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Photoswitching of Enzyme Activity by Laser-Induced pH-Jump

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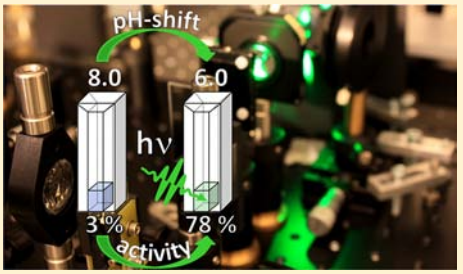
Supporting Information

ABSTRACT: Controlled initiation of biochemical events and in particular of protein activity is a powerful tool in biochemical research. Specifically, optical trigger signals are an attractive approach for remote control of enzyme activity. We present a method for generating optical control of enzyme activity applicable to a widespread range of enzymes. The approach is based on short laser pulses as optical "switches" introducing an instantaneous change of the pH-value for activation of protein function. The pH-jump is induced by proton release from 2-nitrobenzaldehyde. Reaction conditions were chosen to yield a pH-jump of almost 3 units on switching from inactive to active conditions for the enzyme. In this experimental setup, irradiation can be realized without any loss of enzyme activity. Following this change in pH-value, a controlled activation of hydrolytic activity of acid phosphatase is successfully demonstrated. This application provides a general method for photocontrol of enzymatic function for proteins having a significant pH-profile. The kinetic data for the substrate 6-chloro-8-fluoro-4-methylumbelliferone phosphate are determined.



INTRODUCTION

Control and especially controlled activation of biochemical processes is of high interest in today's research.[1−5] The use of trigger signals provides a method for temporary control of protein function, which is essential for detailed investigations of biochemical systems and for steering biomolecular processes. In this context, optical switches offer a number of advantages. For example, due to their lack of chromophores, most cells (except photoreceptors) do not absorb light. Thus, depending on the chosen wavelength, they are not harmed upon irradiation.[4] Furthermore, the use of lasers offers a high spatial and temporal control[6] compared to other methods, such as rapid mixing.[7]

In recent years, light-mediated activation of several inherently photosensitive proteins has been increasingly investigated, leading to detailed kinetic and mechanistic studies. One of the most widely studied examples of photosensitive proteins is the green fluorescent protein. Numerous reports addressing decarboxylation,[8,9] proton transfer,[10,11] or *E*/*Z* isomerization[12−14] have been done, resulting in a wealth of information about its excited-state chemistry (reviewed, e.g., in refs 15, 16). Another well-studied light-driven reaction is the reduction of protochlorophyllide within the chlorophyll biosynthesis by protochlorophyllide oxidoreductase. This enzyme is often used as a model enzyme for studies concerning proton or hydride transfers, conformational changes, or solvent fluctuations.[17−22] DNA-photolyase should also be named as a successful example of investigations of light-activated enzymes.[23−25] Moving on from the inherently photocontrolled proteins, various studies on the photochemical activation of enzymes via the release of caged bioeffectors have been done.[26] The application of caged compounds in biological systems began with the work of Kaplan et al., who reported about photolytic release of ATP in human red blood cell ghosts.[27] A significant amount of excellent work followed this principle, including a diversity of messengers such as ATP, cAMP, glutamate, or other amino acids.[4,26,28] Even external photocontrol via modification of the enzyme and generation of a caged protein were demonstrated.[6,29,30] However, all of these methods are more or less tailored toward a specific class of enzymes depending on the biological effectors. Focusing especially on the modified enzymes, they are also associated with considerable efforts. Thus, more general methods for photocontrol of biological function are needed, as, for instance, Young and Deiters called for in their review.[31]

The use of pH-jump reagents for introducing rapid changes in pH-value is a well-known and established technique. For example, photoacids are employed for reversible pH-jumps,[32−34] or, more frequently applied in biochemical studies, a permanent shift in the pH-value is introduced by a release of caged protons.[5,35,36] Although various pH-jump reagents are known for generating a persistent shift in pH,[1,26,37−39] their application as a tool in biochemical studies is still scarce.[5] This technique is used in diverse protein folding studies as well as for ligand binding kinetics to smaller proteins such as haem proteins.[40−42] In this field, an impressive development took place toward an improvement in time resolution. However, this technique seems to be still restricted mainly to folding studies.

