



The Effect of Visible Light on the Catalytic Activity of PLP-Dependent Enzymes

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Pyridoxal 5'-phosphate (PLP)-dependent enzymes are a versatile class of biocatalysts and feature a variety of industrial applications. However, PLP is light sensitive and can cause inactivation of enzymes in certain light conditions. As most of the PLP-dependent enzymes are usually not handled in dark conditions, we evaluated the effect of visible light on the activity of PLP-dependent enzymes during production as well as transformation. We tested four amine transaminases, from *Chromobacterium violaceum*, *Bacillus megaterium*, *Vibrio fluvialis*

and a variant from *Arthrobacter species* as well as two lysine decarboxylases, from *Selenomonas ruminantium* and the LDCc from *Escherichia coli*. It appeared that five of these six enzymes suffered from a significant decrease in activity by up to 90% when handled in laboratory light conditions. Surprisingly, only the amine transaminase variant from *Arthrobacter species* appeared to be unaffected by light exposure and even showed an activation to 150% relative activity over the course of 6 h regardless of the light conditions.

Introduction

Pyridoxal 5'-phosphate (PLP) is the active form of vitamin B₆ which is involved in numerous key metabolic pathways in living cells.^[1] Since mid of the 20th century, it is known, that PLP serves as a cofactor for enzymatic reactions,^[2] including decarboxylation, de- and transamination, racemization, elimination and replacement.^[3] The unique structure, consisting of a hetero-aromatic pyridine ring and three functional residues, enables PLP to be a versatile coenzyme.^[4] In PLP-dependent enzymes, PLP is usually covalently bonded to a conserved lysine residue in the enzymatic active site by its aldehyde group. This Schiff-base structure is commonly referred to as internal aldimine. In the first step of a catalytic reaction, the internal aldimine breaks up, when PLP forms a new bond with the amino group of the respective substrate. This newly formed structure was declared as external aldimine.^[5] Upon breakage of a bond to the amino group α -carbon, a stabilized carbanionic intermediate is formed, which is supposed to be existent in all PLP-

dependent enzymes.^[6] The subsequent reaction depends on the type of enzyme involved, but mechanistic similarities provide a link between different PLP-dependent enzymes. Sorting these enzymes according to fold types, sequence similarities, regio- and stereospecificities allows a detailed classification.^[7–10] The most prominent structure is fold-type I, which is present mostly in amine transaminases (ATAs) and decarboxylases.^[8] As they catalyze a variety of reactions, PLP-dependent enzymes are extraordinary biocatalysts, which puts them in focus for industrial applications^[11] and makes them subject of a number of structure-function relationship studies.^[4,12–15] An important point, when employing PLP-dependent enzymes, is, that PLP is light sensitive and has chromophoric properties.^[16] Thus, it easily degrades upon illumination. The light-dependent degradation process of free PLP (1) had been analyzed in detail and appeared to be oxygen dependent with the main photoproducts being 4-pyridoxic acid 5'-phosphate (2) and a benzoin like PLP dimer (3) as depicted in Figure 1.^[17,18] However, it was stated, that the dimer (3) was formed later during the isolation process by oxidation of the originally formed pyridoin of PLP.^[18] It already had been suggested, that PLP should be protected from illumination, especially when in solution.^[18] PLP even has the ability to act as a specific photosensitizer. When exposed to light, it has been reported, that PLP can irreversibly inactivate enzymes, when bound as internal aldimine, as in this case histidine residues in

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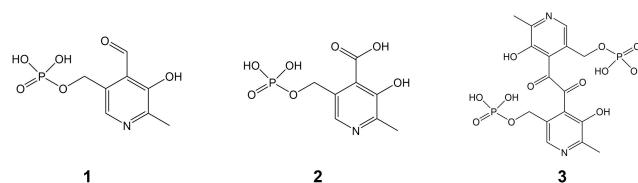
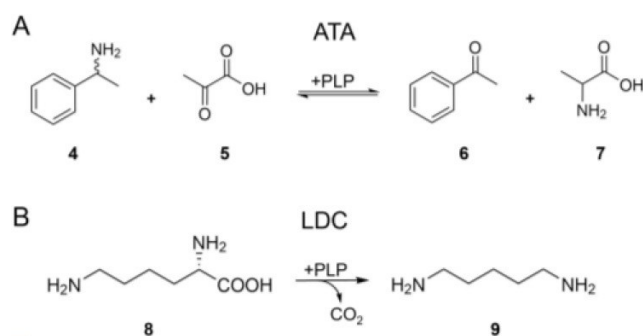


Figure 1. Structures of PLP (1) and its main photoproducts, 4-pyridoxic acid 5'-phosphate (2) and the PLP dimer (3).

the enzymatic active site were destroyed.^[16] In the following, PLP had been applied for the intended photoinactivation of aldolases from rabbit muscles or spinach leaves.^[19,20] During the attempt to increase the thermal and storage stability of the amine transaminase from *Chromobacterium violaceum* (CvATA), Chen and co-workers showed, that after three days of incubation in light, the enzyme was completely inactive, while the enzyme incubated in the dark retained 64% residual activity.^[21] When high concentrations of PLP (5 mM) were added, the residual activity after three days could be improved in both setups. When analyzing the stability of the amine transaminase from *Vibrio fluvialis* (VfATA), the addition of 5 mM PLP led to an increased stability in the dark, while in the light, the enzyme was completely inactive after five days of incubation.^[22] These findings indicate that a light-dependent inactivation of PLP-dependent enzymes is an important issue. However, the inactivation process may vary in different PLP-dependent enzymes and is connected to the unique PLP binding abilities of the respective enzymes. As the loss of the cofactor is a critical limitation to PLP-dependent enzymes,^[23] the characterization and optimization of the PLP binding affinity in general has been subject of a number of studies.^[24–27] In another set of studies, it has been shown, that upon photoexcitation, PLP rapidly forms a reactive triplet state.^[28] Interestingly, when bound to the active site lysine of an aspartate amine transaminase, blue light pulses actually supported the formation of the carbanionic intermediate within microseconds, resulting in an increased catalytic activity.^[29–31] A similar photoactivation has already been described for the enzymatic decarboxylation of 5-hydroxytryptophan.^[32] Although some studies exist, to the best of our knowledge, the effect of light on the stability of PLP-dependent enzymes has not been in scientific focus in the first place. As PLP-dependent enzymes during production and use are presumably most of the time not handled in dark conditions, the question arises, if the influence of light does generally reduce the catalytic activity of PLP-dependent enzymes due to the photoactive properties of PLP. Here, we tested the effect of visible light on the catalytic activity of a number of PLP-dependent enzymes, such as four ATAs and two lysine decarboxylases (LDCs). Both enzyme classes are interesting targets for different industrial fields and frequently applied. ATAs catalyze the transfer of an amino group from an amino donor to an acceptor, which is shown in Scheme 1A for the conversion of (*rac*)- α -methylbenzylamine ((*rac*)- α -MBA; **4**) and pyruvate (**5**) to acetophenone (**6**) and alanine (**7**). In the last years, ATAs have gained attention for the production of high value chemicals, especially chiral amines, which are versatile building blocks for pharmaceuticals.^[33–35] LDCs produce cadaverine (1,5-diamino-pentane; **9**) from the decarboxylation of L-lysine (**8**; Scheme 1B). Cadaverine is a promising compound with many bioactivities and of special interest for the polymer industry, as it represents an innovative platform chemical for the production of bio-based polyamides.^[36–38] In our experiments, we employed the CvATA^[39] and the VfATA^[40] as well as the ATA from *Bacillus megaterium* (BmATA)^[41] and a variant of the ATA from *Arthrobacter species* (AsATAmut11). The AsATAmut11 is a special enzyme, which was engineered to produce



