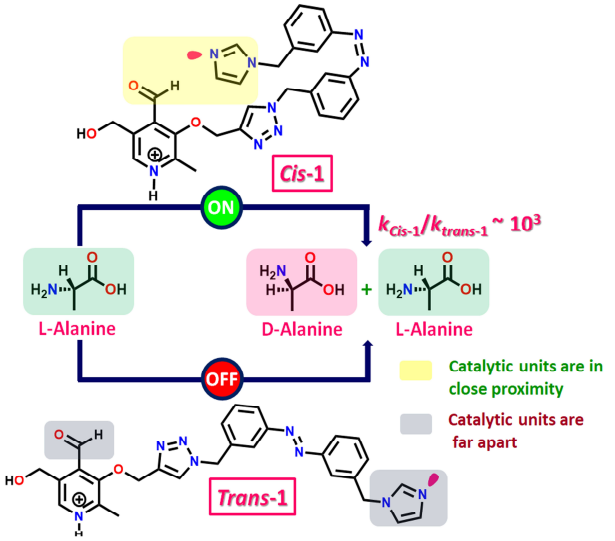

Figure 3. Photoregulation of L-Ala to D-Ala transformation with the PLP-azobenzene-imidazole triad **1**.

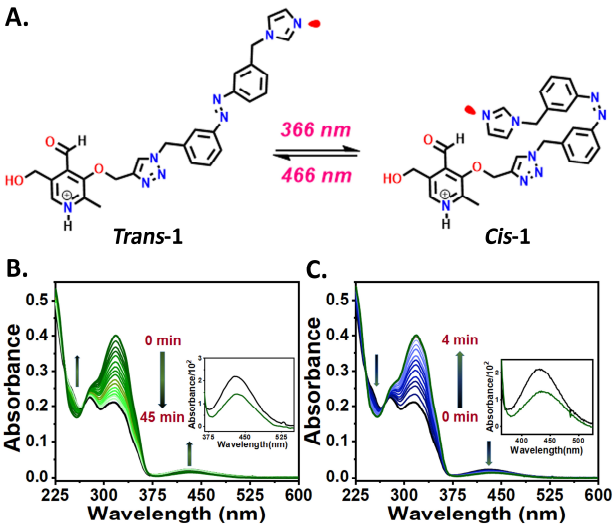

Figure 4. Photoisomerization studies of triad **1** under 366 nm UV light and 466 nm blue light respectively. (A) Schematic re-presentation of photoisomerization; (B) *Trans*-**1** to *cis*-**1** conversion; (C) *cis*-**1** to *trans*-**1** conversion.

that deprotonates the chiral proton of the Schiff's base, followed by the conjugate acid of a second basic residue (Lys) that delivers the proton to resulting prochiral carbanionic intermediate from the opposite face.[31]

The PLP is stabilized by a network of hydrophobic, hydrogen bonding, and other non-covalent interactions. The reaction mechanism was thoroughly established by evidence from X-ray crystal structure[29-30] and detailed spectroscopic studies.

An innovative way to control a bio-inspired activity with light was demonstrated by Branda with the design and demonstration of a *de novo* photochromic pyridoxal phosphate (PLP) mimic.[34-35] Light regulated enzyme mimics of various enzymes such as glycosidase and carbonic anhydrase mimic has been reported recently.[33,18]

In this work, instead of altering the PLP scaffold, a photochromic PLP-azobenzene-imidazole triad was designed to regulate the position of the general basic residue responsible for the racemization using light as the stimuli (Figure 3). In the *cis*-**1** isomer, the imidazole of the histidinyl unit is in close proximity of the racemizable C_α–H of the L-Ala aldimine Schiff's base (Figure S16 and Figure 6B) whereas, in the *trans* form, the basic unit is far and cannot reach the C_α-proton. The *cis* form can, therefore, effectively deprotonate the amino acid leading to its racemization, whereas the *trans*-form is less effective in terms of the deprotonation and, consequently for the racemization as well. Thus, this photochromic PLP-azobenzene-imidazole triad offers a reversible photomodulated control on the activity of the racemase enzyme mimic.