In our work, we demonstrate the optical activation of enzymatic conversion, extending the application of caged protons as an optical trigger and offering a more general and

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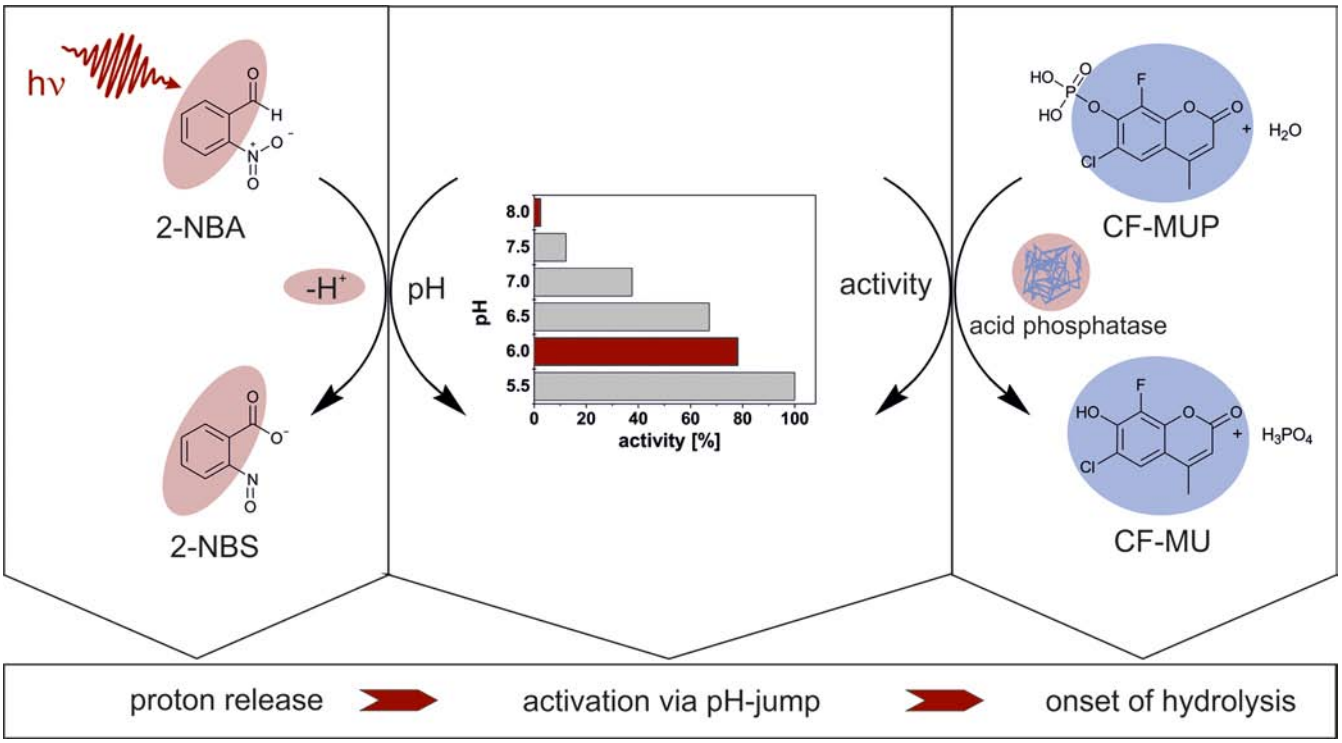
Scheme 1. Principle of the Optical Activation Experiments[a]



[a]The photoexcited intramolecular rearrangement of 2-nitrobenzaldehyde (2-NBA) to 2-nitrosobenzoic acid (2-NBS) causes a proton release and a strong change in pH. As a consequence of the enzymatic pH-profile an activation of hydrolytic activity is achieved and enzymatic conversion of 6-chloro-8-fluoro-4-methylumbelliferone phosphate (CF-MUP) to 6-chloro-8-fluoro-4-methylumbelliferone (CF-MU) is switched on.

flexible alternative to the release of special bioeffectors. In this study, we successfully photoswitch the activity of a hydrolytic enzyme by applying a laser-induced pH-jump to a complex mixture of enzyme, substrate, and phototrigger (Scheme 1). To the best of our knowledge, laser-induced pH-jump for the activation of hydrolytic enzymes and thus controlling the enzymatic conversion has not yet been reported. Protons as a photocontrolled trigger for activation of biochemical processes have several advantages compared to other existing strategies. This method is

(i) applicable to enzymes that are not photosensitive by nature,
(ii) not restricted to the control via special bioeffectors, and
(iii) independent of the substrate's nature and the class of enzyme.