Scheme 1. (A) ATA-catalyzed reaction from (*rac*)- α -MBA (**4**) and pyruvate (**5**) to acetophenone (**6**) and alanine (**7**) involving the cofactor PLP. (B) PLP-dependent decarboxylation of L-lysine (**8**) to cadaverine (**9**) and CO₂ catalyzed by LDCs.

the antidiabetic compound sitagliptin in a manufacturing setting.^[42] Further we tested the LDC from *Selenomonas ruminantium* (SrLDC)^[43] as well as the LDCc from *Escherichia coli* (EcLDC).^[44] After determining strong inactivation of the CvATA with intense blue light illumination, we analyzed the effect of standard laboratory light conditions (combined electric and sun light) on all of the mentioned PLP-dependent enzymes, involving the enzyme production and reactions in varying light and buffer setups. This article is intended to sensitize researchers working with PLP-dependent enzymes to consider the light factor when optimizing reaction conditions and even when producing the enzymes.

Results and Discussion

Blue light inactivation of the CvATA

As PLP absorbs specifically in the blue light region of the visible light spectrum, around 440 nm, it has been reported, that PLP can be stimulated by blue light illumination.^[28,29] To determine changes in the spectrum of free PLP during illumination in different setups, 0.1 mM PLP solutions in HEPES buffer (pH 7.5) were subjected to illumination by a single blue LED (~60 mW/cm²) or by ordinary laboratory and sunlight (~0.25 mW/cm²; Figure 2A). In the following, light conditions consisting of laboratory and sunlight are referred to as “normal light conditions”. The spectrum of the PLP solution kept in the dark did not change over the course of 60 min and showed an absorption maximum at 388 nm. This indicated, that PLP is stable in dark conditions. In both illuminated setups, the spectrum of PLP changed with a new maximum being formed at 288 nm. This photoproduct was isolated by Morrison and Long and was identified as PLP dimer, which reportedly is produced in a benzoin-like self-condensation.^[18] However, the PLP spectrum after blue light illumination showed another peak at 320 nm, which supposedly depicts the photoproduct 4-pyridoxic acid 5'-phosphate, described by Reiber.^[17] It seems like the degradation of PLP and the photoproducts formed over the time-course vary depending on the illumination setup.

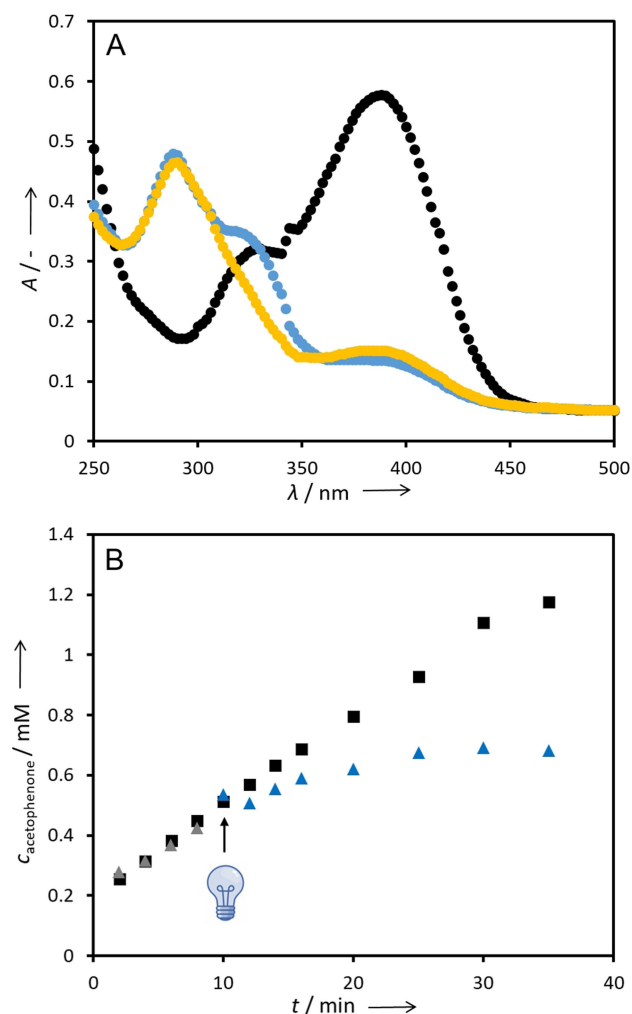


Figure 2. (A) Spectrophotometric analysis of 0.1 mM PLP solutions in 100 mM HEPES buffer (pH 7.5) after 60 min of incubation in the dark (●), in normal light conditions with a light intensity of $\sim 0.25 \text{ mW/cm}^2$ (●) and with blue light illumination of $\sim 60 \text{ mW/cm}^2$ using a single LED (●). Samples were incubated at 22°C and 600 rpm. (B) Acetophenone (6) formation by 0.03 mg/mL purified CvATA over the course of 35 min in the dark (■) and with blue light illumination of $\sim 12 \text{ mW/cm}^2$ using LED strips (60 LEDs; ▲). Before blue light illumination, the enzymatic reaction was initiated in the dark for 10 min (▲), including 30 mM (*rac*)- α -MBA (4), 60 mM pyruvate (5) and 0.1 mM PLP in 100 mM HEPES buffer (pH 7.5) at 22°C and 600 rpm.

