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No evidence for non-resonant optical frequency-induced effects on the intrinsic fluorescence of adenosine-5′-triphosphate and the kinetics of the firefly luciferin–luciferase reaction

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ABSTRACT

A paper by Amat et al. [2] reported that the ATP-driven oxidation of luciferin to electronically excited oxyluciferin catalyzed by luciferase was accelerated when ATP was priorly irradiated at non-resonant optical frequencies (NROF) at 635 and 830 nm. In another paper by Amat et al. [3], increased fluorescence intensities of ATP–Mg complexes, which showed lower fluorescence than ATP when excited at 260 nm, were reported in consequence of concomitant NROF irradiation (i.e., 655 and 830 nm). It was postulated that NROF-induced electric field changes may alter the charge distribution in ATP's phosphate chain, resulting in lowering of the activation energy of its terminal phosphate. Here we use spectrofluorometry to further investigate this hypothesis. The effect of NROF (at 632.8 nm) on the intrinsic fluorescence of non-complexed and Mg-chelated ATP in aqueous solution and the influence of NROF (514.5 nm and 632.8 nm) on the rate of the luciferin–luciferase reaction was studied. We found that neither the intrinsic fluorescence of ATP nor its biochemical behavior during the firefly luciferin–luciferase reaction was affected by laser irradiation with NROF. Consequently, no evidence was found supporting the postulation that NROF-induced alternations on the charge distribution of the phosphate chain affect the reactivity of ATP.

1. Introduction

It is well known that adenosine-5′-triphosphate (ATP) occupies a key position in many biochemical processes as it stores chemical energy that is released in numerous cell metabolic processes. Due to the importance of ATP, small alterations in the biochemical behavior of ATP could have far-reaching biological consequences. In the field of photobiostimulation, enhancement of cellular activity and modulation of tissue response occur in consequence of irradiation with low-energy visible and near infrared light [1].

In a series of papers [2–4], Amat and co-workers described modifications of intrinsic ATP fluorescence and increased ATP-mediated reaction kinetics in isolated enzymatic systems after irradiation of ATP with non-resonant optical frequencies (NROF), i.e., at wavelengths that do not induce an electronic transition to the first excited state (S_1). The authors contended that NROF-induced alterations at the vibrational and/or conformational level might result in lowering of the dephosphorylation energy barrier of ATP's terminal phosphate. A faster energy delivery may thus constitute a fundamental mechanism in photobiostimulation [2–4]. The observed cellular effects were related by Amat et al. [2,3] to the response of ATP to visible and near-infrared laser light.

ATP is weakly fluorescent; relative fluorescence quantum yields [5] and related fluorescence emission intensities of ATP, adenosine-5′-diphosphate (ADP), and adenosine-5′-monophosphate (AMP) were reported in the order of ATP ≫ ADP > AMP when excited at 260 nm [3], indicating an essential influence of the phosphate tail on the fluorescence properties of adenosine. Addition of Mg^{2+} to ATP results in the formation of ATP–Mg complexes and a decreased fluorescence emission intensity [3], possibly due to ionic modifications of the phosphate tail by Mg^{2+} [6]. Interestingly, when excited at 260 nm during concomitant irradiation with NROF, enhancement of the fluorescence intensity

Abbreviations: Ar ion, argon ion; ADP, adenosine-5′-diphosphate; AMP, adenosine-5′-monophosphate; ATP, adenosine-5′-triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HeNe, helium neon; NROF, non-resonant optical frequencies.

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of ATP–Mg complexes was observed and postulated to be a consequence of NROF-induced charge displacements in the phosphate tail that may have reduced the strength of the Mg–phosphates association [3,4]. NROF-induced modifications of the fluorescence emission intensity of ATP samples in the absence of Mg^{2+} were not observed [3].

Considering the prominent impact of the phosphate tail on the intrinsic fluorescence of ATP [3,5], the question arises as to why an NROF-induced displacement of the phosphate-bound charges and a lower activation energy of ATP's highly reactive terminal phosphate, as postulated by Amat et al. [3,4], does not accelerate the rate of spontaneous hydrolysis and the conversion of ATP to di- and mononucleotides. This would translate to lower fluorescence emission intensities due the lower fluorescence quantum yields of ADP and AMP [5], or otherwise modify the intrinsic ATP fluorescence due to alterations in the interaction between the phosphate tail and the adenine moiety. Instead, the alleged charge displacement during NROF irradiation increases the quantum yield.

Additionally, NROF-accelerated ATP-mediated reaction kinetics in isolated enzymatic systems was reported, whereby NROF irradiation at more energetic wavelengths (655 nm and 633 nm) resulted in more profoundly accelerated reaction kinetics compared to longer wavelengths (830 nm) at equal incident irradiances [2–4]. An increased rate of the firefly luciferin–luciferase reaction was observed when ATP, which supplies the reaction with energy, was irradiated with 635 nm and 830 nm laser light before the reaction was initiated [2]. Similarly, the addition of NROF-irradiated ATP to the hexokinase reaction was shown to increase reaction kinetics associated with the conversion of glucose into glucose-6-phosphate by hexokinase in a wavelength-dependent manner [3]. However, the hypothesis that NROF may accelerate the reaction kinetics of hexokinase [3] was recently disproven [7] and other mechanisms that underlie the potential driving force behind photobiostimulation have been proposed [8–10].

In the present work, substantial research was devoted to try to verify the influence of NROF irradiation on the optical properties and the reactivity of ATP. For these purposes, NROF-induced effects on the intrinsic fluorescence of ATP and its biochemical behavior during the firefly luciferin–luciferase reaction were investigated. Fluorescence of non-complexed and Mg-chelated ATP was measured during concomitant laser-irradiation with NROF (632.8 nm) and compared to the fluorescence of non-irradiated control groups. Additionally, the luciferin–luciferase reaction rates were measured spectrofluorometrically under controlled experimental conditions before and after irradiation of ATP with NROF at more energetic wavelengths (514.5 nm and 632.8 nm) and higher irradiances than used in [2]. As reported for the hexokinase reaction [7], no evidence was found for NROF-mediated modulation of ATP's biochemical behavior. Moreover, changes in the fluorescence properties of ATP during NROF irradiation were not observed.