The proteins simply need a sharp activity profile that allows control of performance via pH-value, which is the case for many enzymes even besides the enzyme class of hydrolases.

Our approach offers a possibility for optical control of enzyme activity in a widespread range of enzymes. Hence, the use of protons as photo-controlled trigger for activation of biochemical processes clearly expands the range of enzymes and reactions that can be controlled photochemically.

EXPERIMENTAL SECTION

The overall reaction system is divided into two steps; the pH-jump generation and the enzymatic reaction system (Scheme 1). For the latter, we chose a hydrolytic enzymatic system, as the hydrolysis proceeds without any side reactions, simplifying detection and in particular quantification of the enzymatic conversion. The chosen enzyme, the acid phosphatase (EC 3.1.3.2, type IV-S from potato), is a nonspecific phosphomonoesterase and, with an overall amount of 16.6% carbohydrates, it is categorized as a glycoprotein.[43] The sharp pH-profile of phosphatase activity (Scheme 1) enables us to control the enzyme activity via laser-induced jumps in pH.

The activity of acid phosphatase was analyzed by following the conversion of a novel substrate, introduced by Yang et al., 6-chloro-8-fluoro-4-methylumbelliferone phosphate (CF-MUP) shown in Scheme 1.[44] In comparison to standard substrates like 4-nitrophenyl phosphate or 2-carboxyphenyl phosphate the resulting reaction product 6-chloro-8-fluoro-4-methylumbelliferone (CF-MU) combines two benefits that are important for detection of enzymatic reaction. First of all, CF-MU has a pK_a (4.77) that allows the use in the desired range of pH without changes in absorption properties. Determination of the pK_a-value was done by pH-titration monitored via absorption measurements. For these experiments, an aqueous solution of CF-MU (5 μM concentration) containing 150 mM sodium chloride, to ensure a constant ionic strength, was used. [For a detailed description see Supporting Information, determination of the pK_a of CF-MU and Figure SI1-1.] The observed pK_a of 4.77 ± 0.06 is consistent with known values from literature.[44] Furthermore, the absorption spectra of the substrate as well as that of the formed product do not significantly interfere with the detection of the pH-jump and are not notably disadvantaged by photoconversion of the trigger. Additionally, CF-MU as a fluorophore possesses the potential for a fluorimetric assay even though an absorptive assay was developed for this study. Kinetic data of the enzymatic conversion of CF-MUP by acid phosphatase were recorded and are shown in detail in Figure SI2-1. In this case previous published data are not available for a comparison, and thus, these are the first results of kinetic studies for this new substrate.

For generation of the light-induced pH-jump 2-nitrobenzaldehyde (2-NBA) was chosen. Intramolecular rearrangement of 2-NBA leads to fast formation of nitrosobenzoic acid, a weak acid (expected $pK_a \approx 3.5$[36]) which acts as proton emitter. The proton release occurs within the nanosecond range.[45,46] The mechanism, time regime and kinetics of the photoconversion have been studied in detail.[35,36,45−50]

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In contrast to folding studies, controlling protein activity via laser-induced pH-jumps requires the maintenance of an active enzyme. Therefore the central wavelength, the laser power, and irradiation time have to be chosen carefully. For photoconversion, the second harmonic of a 1 kHz regenerative Ti:sapphire amplifier system was used (λ_{exc} = 388 nm), ensuring that there is no absorption of the excitation wavelength by the enzyme and minimum absorption by the substrate/product mixture. Typically, a power of about 160 mW was applied. An aqueous solution containing 2-NBA as a phototrigger, fluorescein for the detection of the pH-jump, CF-MUP as substrate, and acid phosphatase was irradiated with a beam diameter larger than the sample cross section leading to nearly homogeneous illumination. Hence, approximately half of the power actually irradiated the sample volume. A second beam path with a continuous wave (cw)-light source covering the visible and near-ultraviolet spectral range was used for measuring the sample absorption with a time resolution of 250 ms. [See also Supporting Information SI3 and Figure SI3-1.]