The PLP based azobenzene triad **1** was synthesized by a Cu(I)–catalyzed azide-alkyne "click" cycloaddition reaction between the PLP derivative **2** and the imidazole-azide based azobenzene precursor **3** (Scheme S1). The product was purified by preparative TLC and was characterized thoroughly by various spectroscopic techniques and by the mass spectrometry (Figure S3, S4 and Figure S11). The azobenzene unit was prepared via the reduction of the corresponding *m*-nitrobenzyl azide and *m*-nitrobenzyl imidazole precursors with glucose/NaOH as the reducing agent (scheme S4).[36] The PLP unit was synthesized from the commercially available pyridoxal hydrochloride upon treatment with propargyl bromide under the basic conditions at room temperature. An equilibrium was observed between the hemi-acetal and the aldehyde forms of the coenzyme. The precursor **2** was obtained as crystals in the hemiacetal form (Figure S24, CCDC 1978370). The details of the multistep synthesis of the intermediates compound **2** and **3**, as well as the other precursors have been provided in the supporting information.

The *trans* triad **1** displayed absorption bands with λ_{max} at 230, 250, 319, and 431 nm. The band at 319 nm (ε = 13,300 $M^{-1}cm^{-1}$) corresponds to a π–π* transition, and the band at 431 nm is a symmetry forbidden n–π* band (ε = 470 $M^{-1}cm^{-1}$). Irradiation with 366 nm light under cold conditions (273 K) and monitoring by UV-Vis spectroscopy revealed that *trans*-**1** (30 μM, in CH_3CN) gradually isomerized to the *cis*-**1** upon exposure to UV light (λ=366 nm, irradiation power of 2.3 mW cm^{-2}) within 45 min (Figure 4B). Upon photoisomerization of the *trans*-**1**, the absorption maxima at 319 nm gradually decreased, and a new peak appeared at 431 nm (ε = 730 $M^{-1}.cm^{-1}$) corresponding to the *cis*-**1** isomer. The reversal of the *cis*-**1** to the *trans*-**1** form was achieved upon the exposure of the sample to blue light (λ = 466 nm, irradiation power = 0.5 mW cm^{-2}) (Figure 4C). Under continuous irradiation, the *cis* form was quantitatively converted to the *trans* photoisomer within 250 seconds with an 85% composition of the *cis*-rich photostationary state (PSS), as encountered with the azobenzene photochromic system.[37-38] Reversibility studies also performed for ten cycle and observed no fatigue resistance (Figure S14). The photoswitching behavior of the triad **1** in methanol-d_4 was also studied by the ^{1}H NMR spectroscopy (Figure S13).

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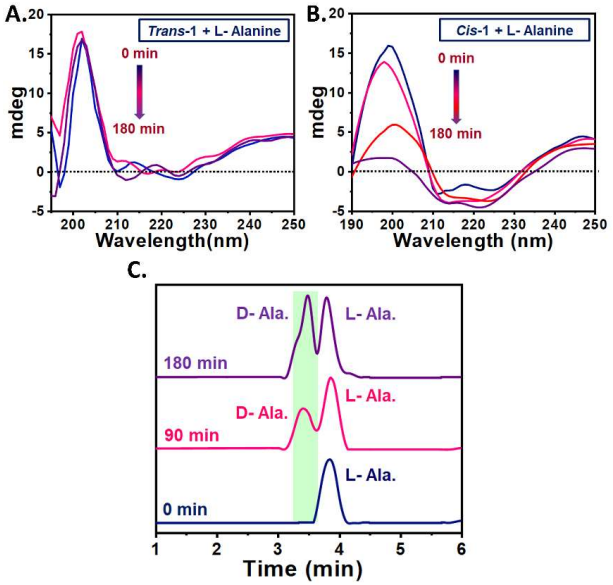

Figure 5. CD spectra for the racemization of L-Alanine with (A) *trans*-**1**, and (B) with *cis*-**1**. (C) HPLC traces of Alanine under the racemization conditions of PLP-conjugated *cis*-**1,** the retention time of L-Alanine and D-Alanine are 3.85 min and 3.45 min respectively in the chiral-HPLC (1:1 isocratic mixture of CH_3CN/water with 1% TFA).