Thus, we observed a main peak appearing at 308 nm in the PLP solution after 5 min illumination with normal light, while after 5 min of blue light illumination most of the PLP dimer was already formed (Figure SI.2). Seemingly, blue light has a particularly strong degrading effect on PLP.

As reported by Chen, et al.^[21] for an illumination test employing the CvATA, the enzyme lost 100% of its activity after incubation in light for three days. Here, we tested the CvATA in an illumination experiment using specific high-intensity blue light exposure (Figure 2B). The enzymatic reaction was initiated in the dark for 10 min before the reaction mixture was subjected to blue light illumination. When the illumination was started, the activity of the CvATA started to decrease significantly within minutes. After 30 min, corresponding to 20 min of

blue light exposure, 100% of the enzyme activity was lost. As none of the other reaction components besides PLP showed a response to blue light exposure (Figure SI.3), it is proposed, that the inactivation effect is mediated by degraded PLP, which acts as a photosensitizer being primarily triggered by blue light. Thus, it could be demonstrated, that it is possible under certain circumstances to inactivate PLP-dependent enzymes completely by illumination with blue light in only 20 min.

Comparison of the activity of PLP-dependent enzymes in three light setups

From Figure 2B could be concluded, that blue light had a strong deactivating effect on PLP-dependent enzymes, but we observed as well in Figure 2A, that after 60 min of illumination, the degradation of free PLP in solution was similar in blue and in normal light. Thus, it seems likely, that illumination with normal light (sun and electric light) might have a strong effect on the activity of PLP-dependent enzymes as well. On one hand, it was not commonly described, that PLP-dependent enzymes and PLP were handled under dark conditions. On the other hand, PLP-dependent enzymes are not generally inactive, as plenty of articles describe successful transformations with PLP-dependent enzymes without protecting the enzyme from light. This means, if PLP-dependent enzymes are negatively affected by normal light ($\sim 0.25 \text{ mW/cm}^2$) during their production and in enzymatic reactions, the effect is not as drastic as compared to specific blue light illumination ($\sim 12 \text{ mW/cm}^2$). To evaluate the effect of normal light on the activity of PLP-dependent enzymes, each enzyme was tested in three light conditions (Figure 3). i) In dark conditions (Figure 3A), the protein production, the purification of the target proteins and

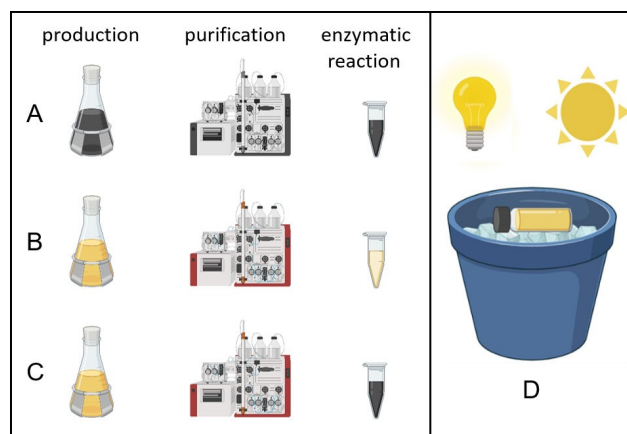


Figure 3. Graphic representation of three tested light setups. (A) Darkest possible conditions during protein production, purification and enzymatic reaction (B) Normal light conditions (sun and electric light, $\sim 0.25 \text{ mW/cm}^2$) during protein production, purification and enzymatic reaction. (C) Mixed conditions, where protein production and purification were done with illumination ($\sim 0.25 \text{ mW/cm}^2$) but the enzymatic reaction was done in dark conditions. (D) Incubation of enzyme (1 h) or PLP solutions (3 h) on ice with normal illumination (sun and electric light, $\sim 0.25 \text{ mW/cm}^2$) prior to the respective enzymatic reaction. Created with biorender.com.

the final enzymatic reactions were conducted in darkest possible conditions. To prevent degradation, PLP solutions were handled solely in the dark as well. Before the respective enzymatic reactions were started, PLP solutions were incubated for 3 h and enzyme solutions for 1 h on ice in dark conditions using ten times concentrated stock solutions to keep it consistent with the following setup. ii) In normal light conditions (Figure 3B), production, purification and enzymatic reaction were performed under the influence of sun and electric light, representing general laboratory (normal) light conditions. Prior to the light exposed enzymatic reactions, PLP solutions were incubated for 3 h and enzyme solutions for 1 h on ice in normal light conditions using ten times concentrated stock solutions, to mimic standard waiting times in laboratory procedures, which might facilitate degradation of PLP and inactivation of PLP-dependent enzymes (Figure 3D). iii) The last tested setup comprised mixed conditions (Figure 3C), where expression and purification were performed in normal light conditions equal to setup B, but the final enzymatic reactions were done in dark conditions equal to setup A, using dark PLP solutions as well. Thus, it ought to be evaluated, if PLP-dependent enzymes are able to regenerate some of their activity, which they might have lost during light exposure.

The activity of six PLP-dependent enzymes in normal light and mixed conditions is depicted relative to their activity in the dark (Figure 4). Due to variations in their response to the different light setups, the enzymes were grouped in three categories. The CvATA and the BmATA (Figure 4A) both showed a significant but moderate response to light exposure. Compared to their activity in dark conditions, the CvATA exhibited 40% reduced activity in light conditions and the

BmATA was 30% less active. In mixed conditions, when undegraded PLP was added for the initial rate reaction, the CvATA and the BmATA were able to restore 30% to 50% of the activity lost during previous light exposure.