2. Materials and methods

2.1. Materials

Adenosine-5′-triphosphate (≥99%, catalogue number A2383, Sigma Aldrich, St. Louis, MO) was used without further purification and dissolved in MilliQ water (Millipore, Billerica, MA) using sterilized 50-mL polypropylene tubes (BD Biosciences, Franklin Lakes, NJ) at concentrations listed separately throughout the text. ATP–Mg solutions were prepared using $MgCl_2$ (≥99.99%, catalogue number 449172, Sigma Aldrich). For pH-dependent experiments, the pH of the solvent (MilliQ) was adjusted with 37 wt.% HCl in water (catalogue number 339253, Sigma Aldrich) or NaOH (catalogue number GA12967, Fluka, Buchs, Switzerland) prior to dissolving the solute. ATP stock solutions were prepared fresh each day and stored on ice.

The reaction components for the firefly luciferin–luciferase reaction were acquired from Sigma–Aldrich (FL-AA ATP Bioluminescent Assay Kit). The ATP Assay Mix is a lyophilized powder containing luciferin, luciferase, $MgSO_4$, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin, and tricine buffer salts (pH = 7.8). The ATP Assay Mix Dilution Buffer contained the same components except for luciferin and luciferase (pH = 7.8), and was used for further dilution of the ATP Assay Mix.

For the luciferin–luciferase kinetics experiments, an Assay Mix stock solution at pH = 7.8 was formulated by dissolving the contents of one vial of ATP Assay Mix in 5.0 mL MilliQ. The solution was swirled gently and kept on ice for 1 h to assure complete dissolution. The Dilution Buffer at pH = 7.8 was prepared by dissolving the contents of one vial of ATP Assay Mix Dilution Buffer in 50 mL of MilliQ and used for the preparation of a 25-fold dilution of the Assay Mix stock solution. The diluted ATP Assay Mix is referred to as 'assay solution' throughout the remainder of the manuscript. All solutions were protected from light and kept on ice.

2.2. Absorption and fluorescence spectroscopy

The absorption spectrum of ATP in MilliQ (40 μM) was obtained with a Cary 50 Bio UV-VIS spectrophotometer (Varian, Palo Alto, CA) in the 200–350 nm range using a 1.0 × 1.0 cm quartz cuvette. Fluorescence emission and excitation spectra were measured on a Perkin Elmer LS50-B luminescence spectrometer (Waltham, MA) at a scan speed of 100 nm/min using pulsed excitation at $\lambda_{ex} = 275 \pm 10$ nm and $\lambda_{em} = 384 \pm 10$ nm. Fluctuations in emitted light intensity due to fluctuations in the exciting light source were corrected by the excitation correction function of the spectrometer in combination with a parallel reference channel. All data were corrected for background (solvent only) and processed with Origin (OriginLab, Northampton, MA). Multiple spectra were recorded ($n > 3$) and used in statistical analysis, but only representative single spectra are presented.

UV–visible absorption spectra of individual reaction components of the luciferin–luciferase reaction, i.e., ATP and the assay solution containing luciferin and luciferase, were measured in the 200–900 nm range using a 2 × 10 mm quartz cuvette. In order to determine the actual absorption at the NROF irradiation wavelengths, i.e., 514.5 nm or 632.8 nm, solutes were dissolved in MilliQ at the final concentrations that were used in the kinetics experiments (assay solution: a 50-fold dilution of the original Assay Mix stock solution as acquired from Sigma–Aldrich; ATP: 0.25 mM). In addition, absorption spectra were recorded at spectrophotometrically justifiable concentrations, i.e., at concentrations at which the optical density of the greatest absorption band is less than 1.5, to ascertain the position of the main absorption peaks relative to the irradiation wavelength. All absorption spectra were corrected for background (solvent only) and processed with Origin.

2.3. Laser irradiation

ATP samples were NROF-irradiated with the 514.5-nm line of a continuous wave argon ion laser (Spectra-Physics 2000-336, Mountain View, CA) or a 632.8-nm continuous wave helium–neon laser (NEC, Tokyo, Japan) guided into a 0.6-mm and 1.0-mm diameter optical fiber, respectively. The optical fiber was positioned in the cuvette by fixation through a hole drilled in the cuvette lid such that the tip was immersed in the solution close to the detection volume in the center of the cuvette (Fig. 1A). The output power at the fiber tip was measured with a power meter (Ophir Optonics, Jerusalem, Israel), and was 10.0 mW for the argon ion laser

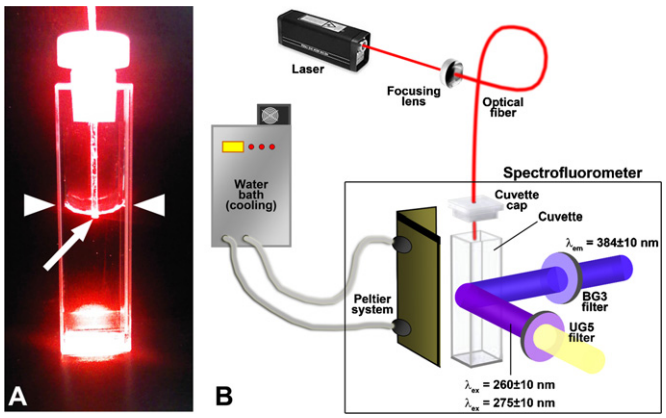

Fig. 1. Specific features of the experimental setup. (A) The solution-containing cuvette was irradiated with laser light, in this example 632.8 nm, via an optical fiber immersed in the solution (arrow). The arrowheads indicate the level of the solution. The fiber was secured in the cuvette through a hole drilled in the cuvette lid. The cuvette was placed in the spectrofluorometer (B) and irradiated at the indicated wavelengths (λ_{ex}). Fluorescence emission (λ_{em}) was measured in scan mode (280 or 295–800 nm) or time-based acquisition mode (384 ± 10 nm). The laser light was focused into an optical fiber through a lens that was guided into the cuvette. The laser was turned on for the NROF experiments and kept off for the control experiments. A Peltier-driven temperature control system ensured that the experiments were performed at a constant temperature.

and 9.0 mW or 12.0 mW for the HeNe laser, resulting in incident irradiances of 3.5 W/cm^2, 1.1 W/cm^2, and 1.5 W/cm^2, respectively.