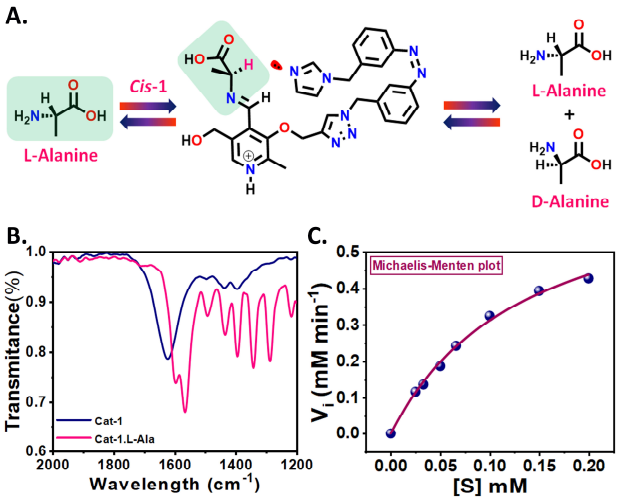

Figure 6. (A) Scheme for the racemization; (B) FTIR spectra of intermediate (triad-**1**, compound 7) Schiff's base with L-alanine; (C) Michaelis-Menten plot of recemization with catalyst-**1**.

In the presence of 366 nm ultraviolet light, the signals at δ 7.83, 7.34 and 7.20 corresponding to the aromatic protons of the azobenzene unit gradually diminished and growth of three new peaks in the upfield region at δ 7.08, 7.02, 6.60 for the same sets of protons were observed.[39-41]

The free PLP and its Schiff's base are in equilibrium with the multiple tautomeric forms, which are not easily distinguishable in the absorption spectra.[42] Nevertheless, various attempts were made to capture the formation of the Schiff base of the triad **1** with L-alanine. Finally, the initial intermediate (compound **7**) formed *in-situ* was detected by FTIR spectroscopy and mass spectrometry (Figure 6B, Figure S16-S17).

The activity of our PLP based enzyme mimic was monitored with the catalyst in both the forms (*trans*-**1** and *cis*-**1**) using circular dichroism (CD) and also by HPLC analysis in the dark as well as under 366 nm constant irradiation (Table S**1**, Figure S19B). Thus, the solution of L-alanine (0.15 mM) in the presence of *trans*-**1** (30 μL; buffered with PBS at pH 7.0) was incubated at 298 K and the aliquots were monitored by CD spectroscopy at various time intervals and no noticeable spectral changes at 201 nm attributed to the L-alanine was observed even after 3 h (Figure 5A). Interestingly, in case of *cis*-**1**, spectral changes were observed owing to the racemization. As a control, similar experiments were carried out with imidazole and without any catalyst in the buffer solution. The outcome of the experiments was found to be close to the *trans*-**1** (Figure S18C and S18D). To substantiate the racemization, the aqueous solution of the reaction with *cis*-**1** was subjected to HPLC analysis with a reverse phase chiral column. Under the HPLC conditions (Figure S20), the peak for the L-alanine standard solution had a retention time of 3.85 min whereas a standard D-alanine had a retention time of 3.45 min (Figure 5C) the PLP-containing triad **1** (both *trans/cis*). However, when the same experiment was carried out using *cis*-**1**, the peak at 201 nm having a positive Cotton effect, commonly observed for the L-alanine in the CD spectra, was found to diminish gradually with time (Figure 5B).

The sequence of the CD spectra after 2h displayed a spectrum close to the baseline with a decline of the characteristic L-alanine peak indicating the racemization (Figure S18). To validate whether racemization has indeed taken place, the aqueous solution of the reaction with *cis*-**1** was subjected to HPLC analysis with a reverse phase chiral column. Under the HPLC conditions (Figure S20), the peak for the L-alanine standard solution had a retention time of 3.85 min whereas a standard D-alanine had a retention time of 3.45 min (Figure 5C).