We could not detect any aggregates or precipitates in the reaction mixture after completed light exposure, which would indicate an unfolding of the illuminated enzymes. The PLP-dependent enzymes in Figure 4B were not influenced by illumination in a negative way. In this particular test, the activity of the VfATA appeared to be within the range of the error for all three illumination setups. Interestingly, the AsATAmut11 showed significant activation in normal light and in mixed conditions. The activity seemed to be about 50% higher, relative to the dark conditions. The last category (Figure 4C) comprises the LDCs, which show a very intense response to illumination. The EcLDC appeared to be 85% less active in the applied light setup compared to the handling in dark conditions and the SrLDC lost 45% of activity. In mixed conditions, the SrLDC could restore 50% of the lost activity but for the EcLDC the increase was only about 10%. As the EcLDC is a relevant enzyme for industry applications,^[37,45,46] the limitation of light exposure to an absolute minimum seems to be critical and might allow for ten times higher activity levels. Of the six PLP-dependent enzymes, that were tested in different light conditions, four showed a significant decrease in activity due to light exposure. These four enzymes were all able to regenerate some of the activity, when undegraded PLP was added for the enzymatic reaction. Thus, the inactivation seen in normal light conditions, cannot only be caused by the photosensitizing effect, that was described by Rippa and Pontremoli,^[16] as this effect was described to be irreversible. Instead, it appears that

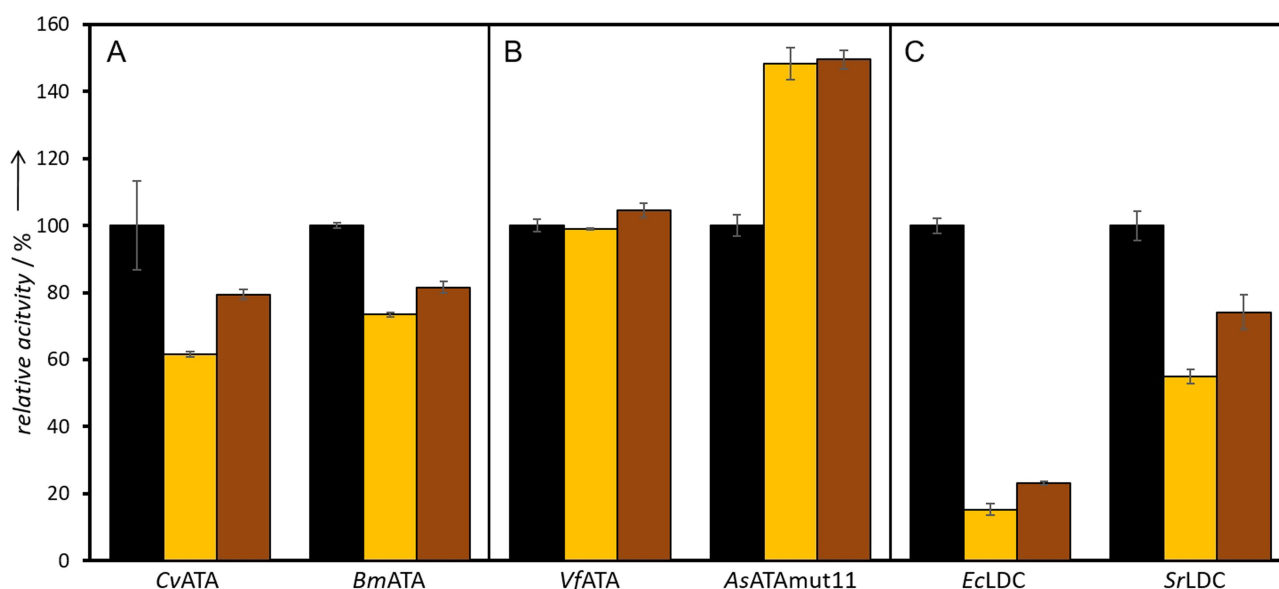


Figure 4. Relative activities of the CvATA (0.5 mg/mL), BmATA (0.1 mg/mL), VfATA (0.05 mg/mL) and AsATAmut11 (1 mg/mL) for the formation of acetophenone (6) and of the EcLDC (0.05 mg/mL) and SrLDC (0.025 mg/mL) for the formation of cadaverine (9) using dark conditions (■), normal light conditions with a light intensity of $\sim 0.25 \text{ mW/cm}^2$ (■) and mixed conditions (■), grouped in three categories (A–C) depending on the level of inactivation. ATA reactions contained 30 mM (*rac*)- α -MBA (4), 60 mM pyruvate (5) and 0.1 mM PLP in 100 mM HEPES buffer (pH 7.5) at 20 °C and 600 rpm. LDC reactions were performed using 10 mM L-lysine (8), 0.1 mM 4-nitrophenol and 0.1 mM PLP in 25 mM HEPES buffer (pH 6.0) at 20 °C. The error bars represent the standard deviation of technical triplicates.

one of the factors involved in restoring activity is the ability of the enzyme to exchange PLP in the active site. When the enzyme has a lower binding affinity towards PLP, PLP in a certain stage of degradation could be easier replaced with fresh PLP than in enzymes with a strong binding affinity for PLP. It has been reported, that the CvATA has a relatively low binding affinity for PLP,^[21] which could explain, that the recovery of activity in the mixed illumination condition with fresh PLP is relatively high (Figure 4A). A similar high recovery rate was seen here for the *SrLDC* (Figure 4C), which is reported to have a flexible binding site and a low PLP affinity as well.^[47] However, proving this theory requires further analysis, while the evaluation of the cofactor binding in PLP-dependent enzymes is subject to other articles.^[12,22,23,26]

The combined effect of different buffer systems and illumination on the activity of PLP-dependent enzymes

The changing spectrum of a PLP solution in HEPES buffer after 1 h normal light exposure has been shown previously (Figure 2A). As a range of other buffers are commonly applied in biocatalysis, we tested, if the already observed changes in the absorption of PLP relate to other buffers after 3 h of normal light exposure as well (Figure 5). Besides HEPES buffer (Figure 5B), we also tested 0.1 mM PLP solutions in water (Figure 5A), 100 mM (pH 7.5) potassium phosphate buffer (Figure 5C), TEA buffer (Figure 5D) and TRIS buffer (Figure 5E). The PLP solution in water (Figure 5A) showed a lower absorption in general, but the overall pattern is comparable to HEPES buffer, where the main peak of free PLP at 388 nm in the dark shifted to 288 nm, indicating the formation of the PLP dimer. Interestingly, a small peak at 288 nm could already be seen in the dark sample, suggesting, that some PLP molecules have degraded, likely caused by the absence of a stabilizing buffer. Though, it has been reported, that pH changes might have an influence on the detection of the photoproducts of PLP.^[17] Changes in the spectrum of a PLP solution in phosphate buffer (Figure 5C) are an exact representation of the changes seen in