The ATP solutions employed in the fluorescence emission measurements (Section 2.5) were irradiated inside the spectrofluorometer (Fig. 1B) for 60 min with the HeNe laser (9.0 mW), accounting for a total light dose of 32.4 J and cumulative radiant exposure of 4.1 kJ/cm^2. Additionally, ATP–Mg solutions were irradiated with the HeNe laser (12.0 mW) for the entire duration of spectrofluorometric acquisition, i.e., 156 s (total light dose of 1.9 J and cumulative radiant exposure of 241.9 J/cm^2). Control experiments were carried out by measuring the intrinsic fluorescence of ATP and ATP–Mg complexes in the absence of NROF under similar experimental conditions. To maximize NROF-induced effects, the fluorescence experiments were carried out at higher incident irradiances with NROF (1.5 W/cm^2) than used by Amat et al. (70 mW/cm^2) [3].

In Section 2.7, oxyluciferin luminescence activity was quantified before and after ATP irradiation with NROF. The ATP solutions were incubated at 37 °C [2] and irradiated for 15 min with the argon ion laser or the HeNe laser (total light dose of 9.0 J or 8.1 J and cumulative radiant exposure of 3.1 kJ/cm^2 or 1.0 kJ/cm^2, respectively). The control experiments with non-irradiated ATP solutions were kept in the dark under similar experimental conditions. In Section 2.8, oxyluciferin luminescence activity was measured before and after the assay solution was irradiated with NROF. The assay solutions were irradiated for 10 min with the argon ion laser or the HeNe laser (total light dose of 6.0 J or 5.4 J and cumulative radiant exposure of 2.1 kJ/cm^2 or 0.7 kJ/cm^2, respectively). The non-irradiated assay solutions were kept in the dark. For the experiments in Section 2.9 the luciferin-luciferase reaction mixture was irradiated with the HeNe laser for the entire duration of spectrofluorometric acquisition, i.e., 60 s. NROF-irradiation of the reaction mixture with 514.5-nm argon ion laser light was not performed because an appropriate bandpass or longpass filter that would attenuate the laser light, but transmit the luminescence signal at 562 nm, was not available. Control experiments were carried out in the absence of NROF irradiation but under the same experimental conditions.

It should be noted that the luciferin–luciferase kinetics experiments were performed at higher-energy wavelengths (514.5 nm and 632.8 nm vs. 635 nm and 830 nm) and higher incident irradiances (3537 mW/cm^2 and 1146 mW/cm^2 vs. 64 mW/cm^2 and 128 mW/cm^2) than used by Amat et al. [2] to maximize NROF-induced effects.

2.4. Spectrofluorometry during NROF irradiation

For the experiments described in Section 2.5, the intrinsic fluorescence of non-complexed ATP was quantified under non-irradiated and NROF-irradiated (632.8 nm) conditions on a Perkin Elmer LS50B luminescence spectrometer in time-based acquisition (fluorescence) mode. For a total duration of 60 min, fluorescence emission was recorded every 2 min using a 30-s signal integration time. The lamp was switched off automatically after each measurement and turned on again 1.0 s before each measurement. The emission wavelength was set to the maximum of the emission spectrum of ATP, i.e., to 384 nm (±10 nm). In the first series of experiments, the samples were excited at the absorption maximum of ATP, i.e., 259 nm (±10 nm). In a second series, the excitation wavelength was set to the excitation maximum of ATP, i.e., to 275 nm (±10 nm).

Additionally, the fluorescence of Mg-chelated ATP was scanned in the 290–550 nm range at a scan speed of 100 nm/min and excitation at 260 nm (±15 nm) [3] during concomitant irradiation with NROF (632.8 nm). Control experiments in the absence of NROF were performed under similar experimental conditions.

Fluctuations in the lamp intensity were corrected by the use of a parallel reference channel. A Schott UG5 bandpass filter (SCHOTT, Mainz, Germany) was fitted between the LS50 lamp and the sample to avoid HeNe laser light entering the reference PMT. In order to eliminate scattered HeNe laser light from the fluorescence signal, a Schott BG3 bandpass filter was placed between the sample and the detector. The cuvette holder was equipped with a PTP-1 Peltier element (Perkin Elmer) for the regulation of the sample temperature and to eliminate temperature fluctuations. The Peltier element was set to 4 °C for the main part of the fluorescence experiments, but a series of measurements was also performed at 22 °C (including the fluorescence experiments with ATP–Mg complexes).

For the luciferin–luciferase kinetics experiments as described in Sections 2.7–2.9, the light emitted during the luciferine–luciferase reaction was quantitated on a LS50B luminescence spectrometer in the time-based acquisition (bioluminescence) mode using a data interval of 2.0 s and a 1.0 s signal integration time for a total duration of 60 s. The Peltier element was set to 25.9 °C [2]. The spectrofluorometer lamp was switched off permanently and the emission wavelength was set to the bioluminescence maximum, i.e., to 562 nm (±20 nm). For the experiment described in Section 2.9, a Corion bandpass filter (Corion p70-550-S-4483-B352, Newport Corporation, Irvine, CA) was placed as emission filter to eliminate scattered HeNe laser light from the oxyluciferin luminescence signal.

2.5. Quantification of the intrinsic fluorescence of ATP in combination with NROF irradiation

In this series of experiments, the fluorescence emission of non-complexed ATP was quantified for a total duration of 60 min under non-irradiated and NROF-irradiated (632.8 nm) conditions, as described in Sections 2.3 and 2.4. ATP stock solutions of 1.0 mM in MilliQ at increasing pH levels were prepared fresh every day and further diluted in a 1 × 1 cm quartz cuvette prior to each measurement at concentrations as described below. The solutions were incubated at the target temperature (4 °C or 22 °C) for 5 min before initializing the experiment. In order to minimize experimental artifacts (slow drift), the non-irradiated and NROF-irradiated experiments were carried out alternately. To eliminate

small differences in ATP concentration, all fluorescence data points were normalized to the fluorescence intensity at $t = 0$ min. Relative fluorescence intensities are presented as mean ± SD of $n = 10$ experiments.

To investigate the influence of pH on ATP fluorescence in the absence or after NROF irradiation, the fluorescence experiments were performed at pH = 2.5, 5.2, and 10.8. In order to eliminate pH differences between the ATP solutions within the same experimental group, a stock solution of MilliQ was prepared at the desired pH prior to dissolving the solute and stored at the target temperature. The stock solution of MilliQ was employed for both the preparation of 1.0 mM ATP stock solutions and for further dilutions of the ATP stock solution.