For quantification of the changes in pH, fluorescein was used as an indicator. The use of dyes like fluorescein for the purpose of monitoring proton transfer reactions in proteinogenic systems via change of absorbance has been described by Gutman et al.[51−54] The proton release induced by photorearrangement of 2-NBA can be observed by the changing ratio of protonated and deprotonated species of fluorescein. Because of its four prototropic forms fluorescein has matching pH-dependent absorption properties [see Figure SI3-2], as a pH-jump from basic to slight acidic conditions, with a range of 2−3 units, is striven for. Hence, the use of a 3 μM aqueous fluorescein solution enables the detection of the induced pH-jump by following the decreasing absorption at 490 nm.

RESULTS AND DISCUSSION

The pH-jumps shown in Figure 1 are achieved under parameters ensuring stable conditions for the enzyme. Excitation at 388 nm for about 250 ms leads to a jump in pH of approximately 2−3 pH-units, and a stable pH-value is given for more than 30 s after excitation. The magnitude of the jump in pH strongly depends on the composition of the overall system. The enzyme and the substrate both hold hydroxyl groups that can act as proton scavengers, thereby lowering the resulting pH-jump. Hence, for these experiments we avoided excessive concentrations of both. The addition of 83.3 μM of substrate reduces the proton release by approximately 0.6 pH-unit, which corresponds to a decrease of about 7.4 μM of protons.

The concentrations of substrate and enzyme we have chosen to apply are a compromise to detect the enzymatic conversion sufficiently without providing a buffer capacity that is too high for generating the desired pH-jump. As a result of the enzymes pH-profile, this laser-induced pH-jump leads to photoswitching of enzymes activity. Simultaneously to the pH-jump, the onset of enzymatic reaction was analyzed. The enzymatic conversion was monitored red-shifted to the absorption maximum of CF-MU at 370 nm to minimize interferences with absorption changes produced by photoconversion. The complex processing of the sample and the resulting time profile of the pH-jump experiments is pictured in Figure 2B−D. [For detailed spectra, see Figure SI.3-3.]

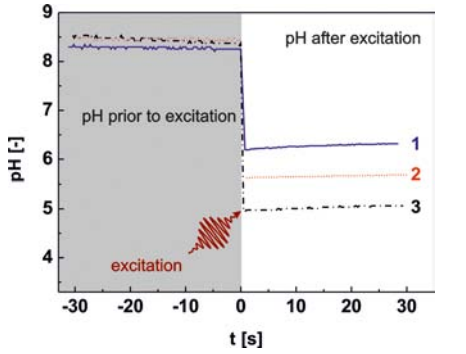

Figure 1. Time profile of pH-jump experiments as received from the absorption time trace of fluorescein at 490 nm: the excitation beam at 388 nm with a power of 80 mW was applied to the sample volume for a defined time of 250 ms. 1, 500 μM 2-NBA, 83.3 μM CF-MUP, and 0.08 mg/mL acid phosphatase; 2, 500 μM 2-NBA and 83.3 μM CF-MUP; 3, 500 μM 2-NBA; 3 μM fluorescein was additionally included in all mixtures.