HEPES buffer, assuming the degradation mechanisms are equal. However, the degradation in TEA buffer (Figure 5D) seemed to behave differently, as the main photoproduct after 3 h of normal light exposure appeared to be the 4-pyridoxic acid 5'-phosphate (320 nm). Based on this spectrum, the PLP dimer (288 nm) was marginally formed. The spectra of a PLP solution in TRIS buffer (Figure 5E) exhibited a completely different pattern. After dark incubation, the main peak of PLP could not be observed at 388 nm but at 410 nm. Further, there was another prominent peak at 275 nm. After 3 h of light exposure, the spectrum of free PLP in TRIS buffer did barely change, revealing only a minor additional peak at 340 nm. Even when the normal light exposure was extended to 24 h, the spectrum did not exhibit any other changes. This could either imply, that TRIS buffer is able to prevent the degradation of PLP in solution, or that the combination of the TRIS buffer salt and PLP create an overlying absorbance, which covers the detection of certain photoproducts. Since TRIS buffer was used for protein purification in previous experiments (Figure 4), it would be very beneficial to maintain the integrity of PLP during the purification process.

The most interesting buffers to test for enzymatic reactions with different light setups compared to HEPES buffer appeared to be potassium phosphate buffer and TRIS buffer, as these are all compatible with our enzymes. From the spectra (Figure 5) it was expected that the enzyme inactivation in phosphate buffer might be similar to HEPES buffer, while the inactivation in TRIS buffer might be prevented. Therefore, the specific activities in dark and normal light conditions were tested in 100 mM (pH 7.5) HEPES, TRIS and phosphate buffer for the CvATA and the VfATA (Figure 6). Protein purification was performed using the respective buffers as well, except for the protein used for the transformations in HEPES buffer.

As protein purification in HEPES buffer was not possible in this case due to protein degradation, the enzymes used for the transformation in HEPES buffer were purified in TRIS buffer as well. Regarding the CvATA, the inactivation was similar in the three tested buffers. As already observed in Figure 4A, after light exposure in HEPES buffer, the activity was reduced by

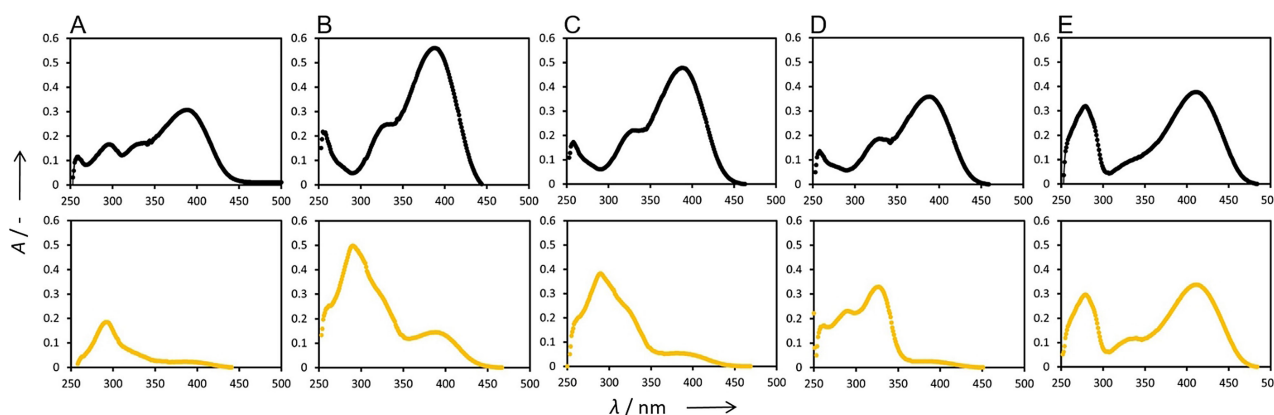


Figure 5. Comparison of spectra from 0.1 mM PLP solution in water (A), or different buffers (100 mM, pH 7.5 each), such as HEPES (B), potassium phosphate (C), TEA (D) or TRIS (E) after 3 h incubation on ice in the dark (●) or with normal light exposure of $\sim 0.25 \text{ mW/cm}^2$ intensity (○).

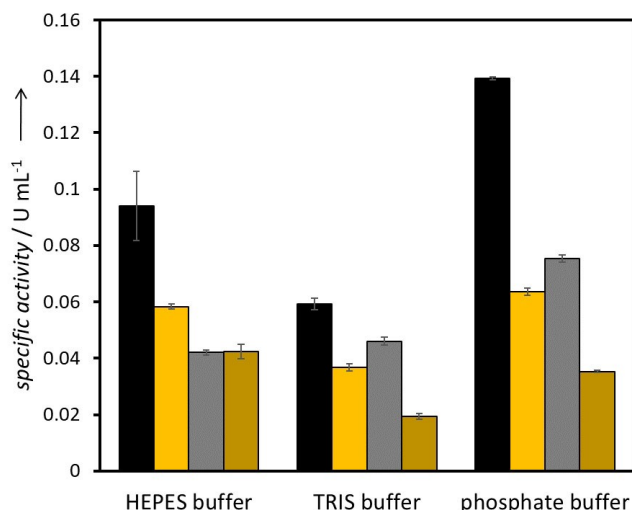


Figure 6. Specific activities towards the formation of acetophenone (**6**) for the CvATA (0.5 mg/mL) in dark conditions (■) or in normal light conditions with a light intensity of ~ 0.25 mW/cm² (■) and for the VfATA (0.05 mg/mL) in dark conditions (■) or in normal light conditions with a light intensity of ~ 0.25 mW/cm² (■) in 100 mM (pH 7.5) HEPES, TRIS and potassium phosphate buffer. Enzymatic reactions contained 30 mM (*rac*)- α -MBA (**4**), 60 mM pyruvate (**5**) and 0.1 mM PLP at 20 °C and 600 rpm. The error bars represent the standard deviation of technical triplicates.