The samples at low pH were prepared by adjusting the pH of 1.0 L MilliQ by the addition of 830 μL of 37 wt.% HCl (pH = 2.08), resulting in a 20 μM ATP solution (after sample preparation and dilution) with a pH of 2.5 at a temperature of 4 °C. Because the fluorescence quantum yield is significantly higher at low pH [5], the ATP concentration was twofold lower compared to the experiments at higher pH values in order to avoid detector saturation at low pH levels. The 40 μM ATP solutions at pH = 5.2 were prepared by using MilliQ without pH adjustments, whereas the 40 μM ATP solutions at pH = 10.8 were prepared from MilliQ to which 12.65 mg NaOH (per L MilliQ) had been added.

Additionally, the light emission of Mg-chelated ATP was investigated in the absence or during NROF irradiation (632.8 nm) by quantifying the intrinsic ATP fluorescence as described in Sections 2.3 and 2.4. ATP–Mg solutions were prepared in MilliQ at an ATP concentration of 40 μM and Mg^{2+} concentrations of 40 μM and 400 μM. Fluorescence intensities were determined by calculation of the area under the emission curve between 310 and 490 nm and are presented in box plots comprising the mean fluorescence intensity, the range of the intensities, and the standard deviation of $n = 5$ experiments. The non-irradiated and NROF-irradiated experiments were carried out alternately at 22 °C.

2.6. The luciferin–luciferase reaction

Luciferase catalyzes the oxidation of luciferin into a triplet state (T_1)-excited oxyluciferin product which then decays to the ground state with the emission of yellow-green light [11–13]. The energy for the reaction is provided by ATP as it is converted to AMP:

$$\text{ATP} + \text{luciferin} \overset{\text{Firefly Luciferase}}{\underset{Mg^{2+}}{\rightleftarrows}} \text{adenyl-luciferin} + PP_i \quad (1)$$

$$\text{adenyl-luciferin} + O_2 \rightarrow \text{oxyluciferin} + \text{AMP} + CO_2 + h\nu \quad (2)$$

The formation of the enzyme-bound adenyl-luciferin in reaction (1) is a reversible process and the equilibrium lies far to the right, while reaction (2) is essentially irreversible [14,15]. If the enzymatic reaction is started by the addition of excess ATP to a solution containing luciferase and saturating amounts of the other reaction components, a strong bioluminescence signal is obtained that decays within ~10 s to a low level of light production. This continuous low production of light persists for a relatively long time [16]. It has been shown that the intensity of the initial bioluminescence signal and the decay rate increase significantly at higher ATP concentrations [11,16]. These observations are attributable to the existence of two distinguishable kinetic pathways due to the presence of two catalytically active ATP binding sites of luciferase, i.e., a low affinity one that is rapidly inhibited by the product after a single catalytic event and hence inactivated after the initial flash of light, and a higher affinity site that catalyzes the continuous low production of light [11,17–21].

The extreme specificity of luciferase for ATP [22] allows the investigation of slight modifications of the biochemical behavior of ATP by means of the luciferin–luciferase reaction. In the present work, the influence of ATP irradiation with NROF on the rate of the luciferin–luciferase reaction was studied by measuring the emission intensity luminometrically with and without NROF-irradiation of ATP, the assay solution without ATP, or the entire reaction mixture. The rate of product formation was derived from analysis of the time course of the initial flash light emission rather than the luminescence peak areas. Because every catalytic event results in the emission of a photon, an accelerated reaction would not alter the area under the luminescence curve as the total number of photons that are finally emitted are not influenced. In contrast, enhanced reaction kinetics yield significantly increased initial light intensities and increased decay rates, resulting in an accelerated extinction of light as the rate of photon emission by the electronically excited oxyluciferin product increases. However, for completeness, both the time courses of the initial flash light emission and the peak areas under the luminescence curve are shown.

The optimal ATP concentration as used in the kinetic experiments was determined on the basis of a maximum initial bioluminescence intensity and a quantifiable decay rate. As described above, it has been shown previously [11,16] that the time course of light emission observed after initiation of the reaction by the addition of excess ATP is strongly influenced by the ATP concentration. At higher concentrations, the intensity of the initial flash light and its decay rate increase significantly [16], resulting in a greater peak amplitude and slope [11,16]. Enhanced reaction kinetics would result in a different bioluminescence trace with a greater peak amplitude and slope. In light of these considerations, the ATP concentration was chosen such that the amplitude of the initial bioluminescence peak and its decay rate were maximal. This allowed optimal quantification of the differences between the luciferin–luciferase reaction kinetics of non-irradiated and NROF-irradiated experimental groups.

The time course of light emission was continuously monitored by the spectrofluorometer immediately after all reagents were added. The measured luminescence intensities were normalized by dividing all data points by the intensity at $t = 60$ s. The data points of the luminescence time course are presented as mean ± SD of $n = 5$ experiments for each experimental group. The areas under the luminescence curves were obtained by determining the integral of all individual measured time courses of light emission ($n = 5$) in Origin, after which the average value and standard deviation of the individual peak areas were determined. Peak areas are presented as mean ± SD of $n = 5$ experiments for each experimental group.

2.7. Protocol luciferin–luciferase reaction in combination with NROF irradiation of ATP

In this series of experiments ATP was NROF-irradiated (514.5 nm or 632.8 nm) before the reaction was initiated. An ATP solution was prepared at a concentration of 0.50 mM in MilliQ and 1.0 mL was transferred to a 5 × 10 mm quartz cuvette before initiation of the reaction. The ATP solution was kept at 37 °C [2] for 15 min under either non-irradiated or NROF-irradiated conditions (Section 2.3). Non-irradiated ATP controls were kept in the dark in order to rule out ambient light effects. The end of the incubation time of the ATP solution coincided with the end of the incubation time of the assay solution as described below.

A 2 × 10 mm quartz cuvette containing 200 μL of the assay solution (25-fold dilution, Section 2.1), was incubated for 5 min at 25.9 °C [2] and placed in the cuvette holder of the spectrofluorometer directly before initiation of the reaction. The reaction was started by manual injection of 200 μL of the 0.50 mM ATP solution. The final concentrations of the reaction components were a 50-fold final dilution of the assay solution and 0.25 mM ATP.