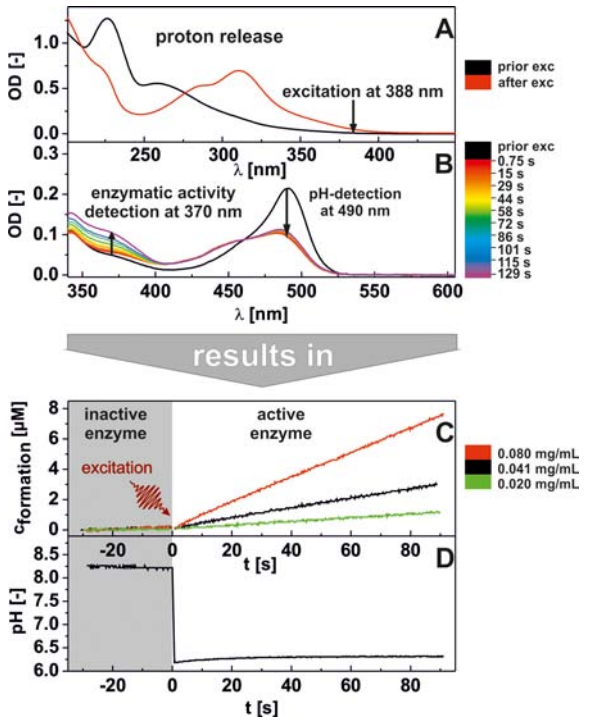

Figure 2. (A) Absorption spectra of an aqueous 100 μM 2-NBA solution prior and after excitation. Optical excitation at 270 nm with a power of 0.23 mW was applied to the sample volume for a defined time of 300 s. (B) Monitoring of time-resolved absorption spectra of the pH-jump-system. Optical excitation at 388 nm with a power of 80 mW was applied to the sample volume for a defined time of 250 ms; the solution contains 500 μM 2-NBA for the proton release and 3 μM fluorescein for detection of the pH-value, enzymatic conversion using 83.3 μM of substrate CF-MUP and 0.08 mg/mL acid phosphatase (the absorption of 2-NBA prior and after excitation was mathematically eliminated). (C) Resulting activation of hydrolytic conversion of CF-MUP by acid phosphatase at different enzyme concentrations controlled by optical excitation (conditions like B) (red line 0.08 mg/mL, black line 0.041 mg/mL, green line 0.02 mg/mL acid phosphatase in 150 mM NaCl). (D) Resulting pH-jump by optical excitation (conditions like B).

Figure 2C shows the time-dependent rise in product formation resulting from the activation of acid phosphatase by the laser-induced pH-jump, demonstrating external control of enzymatic activity. Prior to excitation, there is no significant hydrolytic activity apparent. After the photoexcited pH-jump, the onset of hydrolytic conversion is detected. Upon activation, the enzymatic conversion runs with a reaction rate of 0.06 μmol/(min·mg$_{enzyme}$) for an enzyme concentration of 0.08 mg/mL, which corresponds to an increased conversion rate by a factor of 8 compared to the reaction rate prior to laser excitation. Monitoring the reaction over a period of 600 s a linear progress of

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reaction can be observed (data not shown). It is the laser-induced pH-jump that leads to activation of the enzyme and as a consequence to enzymatic conversion of CF-MUP as the following test demonstrates. The presence of enzyme or substrate alone did not yield an activation of enzymatic conversion. Therefore, samples containing only 0.041 mg/mL enzyme and only 83.3 μM CF-MUP besides the 2-NBA were analyzed displaying no significant enhancement of change in absorption signal.

For further characterization, the amount of enzyme added was varied (Figures 2C and 3). Upon decreasing the concentration

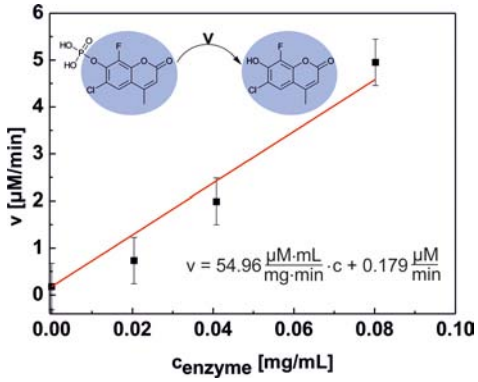

Figure 3. Plot of reaction rate vs enzyme concentration.

from 0.08 mg/mL to 0.041 and 0.02 mg/mL, the reaction rate dropped accordingly, showing a linear behavior between observed reaction rate and enzyme concentration, as Figure 3 proves. Here, calculated from the slope, an average activity of 55 μM·mL/(mg·min) is obtained, which corresponds to 0.055 μmol/(min·mg$_{enzyme}$), equivalent to the single value determined for 0.08 mg/mL.