40% compared to the activity under dark conditions. TRIS buffer could not prevent the inactivation of the CvATA, under normal illumination the activity was reduced by 30%. Thus, the activity decrease due to light exposure was slightly less compared to HEPES buffer, but still present. Compared to the other buffers, the specific activity in TRIS buffer was reduced under dark conditions. In phosphate buffer, the CvATA showed a significantly higher specific activity under dark conditions. However, the inactivation after normal light exposure was significantly higher (50%) as well. The VfATA did not show any inactivation after light exposure in HEPES buffer previously (Figure 4B), which could be confirmed in this experiment (Figure 6). However, illumination of the VfATA in TRIS buffer and in phosphate buffer, resulted in an inactivation by 50% compared to the dark approaches. The specific activity in phosphate buffer was again highest in relation to the other buffers. While the light-mediated inactivation of the CvATA was similar in all three buffer systems, the VfATA exhibited strong inactivation in TRIS and phosphate buffer, however, HEPES buffer seemed to prevent light-mediated inactivation exclusively in case of the VfATA. About any interaction between HEPES buffer and the VfATA, which might facilitate a higher stability of PLP in the enzymatic active site, can only be speculated. It can be assumed, that the application of TRIS instead of phosphate buffer during the production process of the enzymes does not prevent light mediated inactivation. Thus, it has been shown, that the specific activity of enzymes varies depending on the employed buffer system. In this case, highest specific activities were achieved in potassium phosphate buffer under dark conditions. When comparing the enzyme activities under light exposure in TRIS buffer with the

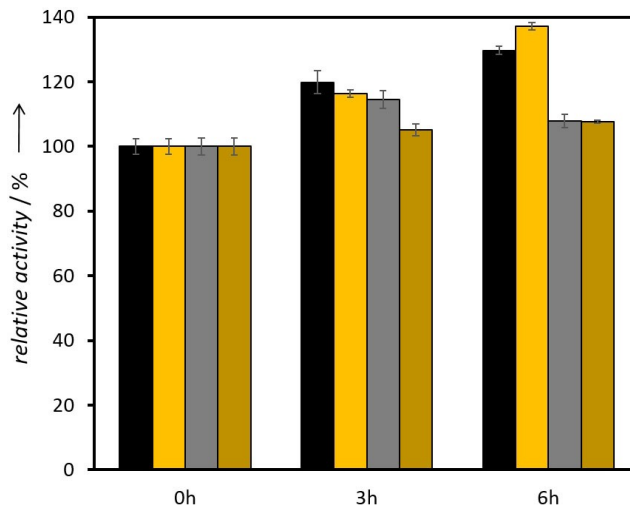


Figure 7. Relative activity of the AsATAmut11 (1 mg/mL) for the formation of acetophenone (**6**) over an incubation period of 6 h using 100 mM HEPES buffer (pH 7.5) in dark (■) and in light conditions with a light intensity of ~ 0.25 mW/cm² (■), or using 100 mM potassium phosphate buffer (pH 7.5) in dark (■) and in light conditions with a light intensity of ~ 0.25 mW/cm² (■). Enzymatic reactions contained 30 mM (*rac*)- α -MBA (**4**), 60 mM pyruvate (**5**) and 0.1 mM PLP at 20 °C and 600 rpm. The error bars represent the standard deviation of the technical triplicates.

activity in dark phosphate buffer, we could demonstrate, that the specific activity of PLP-dependent enzymes could be increased about four-fold, by adjusting buffer and light conditions.

Activation of the AsATAmut11

Another special case is represented by the AsATAmut11. From Figure 4B could be recognized, that after 1 h of incubation in normal light and mixed conditions, the AsATAmut11 showed drastically increased activity. These results were re-evaluated by testing the activation of the AsATAmut11 in normal light conditions for a period of 6 h in HEPES buffer (Figure 7). The activity in dark conditions, incubated for 6 h, served as control. A key observation was that the previously noted activation did not only occur in illuminated conditions, but as well in dark conditions. Thus, activation was not by light, but by incubation of the AsATAmut11 in buffer. Accordingly, the cause of activation in our case is different from the case of Hill, et al. who used a special illumination setup for the activation of a PLP-dependent aspartate aminotransferase.^[28–30] In dark conditions (HEPES buffer), the activity increased by 35% within 6 h of incubation relative to the activity after 0 h of incubation. In normal light conditions (HEPES buffer), the activity seemed to increase until a maximum activity of 138% was reached after 6 h of incubation. However, after 24 h of incubation in normal light conditions, the activity had decreased again, reaching a level of 125% of activity, while in dark conditions the activity remained at the same level over the course of 24 h of incubation (Figure SI.4). As the results with light exposure come

with a notable error, it remains unclear if light has an additional beneficial effect on the activation of the AsATAmut11. Another experiment with 30 min of targeted blue light illumination, as follow up to 6 h incubation in dark conditions, had no negative impact on the activity level of the AsATAmut11 likewise, highlighting the superior stability of this enzyme under light exposure. As in case of the VfATA a variation in the reaction buffer led to different inactivation characteristics, the activation of the AsATAmut11 in HEPES buffer was compared to phosphate buffer (Figure 7). In previous experiments employing the CvATA and the VfATA (Figure 6), the application of 100 mM potassium phosphate buffer (pH 7.5) mediated a higher specific activity in the dark, compared to other buffers, combined with a strong deactivating effect upon illumination (Figure 6). In phosphate buffer, the activation of the AsATAmut11 in dark as well as in light conditions was drastically reduced. After 3 h of incubation in dark phosphate buffer, the relative activity was increased by 15 %. For samples taken after 3 h of incubation in light or after 6 h incubation in light and dark, the activation of the AsATAmut11 in phosphate buffer was limited to an increase of 5 %. Thus, by employing phosphate buffer the activity of the AsATAmut11 could not be further increased. After seeing the general activation in the dark over time for the AsATAmut11, we also checked the activity of CvATA over the course of 6 h of incubation in 100 mM HEPES buffer (pH 7.5) under dark conditions, however, an activation could not be noticed (Figure SI.5). Moreover, the relative activity slightly declined by 5 % during the incubation period. The incubation over time was performed using ten times concentrated enzyme stock solutions (10 mg/mL for AsATAmut11, 5 mg/mL for CvATA). It has been previously shown for the CvATA, that the residual activity of the enzyme could be increased by 10 %, when a 5 mg/mL enzyme solution was incubated in 50 mM HEPES buffer (pH 8.2) for 24 h. In this study by Chen *et al.*, the effect was not seen for the incubation of enzyme solutions with lower concentrations.^[48] Similar to the activation of the AsATAmut11 described herein, an activation by 29 % was reported for the incubation of freshly purified CvATA overnight in 50 mM HEPES buffer (pH 8.3). It was discussed, that the result might be due to complete dimerization to form active enzyme.^[49] Further, it is known, that the supplementation of PLP (> 0.25 mM) as well as the addition of co-solvents such as DMSO, methanol and glycerol can increase the stability of ATAs in aqueous solutions.^[22,39,48,50] Thus, enzyme activation seems to be dependent on the selected ATA and the incubation conditions such as buffer concentration and pH. However, an inactivation by light of the AsATAmut11 could not be observed, here. Certainly, the AsATAmut11 appeared to exhibit extraordinary stability in the applied reaction setups. Even through intense and targeted blue light illumination and after 24 h of normal light exposure, the enzyme still displayed increased activity of about 125 %. Besides the conversions achieved in sitagliptin synthesis,^[42] the high stability to light and the activation observed in this work are great features of the AsATAmut11. However, the mechanism which enables the AsATAmut11 to maintain high stability in various light and reaction setups has yet to be evaluated, but it could be associated with the introduced mutations.^[42]