Ten measurements were carried out with non-irradiated ATP, five with NROF-irradiated ATP at 514.5 nm, and five with NROF-irradiated ATP at 632.8 nm. Non-irradiated and NROF-irradiated experiments were performed alternately to minimize experimental artifacts. To exclude background light emission, blank measurements were performed by the addition of 200 μL of MilliQ instead of ATP under similar experimental conditions. In order to eliminate the effects of the described reactivity in time [23], the signal was normalized to the luminescence intensity at $t = 60$ s. All data are presented as mean ± SD.

2.8. Protocol luciferin–luciferase reaction in combination with NROF irradiation of the assay solution

In this series of experiments the assay solution was NROF-irradiated (514.5 nm or 632.8 nm) separately before the reaction was initiated. The assay solution consisted of a 25-fold dilution of the ATP Assay Mix stock solution at pH = 7.8 (Section 2.1). A 200 μL aliquot of the assay solution was transferred to a 0.2 × 1.0 cm quartz cuvette and kept for 10 min at 25.9 °C in the cuvette holder of the spectrofluorometer under either non-irradiated or NROF-irradiated conditions (Section 2.3). The non-irradiated assay solutions were kept in the dark in order to rule out ambient light effects. The end of the incubation time of the assay solution coincided with the end of the incubation time of the 0.50 mM ATP solution that had been kept at 25.9 °C for 5 min. The reaction was initiated by the addition of 200 μL of the 0.50 mM ATP solution during luminescence acquisition. Other features of the analytical procedure, including sample handling, sample size, and the final concentrations, are detailed in Section 2.7.

2.9. Protocol luciferin–luciferase reaction in combination with NROF irradiation of the reaction mixture

In this series of experiments the entire reaction mixture was irradiated with NROF (632.8 nm) during the progression of the reaction. A 200 μL aliquot of the assay solution was transferred to a 0.2 × 1.0 cm quartz cuvette and kept for 10 min at 25.9 °C in the cuvette holder of the spectrofluorometer. The laser was switched on directly before the initiation of the reaction by the addition of 200 μL of the 0.50 mM ATP solution or remained off during the non-irradiated experiments. Other features of the experimental procedure, including sample handling, sample size, and the final concentrations, are detailed in Section 2.7.

2.10. Reaction rate calculations and statistical analysis

The reaction rates, k_1 and k_2, were calculated in Origin after luminescence background subtraction. The luminescence decay traces were fitted with a second order exponential function, from which the values of k_1 and k_2 were derived.

Differences between ordinal variables were analyzed with an unpaired, homoscedastic student's t-test when the data were normally distributed or with a Mann–Whitney test for non-Gaussian data sets. Normality was tested with a D'Agostino & Pearson omnibus normality test. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Absorption, excitation, and emission spectra of ATP

The absorption and fluorescence excitation and emission spectra of ATP in aqueous solvent were normalized to maximum absorbance and emission intensity, respectively, and are presented in Fig. 2. ATP exhibited absorption peaks at 207 nm and 259 nm that

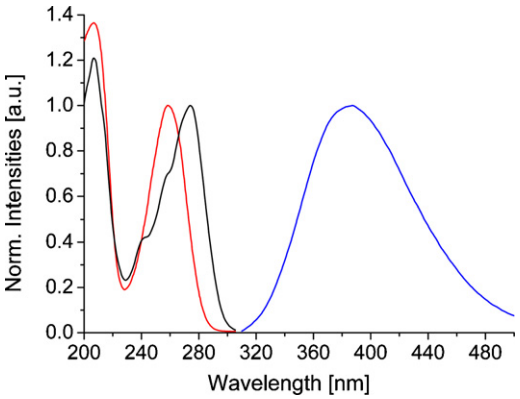

Fig. 2. Absorption (—), excitation (—), and emission (—) spectra of ATP in MilliQ (40 μM). The spectra were normalized to maximum values in the 230–500 nm range to enable comparison of absorption and excitation spectra. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

are ascribable to the adenine moiety [24]. Fluorescence excitation maxima were observed at 207 nm and 275 nm. ATP shows fluorescence in the 310–500 nm region when excited at 275 nm, with an emission maximum at 384 nm. The shape of the emission spectrum was not altered when ATP was excited at its absorption maximum (259 nm). However, the relative intensity was significantly lower at the latter wavelength.

The absorption maximum (259 nm) was significantly blue-shifted compared to the excitation maximum (275 nm). A possible explanation for this discrepancy is the coexistence of two different tautomeric species of ATP; an abundant population of ATP tautomers with a low quantum yield (i.e., high absorption peak vs. negligible excitation peak) and a very small but highly fluorescent population of ATP tautomers (i.e., negligible absorption peak vs. high excitation peak) [5]. For this reason, the series of experiments regarding NROF-induced effects on the intrinsic fluorescence of ATP (Section 3.2) were performed at an excitation wavelength of 259 and 275 nm.

3.2. Influence of NROF irradiation on the intrinsic fluorescence of ATP and hydrolysis rate

Amat et al. [3] reported NROF-induced increases in the fluorescence emission intensity ($\lambda_{ex} = 260$ nm) of ATP–Mg complexes, but not in non-complexed ATP. It was postulated that the NROF-induced electric field produces charge displacement in the phosphate tail that alters the strength of the Mg^{2+} binding, which consequently leads to enhanced fluorescence intensities [3]. In this study, a series of experiments was performed to further investigate the fluorescence of non-complexed and Mg-chelated ATP under non-irradiated and NROF-irradiated conditions.

The results (Fig. 3) demonstrate that no differences were observed in the intrinsic fluorescence intensity of non-irradiated and NROF-irradiated non-complexed ATP at 4 °C ($p > 0.05$). At low pH (Fig. 3A and B), ATP fluorescence exhibited a slight fluorescence decay that was not observed under non-adjusted pH conditions (Fig. 3C and D). At high pH (data not shown), ATP emitted virtually no fluorescence. The NROF measurements at low pH were repeated at 22 °C (data not shown), but no significant differences in peak positions, peak intensities, or fluorescence kinetics were observed compared to the measurements performed at 4 °C.

