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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