Conclusion

Herein, we evaluated the effect of visible light on the activity of six PLP-dependent enzymes. We could observe that the photo-degradation of free PLP in solution under normal light exposure (sun and electric light) is similar to specific blue light illumination. By exposing the amine transaminase CvATA to targeted and highly energetic blue light illumination, we were able to inactivate the enzyme completely within 20 min of illumination. Tests with PLP-dependent enzymes in three different light setups revealed, that even under normal light exposure the CvATA and the BmATA exhibited a loss of about 40 % activity, compared to their activity in dark conditions. In case of the EcLDC and the SrLDC, an even stronger response to light was observed, resulting in an inactivation by up to 90 %. Experiments with different buffer systems showed, that the specific activity of PLP-dependent enzymes, especially of the VfATA, is not only depending on light conditions alone but also on the selected reaction buffer and certainly a number of additional factors. The AsATAmut11 represented an interesting case, as the enzyme seemed to be resistant to illumination and an inactivation was not observed for different light and buffer setups. Moreover, by incubation in HEPES buffer for a minimum of 6 h, the AsATAmut11 exhibited an activation of up to 150 %, the underlying mechanism yet remains unclear. This increased light stability is certainly, amongst others, one of the features, making this amine transaminase mutant so potent. Based on our results, we recommend to anyone working with PLP-dependent enzymes, to select a reaction system with a suitable buffer carefully and to evaluate the light sensitivity of the respective enzyme, not only during the enzymatic reaction but also during enzyme production and purification. Thus, it might be possible to increase the activity of certain PLP-dependent enzymes easily by up to ten times.

Experimental Section

All chemicals were purchased from Sigma-Aldrich, Roth and Merck.

Expression vectors and transformation

The vectors for protein expression pET29a_CvATA, pASK-IBA-35_VfATA, pET21a_BmATA and pET21a_AsATAmut11 were created by Erdmann, *et al.*^[51] For LDC expression, the vector pkk32_EcLDC was used, which was created by Kira Küsters (group of Marco Oldiges; IBG-1, Forschungszentrum Jülich GmbH, Jülich, Germany and ABBt, RWTH Aachen University, Aachen, Germany) and contained the LDCc gene from *E. coli* employed by Kloss, *et al.*^[52] and the vector pET22b_SrLDC, which was constructed by Baud, *et al.*^[53] Vector details are given in the Supporting Information. *E. coli* BL21(DE3) cells (Merck, Darmstadt, Germany) were transformed with the respective expression plasmid by adding 1 μ L plasmid solution (100 ng/ μ L) to 100 μ L bacterial solution ($OD_{600} = \sim 12$, in 80 mM $CaCl_2$, 20 % (w/v) glycerol). After 30 min incubation on ice, a heat shock was performed at 42 °C for 40 s and the cells were stored on ice for 2 min. Then, 900 μ L S.O.C. medium (Thermo Fisher Scientific, Waltham, MA, USA) was added and the cells were incubated for 1 h at 37 °C and 700 rpm in a thermomixer (comfort 5335r, Eppendorf, Hamburg, Germany). The cells were plated on lysogeny broth (LB)

agar with 100 µg/mL ampicillin for all constructs, except for transformations using pET29a or pKK32 vectors, here 50 µg/mL kanamycin was used, and incubated overnight at 37 °C.

Production and purification of enzymes

For all enzymes, the production was performed under normal light and under dark conditions. For normal light conditions, windows were left uncovered and the electric light was switched on but no specific or targeted illumination occurred. In dark conditions, illumination was avoided as much as possible. Flasks and solutions were wrapped in aluminum foil, windows were covered, and the electric light was switched off. The target enzymes were produced in shaking flasks with a filling volume of up to 15%. A single colony from the respective overnight plate was transferred to 50 mL LB medium (with 100 µg/mL ampicillin for all constructs, except for cells transformed with pET29a or pKK32 vectors, here 50 µg/mL kanamycin was used) and the precultures were cultivated overnight at 37 °C and 150 rpm (Infors HT, Bottmingen, Switzerland). For the main culture, auto induction (AI) medium was used, which was incubated for 48 h at 37 °C (decreased to 20 °C after 2 h) and 150 rpm (Infors HT, Bottmingen, Switzerland). Cells were harvested by centrifugation (Avanti J-20 XP, Rotor JLA-8.1000, Beckman Coulter, Brea, CA, USA, 30 min, 8,000 rpm, 4 °C) and the resulting cell pellets were frozen. For the purification, frozen cells containing the respective enzymes were thawed and resuspended (30% (w/v)) in TRIS buffer (50 mM, pH 7.5, 0.2 mM PLP) with 1 mg/mL lysozyme (Merck, Darmstadt, Germany) and 10 units/mL benzonase (Merck, Darmstadt, Germany) for 30 min on ice. The cells were disrupted by ultrasonication (Digital Sonifier 450, Emerson Electric Co., Ferguson, MO, USA) on ice for a total sonication time of 5 min (2 s pulse, 8 s pause) at 60% intensity. After centrifugation (Avanti J-20 XP, Rotor JA-25.50, Beckman Coulter, Brea, CA, USA, 45 min, 20,000 rpm, 4 °C), the supernatant was applied to a Ni-NTA Superflow resin (Qiagen, Hilden, Germany), pre-equilibrated with TRIS buffer (50 mM, pH 7.5, 0.2 mM PLP) using an ÄKTA pure chromatography system (GE Healthcare, Bosten, MA, USA). After a washing step with an appropriate washing buffer (50 mM TRIS buffer, pH 7.5, 0.2 mM PLP, 25 mM imidazole), the His₆-tagged proteins were eluted (50 mM TRIS buffer, pH 7.5, 0.2 mM PLP, 300 mM imidazole). Relevant protein samples were pooled and desalted on a HiTrapTM Sephadex G-25 resin (GE Healthcare, Bosten, MA, USA), pre-equilibrated with TRIS buffer (10 mM, pH 7.5, 0.2 mM PLP). For enzymatic reactions with the CvATA and VfATA in potassium phosphate buffer, potassium phosphate buffer was used instead of TRIS buffer during the production and purification process. The protein concentration of the pooled relevant samples was adjusted to 1 mg/mL with water. After freezing the protein solutions overnight, it was submitted to lyophilization (Alpha 1-4 LD Plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