The aliquot taken out of the reaction after 1.5 h of the reaction in the presence of the *cis*-**1** PLP-conjugate displayed two peaks – one corresponding to the L-alanine and the other corresponding to the D-alanine with an enantiomeric ratio of 70:30 (±2) (L>D). The aliquot collected after 3 h of the reaction displayed an enantiomeric ratio of 52/48 (±2) (L:D) of the two isomers indicating the racemization. The rate of racemization with the *cis*-**1** PLP-conjugate was 1.5 x 10^3 times faster compared to the *trans*-**1** (Table S1, Figure S19). To quantify the intramolecular catalytic efficiency of the imidazole unit present in the active catalyst, compared to the intermolecular reaction with imidazole, the effective molarity (k_{intra}/k_{inter}) was estimated and found to be 1.91

Table 1. Kinetic parameters of racemization with the catalyst (*cis*-**1**) in PBS buffer, pH 7.0

k_{cat} (min^{-1})	k_{uncat} (min^{-1})	k_{cat}/k_{uncat}	K_m (mM)	k_{cat}/K_m ($min^{-1}mM^{-1}$)
0.3915	0.00002	1.8×10^4	0.13	3.25

x 10^3 M. The kinetic parameters of the racemization process were also determined using the *cis*-catalyst with different substrate concentrations. The kinetics data exhibited a Michaelis-Menten behaviour (Figure 6C). Albeit the natural enzymes are far superior compared to this mimic (Table S2), the catalyst *cis*-**1** displays an enzyme-like activity as is evident from the data in Table **1**.

Although establishment of the details of the mechanism including the rate-determining step require further studies, a key step for the racemase activity is the base-induced deprotonation from the amino acid-PLP aldimine. For our active-site mimic, in the *cis* isomer the base reaches an optimum orientation and the distance for an intramolecular deprotonation from the aldimine, whereas, in the *trans*-isomer, the base and the aldimine units are far apart. This makes the only *cis*-isomer an efficient active-form and the activity could thus be turned on and off by light (Figure 6A). Various thermodynamic parameters, including ΔE_{act}, $\Delta H^{\#}$, $\Delta S^{\#}$ and $t_{1/2}$ were calculated from the variable temperature kinetics data. (Figure S22A and S22B, Table S3).

In summary, this work reports an active site mimic[44] of the racemase enzyme. This activity can be turned on and off reversibly with light. The racemase mechanism involves a deprotonation of the C_α–H of the amino acid-PLP Schiff's base to generate the ketimine intermediate that is subsequently reprotonated. We have designed a light controlled dynamic catalyst using the *cis-trans* photoisomerization of an azobenzene photoswitch. The active site mimic consists of a base-photoswitch-PLP triad. When the azobenzene was photoisomerized, the relative position between the imidazole base and the reaction centre changed. In the *trans* form, the imidazole unit was too distant to reach the C_α–H proton of the PLP-bound alanine substrate. Whereas, in the photogenerated *cis* isomer, the imidazole reached was placed within the proximity of the C_α–H proton such that the deprotonation could easily take place. The reprotonation of the resulting highly stabilized, planar carbanion takes place from either prochiral faces generating a racemic mixture. Thus the catalyst displays a photoregulated racemase activity.

Acknowledgements

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Keywords: biomimetic, enzyme catalysis, photochemistry, azobenzene, photochromism.

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Layout 1:

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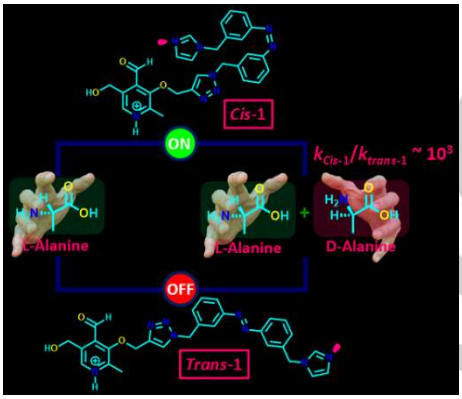

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