The addition of Mg^{2+} to the ATP solutions resulted in a relative decrease of intrinsic ATP fluorescence in a Mg^{2+}-concentration dependent manner (Fig. 4), as reported previously [3]. However, the results presented in this study demonstrate that there is no

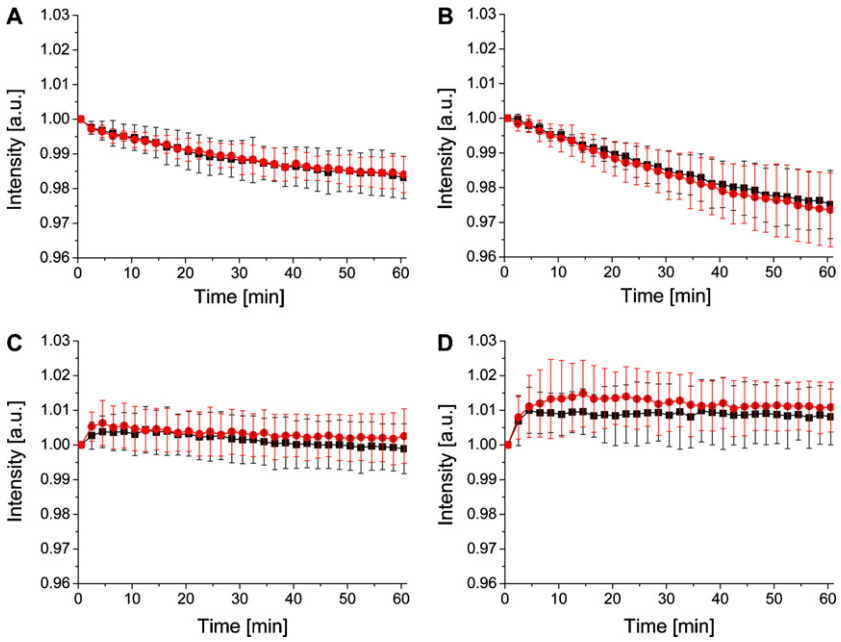

Fig. 3. Fluorescence intensities of (■) non-irradiated and (●) NROF-irradiated (632.8 nm) non-complexed ATP at (A) low pH while excited at 275 nm, (B) low pH while excited at 259 nm, (C) non-adjusted pH while excited at 275 nm, and (D) non-adjusted pH while excited at 259 nm. All fluorescence intensities were normalized to the intensity at $t = 0$ min and are presented as mean ± SD of $n = 10$ experiments per group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

significant difference in ATP fluorescence between the NROF-irradiated and the non-irradiated samples when excited with 260 nm light at 22 °C ($p > 0.05$).

3.3. Absorption spectra of the individual luciferin-luciferase reaction components

In order to ascertain that 514.5-nm and 632.8-nm irradiation could be classified as NROF, the UV and visible absorption spectra of the individual reaction components, i.e., ATP and the assay solution containing luciferin and luciferase, were acquired at spectrophotometrically justifiable solute concentrations at which the absorption peaks in the UV range could be optimally measured and at concentrations as used in the kinetics experiments (Fig. 5). ATP exhibited absorption below 260 nm, whereas the assay solution showed notable absorption below 250 nm and minor peaks at 280 nm and 325 nm. Absorption in the visible spectrum was negligible for all compounds, confirming that irradiation of the compounds with 514.5-nm and 632.8-nm laser light can be classified as NROF-irradiation.

3.4. Reaction kinetics of the ATP induced luciferin–luciferase reaction

The kinetics experiments were performed by the addition of excess ATP, either non-irradiated or NROF-irradiated, to a solution containing luciferase and the other reaction components at saturated concentrations.

3.4.1. Reaction kinetics of NROF-irradiated ATP

Fig. 6 depicts the luminescence emission traces and the areas under the curve following addition of non-irradiated or NROF-irradiated (514.5 nm and 632.8 nm) ATP to the assay solution. Irradiation of ATP with NROF did not affect the time course of light emission or the area under the curve compared to the non-irradiated (control) group ($p > 0.05$), indicating that NROF-irradiation of ATP did not speed up the reaction kinetics.

3.4.2. Reaction kinetics of the NROF-irradiated assay solution

NROF irradiation (514.5 nm and 632.8 nm) of the assay solution was performed separately before initiation of the reaction to determine whether NROF could affect the reaction kinetics via components other than ATP (Fig. 7). No significant differences were observed in luminescence decay between non-irradiated and NROF groups at either wavelength ($p > 0.05$), demonstrating that NROF-irradiation of the assay solution did not modify the reaction kinetics.

3.4.3. Reaction kinetics of the NROF-irradiated reaction mixture

As a final test to rule out the effect of NROF-irradiation on the luciferin–luciferase reaction, the entire reaction mixture was subjected to NROF irradiation for the total duration of the reaction. By

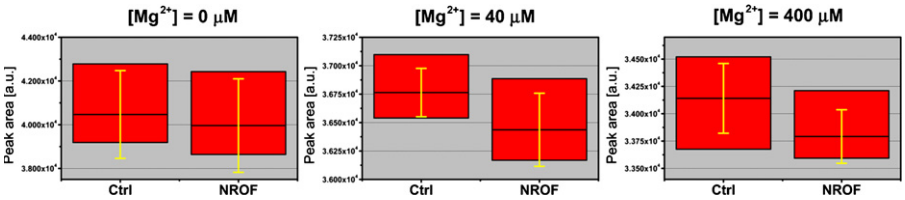

Fig. 4. Fluorescence intensities of non-irradiated and NROF-irradiated (632.8 nm) ATP–Mg complexes at different concentrations of Mg^{2+} when excited at 260 nm. The box plots present the mean fluorescence intensity (central line of bar), the range of the reaction rates (maximum, top line bar; minimum, bottom line of bar), and standard deviations (error bars) of $n = 5$ experiments.