ATA initial rate reactions and analytics

The ATA activities were tested in different light setups, which were explained above. Illumination of enzyme and PLP solutions occurred using ten times concentrated stock solutions. The transformations were performed in 1 mL scale in 100 mM HEPES buffer, TRIS buffer or potassium phosphate buffer (pH 7.5) containing 0.1 mM PLP, 30 mM (*rac*)- α -MBA (4) and 60 mM pyruvate (5). For an optimal initial rate measurement, the enzyme concentrations were set to 0.5 mg/mL for CvATA, 0.05 mg/mL for VfATA, 0.1 mg/mL for BmATA and 1 mg/mL for AsATAmut11. The protein concentrations were determined according to Bradford^[54] after diluting relevant samples 1:100 (v/v) in the respective buffer. Initial rate reactions were incubated for a duration of 30 min at 20 °C and 600 rpm in

transparent or amber colored microcentrifuge tubes in a thermomixer (comfort 5335r, Eppendorf, Hamburg, Germany). Product formation was detected via HPLC analysis. The enzymatic reaction in samples taken during the initial rate experiments was quenched 1:20 (v/v) in a mixture of 45% acetonitrile, 55% H₂O and 0.1% trifluoroacetic acid (v/v). The samples were analyzed by reversed phase chromatography using an Ultimate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a diode array detector and a LiChrospher[®] 100 RP-18 column (Merck, Darmstadt, Germany). The analysis was carried out isocratically with a flow rate of 1.5 mL/min at 25 °C for 10 min using a solvent mixture of 45% acetonitrile, 55% H₂O and 0.1% trifluoroacetic acid (v/v). A 10 µL sample was injected and the absorption of acetophenone (6) was detected at 254 nm with a retention time of 4.3 min. A slope was generated from the resulting peak areas, corresponding to the relative activity of the respective ATA. For specific activity determination, the concentration of the formed acetophenone (6) was calculated from the peak areas using a calibration curve (Figure SI.1). The activity is given in U mL⁻¹, which is defined as the amount of enzyme in 1 mL reaction solution, which catalyzes the formation of 1 µmol acetophenone (6) per minute. The error bars in the respective diagrams give information about the standard deviation of technical triplicates.

LDC initial rate reactions and analytics

The relative LDC activities in different light setups were measured via a newly developed colorimetric assay using the indicator 4-nitrophenol for the decarboxylation reaction from L-lysine (8) to cadaverine (9; manuscript in preparation). Illumination of enzyme and PLP solutions was done as described previously using ten times concentrated stock solutions. The corresponding illumination setup is depicted in Figure SI.6.A–B. Colorimetric measurements and initial rate reactions were performed using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Kyōto, Japan) in a 1.5 mL cuvette at 20 °C, measuring the absorbance at 400 nm in 10 s intervals. Before the enzymatic reactions in 1 mL scale were started, 10 mM L-lysine (8) and 0.1 mM PLP were pre-incubated in 25 mM HEPES buffer (pH 6.0) for 4 min. Then, 0.1 mM 4-nitrophenol was added and the initial rate measurement was started by addition of 0.05 mg/mL EcLDC or 0.025 mg/mL SrLDC. After manual stirring for a couple of seconds, a blank measurement was performed and then the photometric measurement was started, which lasted 30 min. The error bars in the respective diagrams give information about the standard deviation of the technical triplicates.

Blue light inactivation of the CvATA

Blue light experiments with the CvATA were performed for initial rate reactions as described above. A reaction solution containing 0.03 mg/mL enzyme was incubated for 10 min in an amber colored microcentrifuge tube in a thermomixer (comfort 5335r, Eppendorf, Hamburg, Germany; 600 rpm, 22 °C), before the solution was subjected to blue light illumination for 25 min. Inactivation experiments were carried out in a stirred 2 mL glass vessel (600 rpm), blue LED strips (60 LEDs, 450 nm, X105-0200, revoART GmbH, Markkleeberg, Germany; ~12 mW/cm²) were used for illumination. The corresponding blue light setup is depicted in Figure SI.6.E–F. Light intensities at 450 nm were determined with a distance of 1 cm from the light source using an energy meter PM100D equipped with a S302 C sensor (Thorlabs, Newton, NJ, USA). As the energy meter shows sensitivity not only for light but as well for temperature changes, the measured light intensities might be error prone. The reaction setup was cooled with ice to compensate for a temperature increase mediated by the LED strips. The temperature

was monitored and kept at an average value of 22 °C. For the sake of comparison, the corresponding dark control was handled at the exact same temperature conditions. Samples taken during the initial rate reaction were analyzed via HPLC as described above. From the resulting peak areas, the corresponding concentration of acetophenone (6) was calculated using the mentioned calibration curve.

Spectrophotometric analysis of PLP solutions

The spectrophotometric analysis of PLP solutions was done in a 1.5 mL cuvette at 20 °C using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Kyōto, Japan), measuring the spectrum from 250 nm to 500 nm. Test samples in 1 mL scale contained 0.1 mM PLP dissolved in 100 mM HEPES, TRIS, potassium phosphate, TEA buffer (all pH 7.5) or in water. 1 h blue light illumination was carried out in a stirred 1.5 mL quartz glass cuvette (Hellma GmbH & Co. KG, Müllheim, Deutschland; 600 rpm) using a blue LED (450 nm, royal blue, XP-E2 SMD-LED, Star-PCB; Cree, Durham, NC, USA; ~60 mW/cm²). The corresponding single blue LED setup is depicted in Figure S1.6.C–D. The light intensity at 450 nm was measured as described above. The setup was cooled with ice to keep the solution at an average temperature of 22 °C. Illumination of PLP solutions in normal light conditions was done as described above.

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Conflict of Interest

The authors declare no conflict of interest.

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