Comparing the observed reaction rate to that expected from the kinetic data, only 15% of the activity was found. This decrease arises from the difference in reaction conditions like the temperature, the incubation period at basic milieu, the absence of buffer and the present concentration of 2-NBA. In order to demonstrate whether the applied method offers 100% of the possible activation, which means that the maximal achievable formation rates, under given conditions, are observed after excitation, a number of experiments were conducted. This was executed by measuring the phosphatase activity in a conventional assay and after laser pulse activation. Starting at pH 8, an enzymatic activity of only 0.014 μmol/(min·mg$_{enzyme}$) was detected. Irradiation of the sample results in changing pH up to 6 and as a consequence, the activity raises by eight times up to 0.109 μmol/(min·mg$_{enzyme}$). The 8-fold activation rate is consistent with the previously presented pH-jump experiments. For proof of reproducibility, the experiment was repeated several times. In an equivalent experiment, using the similar sample composition, the enzymatic reaction was monitored at a pH of 6 initially without applying laser irradiation. Here an enzymatic activity of 0.101 μmol/(min·mg$_{enzyme}$) was detected, clearly indicating that the enzyme can be fully activated by application of laser pulses [see Supporting Information, Table SI3-1 for a summary of the results].

In order to prove that there is no significant influence on enzymes activity, the hydrolytic reaction rate of acid phosphatase (see Figure 4) without 2-NBA was assayed at a pH-value of 5.5 with and without irradiation as well as before and after

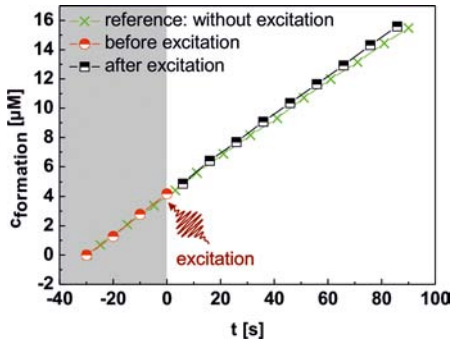

Figure 4. Influence of irradiation on the enzymatic activity: enzymatic conversion was detected at 370 nm using 83.3 μM substrate CF-MUP and 0.042 mg/mL acid phosphatase in 150 mM acetate buffer pH 5.5 The excitation beam at 388 nm with a power of 80 mW was applied for a defined time of 250 ms.

irradiation. As shown by these measurements, irradiation can be realized without any loss of activity. In the case of the reference sample without irradiation as well as for the samples before and after irradiation for about 250 ms, comparable activities in the range of 0.2 μmol/(min·mg$_{enzyme}$) were observed. This means the enzyme shows high compatibility to the irradiation wavelength and time needed for the activation experiments.

CONCLUSIONS

In conclusion, we have presented a method of controlled activation of hydrolytic enzymes simply by photoswitching the pH of the environment. This system would be applicable to various enzymes, whereby it is not restricted to a natural photosensitivity or the application of special bioeffectors. Promising aspirants providing the possibility for remote control via proton release are, for example, β-glucosidases. [For a more detailed overview see Supporting Information, Table SI4-1.] But candidates are not only found within the hydrolases; also laccases, catalases, or decarboxylases are conceivable, underlining the versatility of this system. The spatial control that is achievable by photoregulation of protein activity is a further advantage that this simple one-pot arrangement holds compared to, for example, mixing techniques. Moreover, combinations of the pH-jump system with different analytical methods like NMR, vibrational studies, or electron paramagnetic resonance measurements provide a flexible design of experiments to study diverse biological events. We are aware of the fact that the time scale of the presented measurements of photoswitching enzyme activity competes with simple rapid stop-flow measurements. A significant advantage of the presented system is the large potential laser-induced pH-jumps offer in controlled activation of enzymes overtaking the time regime of mixing methods. By reducing the irradiated volume and increasing the sensitivity, the presented approach has the potential for a time resolution of nanoseconds, which is given by the response of 2-NBA.[46,55] This laser-induced pH-jump activation would be highly informative for detailed mechanistic and dynamic investigations of biochemical systems. It represents a sufficiently simple method that is versatile and applicable to all enzymes showing a significant pH-profile, and it will open a new window for biological and biochemical studies with the need for remote control of enzyme activity.

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■ ASSOCIATED CONTENT

Supporting Information

Materials, determination of the pK_a of CF-MU, determination of the kinetics of acid phosphatase, setup and procedure for measuring enzyme activation by a photo-switched pH-jump, and promising candidates offering a remote control based on the presented method. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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