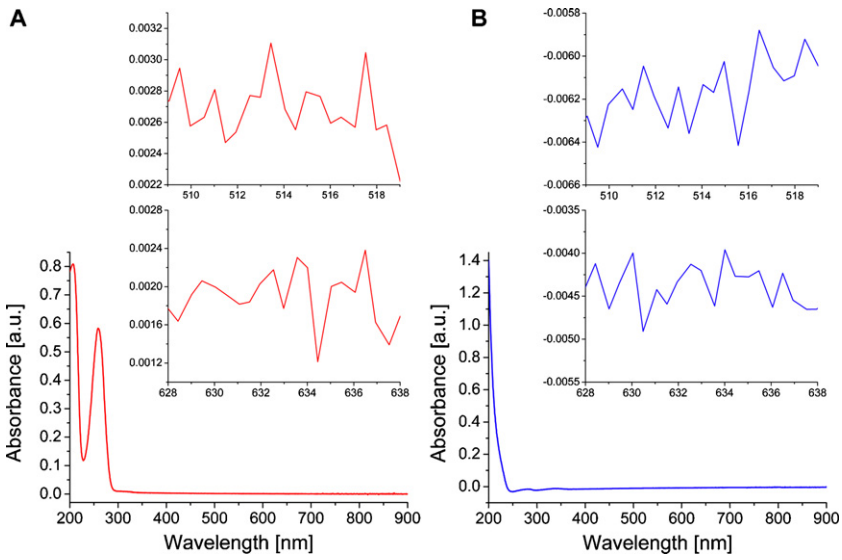

Fig. 5. UV and visible absorption spectra of (A) ATP, and (B) the assay solution containing luciferin and luciferase. The complete, background-subtracted absorption spectra were obtained at spectrophotometrically justifiable solute concentrations, whereas the insert provides delimited absorption spectra of the compounds in the NROF wavelength ranges at the concentration used in the reaction mixture.

these means, any NROF-induced effect resulting from the interplay of multiple NROF-irradiated reaction components could be investigated.

The time course of light emission and the corresponding areas under the luminescence curves for non-irradiated and NROF-irradiated (632.8 nm) reaction mixtures are presented in Fig. 8. Irradiation of the reaction mixture during the progression of the reaction did not affect the time course of light emission or the area under the curve ($p > 0.05$), indicating that NROF-induced alterations of the reaction kinetics are absent at every level of the luciferin-luciferase reaction.

4. Discussion

4.1. The intrinsic fluorescence of ATP during NROF irradiation

The light emission observed when non-complexed ATP was excited at 259 nm or 275 nm during laser-irradiation with NROF (632.8 nm) was not significantly different compared to non-irradiated ATP, demonstrating that the intrinsic fluorescence emission intensity of ATP is not altered by laser-irradiation with NROF. Additionally, our results demonstrate that NROF irradiation (632.8 nm) of Mg-chelated ATP, when excited at 260 nm, did

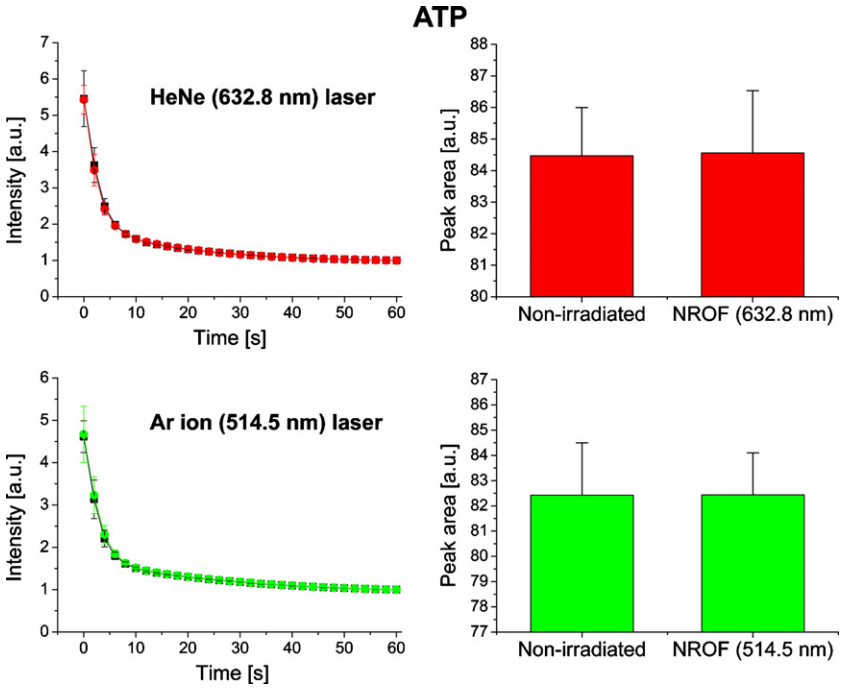

Fig. 6. Time course of light emission fitted with a second order exponential fit after the addition of (■) non-irradiated ($R^2 = 0.9936$), (●) NROF-irradiated (632.8 nm) ($R^2 = 0.9989$), and (●) NROF-irradiated (514.5 nm) ($R^2 = 0.9999$) ATP to the reaction mixture. All luminescence intensities were normalized by dividing the data points by the intensity at $t = 60$ s and are presented as mean ± SD of $n = 5$ experiments. The bar charts show the mean ± SD area under the normalized luminescence curves. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

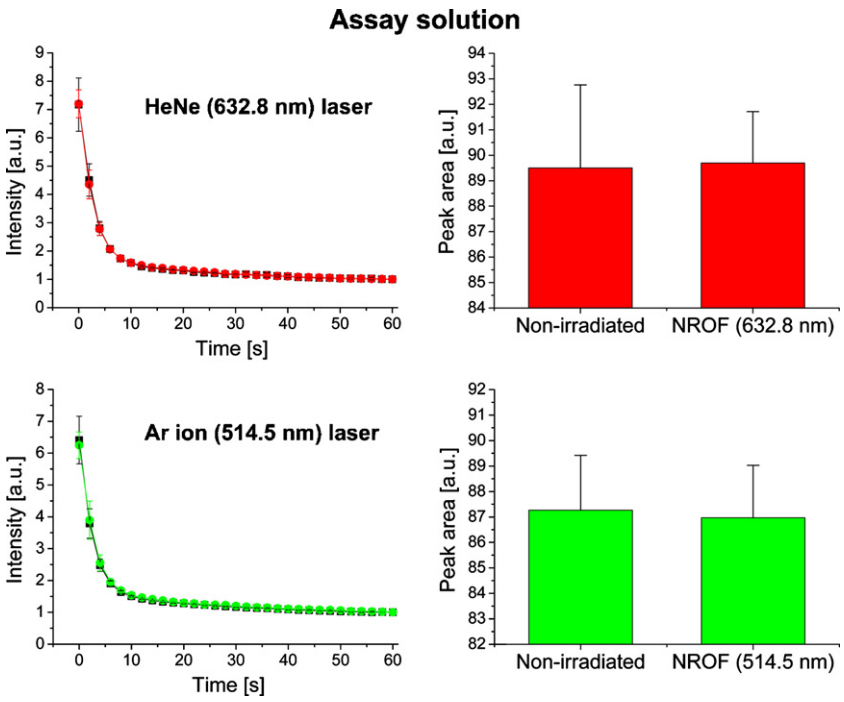

Fig. 7. Time course of light emission fitted with a second order exponential fit after the assay solution was (■) non-irradiated (R^2 = 0.9971), (●) NROF-irradiated at 632.8 nm (R^2 = 0.9983), and (●) NROF-irradiated at 514.5 nm (R^2 = 0.9997), before the reaction was initiated by the addition of ATP. All luminescence intensities were normalized by dividing the data points by the intensity at t = 60 s and are presented as mean ± SD of n = 5 experiments. The bar charts show the mean ± SD area under the normalized luminescence curves. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

not result in an enhanced ATP fluorescence, even at significantly higher incident NROF irradiances and more energetically NROF wavelengths than used in [3].

The slow decay of the intrinsic fluorescence in time observed for non-complexed ATP at low pH might be related to spontaneous hydrolysis, as it is known from literature that the hydrolysis rate of ATP to ADP, AMP and inorganic phosphate is strongly pH-dependent [25] and significantly increases at decreasing pH levels [26,27]. This would result in lower fluorescence emission intensities in time due the relatively lower fluorescence quantum yields of ADP and AMP [5].

Amat et al. [3] reported NROF-induced increases in the fluorescence intensity of ATP–Mg complexes (λ_{ex} = 260 nm) and proposed that irradiation with NROF can induce modifications of electronic transition states when Mg^{2+} is chelated to ATP's terminal phosphate [3,4]. Due to the prominent impact of the phosphate tail on the intrinsic fluorescence of ATP [3,5], any charge displacement would be revealed directly by changes in fluorescence. However, in this study, no evidence was found that irradiation with NROF affects the light emission of ATP by alterations in molecular/electronic constituents of ATP–Mg complexes.

Conclusively, the results presented in this study unequivocally demonstrate that excitation of ATP during concomitant laser-irradiation with NROF (632.8 nm) did not modify the intrinsic fluorescence properties of both non-complexed and Mg-chelated ATP and that the assumptions related to NROF-induced charge displacements in the phosphate tail, as hypothesized in [2–4], could not be confirmed even though higher irradiation intensities were used.

4.2. Luciferin–luciferase reaction kinetics

The kinetics experiments as presented in this study demonstrated that the luciferin-luciferase reaction rates associated with

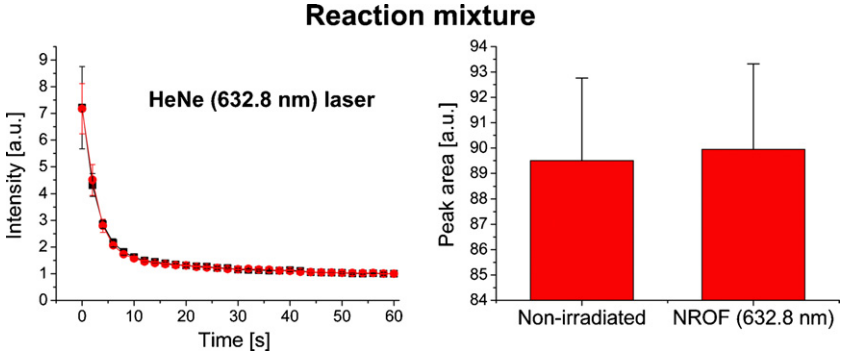

Fig. 8. Time course of light emission fitted with a second order exponential fit of the (■) non-irradiated (R^2 = 0.9971) and (●) NROF-irradiated (632.8 nm) (R^2 = 0.9987) reaction mixture. All luminescence intensities were normalized by dividing the data points by the intensity at t = 60 s and are presented as mean ± SD of n = 5 experiments. The bar charts show the mean ± SD area under the normalized luminescence curves. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the oxidation of luciferin into an electronically excited oxyluciferin product catalyzed by luciferase were not accelerated by NROF irradiation, even when more energetically NROF wavelengths and significantly higher incident irradiances were employed than used in [2]. NROF-induced influences on the luciferin–luciferase reaction kinetics were absent in all experimental groups, i.e., individual NROF irradiation of ATP or the enzyme–substrate mixture before initiation of the reaction as well as NROF irradiation of the entire reaction mixture during the progression of the reaction.

These results are inconsistent with those published by Amat et al. [2], who observed a significant increase in initial emission peak amplitude but simultaneously a significant increase in the area under the luminescence curve in consequence to NROF irradiation of ATP before initiation of the luciferin-luciferase reaction. However, the combination of an increased peak amplitude and an increased luminescence peak area reflects errors in the experimental procedure rather than an altered reaction rate. The reaction rate is derived from the initial light intensity (peak amplitude) and the slope of light extinction curves (decay rate). Higher reaction rates exhibit a greater peak amplitude and slope, whereby the luminescence trace is rather short-tailed. Contrastingly, slower reaction rates produce a lower peak amplitude and slope, as a result of which the luminescence trace is rather tapered. The area under the luminescence curve, however, should at all times remain constant when the concentration of reagents is unaltered. Consequently, the conclusions drawn in [2] are, in our opinion, not valid because they are based on experiments in which the concentration of reagents, and thus the area under the luminescence curve, was incongruent between measurements.

On the other hand, the data presented in this paper support the conclusions derived in a previous study [7], in which no evidence was found for NROF-induced accelerated reaction rates associated with the conversion of glucose to glucose-6-phosphate by hexokinase.

5. Conclusion

This work has demonstrated that neither the intrinsic fluorescence of non-complexed and Mg-chelated ATP nor its biochemical behavior during the luciferin–luciferase reaction are affected by laser irradiation with NROF. Our previous work [7] and the present study hence disprove the hypothesis that NROF irradiation accelerates reaction kinetics by lowering the activation energy for the dephosphorylation of ATP's terminal phosphate in two ATP-driven enzyme systems.

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