Journal of Photochemistry and Photobiology A: Chemistry 213 (2010) 147–151

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology A: Chemistry

journal homepage: www.elsevier.com/locate/jphotochem

Use of cyclodextrins as scavengers of inhibitory photo-products in light controlled *in vitro* synthesis of RNA

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ARTICLE INFO

Article history:
Received 22 March 2010
Received in revised form 14 May 2010
Accepted 23 May 2010
Available online 31 May 2010

Keywords:
Caged nucleotides
Coumarin
In vitro transcription
Cyclodextrin
RNA

ABSTRACT

We recently reported on the use of caged nucleotides to attain full control of enzymatic polymerization of RNA solely by light. In the absence of light no RNA formation was possible due to the efficient caging by the coumarin moiety; after irradiation, caged ATP was released with quantitative precision and RNA polymerization was resumed. As photolabile protecting group [7-(diethylamino)coumarin-4-yl]methyl] (DEACM) was used due to its high absorbance in the visible region of the spectrum, fast deprotection kinetics and absence of radical intermediates. However, the 7-diethylamino-4-hydroxymethylcoumarin photo-product (DEACM-OH) was shown to inhibit the transcription reaction for concentrations higher than 30 μM [5]. This inhibition has been associated with poor water solubility, which is commonly dealt with via cumbersome chemical modifications of the protecting moiety. To overcome inhibition, we evaluated the use of molecular scavengers to sequester DEACM-OH formed after irradiation. Determination of association constants of coumarin with β-cyclodextrins allowed the assessment of its capability to remove free coumarin molecules from solution. The influence of β-cyclodextrin in transcription reaction was also assessed. Results show that β-cyclodextrin can be successfully used as scavenger as it increases the DEACM-OH threshold concentration for inhibition, amplifying the efficiency of light controlled *in vitro* transcription.

1. Introduction

Controlled temporal and spatial release of biomolecules from photolabile precursors, commonly known as *caged* molecules, is of extreme relevance as a tool for bio-molecular studies [1–4]. The cage (chemical modification with a photolabile protecting group) renders the molecule of interest biologically inactive; upon irradiation with light of a suitable wavelength, the biologically active molecule is released, generating a time-controlled burst in concentration with tight spatial control. For this purpose, the selected cage molecule ought to fulfill several critical conditions: (i) the efficiency of uncaging should be high in order to avoid long irradiation times and deleterious effects to the biological samples; (ii) the rate of uncaging must be faster than the process under study, i.e. the rate at which the biomolecule is released should not be the rate limiting step; (iii) easy to synthesize; and (iv) the resulting by-products should not hamper the desired reaction.

We have previously described the use of ATP nucleotides caged with [7-(diethylamino)coumarin-4-yl]methyl (DEACM-ATP) for light-controlled *in vitro* transcription reactions, where the quantity of RNA being polymerized could be controlled through DEACM-ATP irradiation [5]. Coumarin derivatives present high molar absorption coefficients, high photochemical quantum yields and absorptions in the visible region of the spectrum (<420 nm), with the advantage of fast photocleavage kinetics and wavelength tunability through changes in the position and/or nature of the chemical residues attached to the coumarin moiety [6–9]. In most aspects, coumarin cages, and in particular DEACM, follow the above mentioned selection requirements. However, following irradiation, high concentrations of photo-by-product 7-diethylamino-4-hydroxymethylcoumarin (DEACM-OH) inhibited the transcription reaction [5]. This effect was attributed to the poor coumarin solubility in water leading to the partitioning of the hydrophobic DEACM-OH molecule to the T7 RNA Polymerase, causing inhibition. To circumvent the solubility issue, hydroxyl or acetate groups can be added to the coumarin moiety that due to their anionic characteristics at physiological pH increases considerably the coumarin water solubility [10,11]. Although it might constitute a robust process of reducing the inhibition, it requires additional synthesis steps, on top of an already complex synthetic route.

Here, we present an alternative supramolecular approach to decrease the inhibitory effect of water insoluble DEACM photo-by-products in enzymatic reactions – using β-cyclodextrin molecules

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doi:10.1016/j.jphotochem.2010.05.014

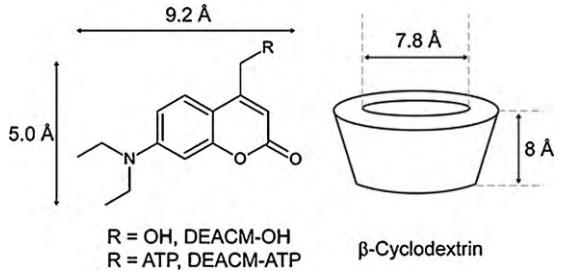

Fig. 1. DEACM photo-by-products (DEACM-OH and DEACM-ATP) and β-cyclodextrin. Potentially, the β-cyclodextrin has the ability to include molecules of organic compounds, such as the coumarin rings of DEACM products, into its hydrophobic cavity. The β-cyclodextrin cavity [12,13] has a 7.8 Å diameter that is suitable to accommodate the complexation of the DEACM along its long axis (diameter 5 Å). Due to the larger long axis size of the coumarin ring (9.2 Å *vs.* 8 Å), and the presence of the –OH or –ATP moieties, it is expected the formation of a 1:1 complex where the 7-diethylamino benzene moiety is inside the cyclodextrin cavity.

as molecular scavengers (see Fig. 1). β-Cyclodextrin is a cyclic oligosaccharide composed by 7 α-D-glucopyranose units, forming a hydrophobic cavity that is suitable for complexation of hydrophobic small molecules [12].

Using cyclodextrins in the reaction mixture, we were able to increase the concentration of released substrate (ATP), while scavenging the DEACM-OH photo-product generated after DEACM-ATP photocleavage. When added to the light-controlled transcription reaction, it was possible to channel the partitioning of DEACM-OH molecules into the formation of coumarin–cyclodextrin complex, thus reducing inhibition. We believe that this strategy can be easily extended to other coumarin derivatives, and any other similar compound for that matter, for *in vitro* reactions, i.e. reducing photo-product inhibition due to poor water solubility without further synthesis steps.

2. Materials and methods

2.1. General information

All chemicals were purchased from Sigma–Aldrich in the highest purity available and used without further purification. T7 RNA Polymerase was purchased from Fermentas (Vilnius, Lithuania). All oligonucleotides were purchased from STAB Vida (Lisbon, Portugal). Irradiations were performed in a monochromated (model 1681 0.22 m monochromator included in a SPEX Fluorolog spectrofluorimeter) 150 W Xe lamp at 390 nm, with a bandpass of 15 nm. A Hitachi-Merck HPLC L6200A Pump with an L-4500 Diode Array Detector using a RP-18 end-capped (Purospher Star, Merck) analytical column (4.6 mm × 15 mm, 5 μm) was employed for DEACM-ATP and DEACM-OH detection in photolysis and photochemical quantum yield determinations. Eluent A was triethylammonium acetate buffer in water, 5 mM, pH 6.9; eluent B was methanol. The gradient used started with 35% of B in A, from 0 min to 3 min; with an increase to 100% B after 6 min, and finished after 15 min at 100% of B. Separations were run at a flow rate of 0.9 mL/min and the column temperature was 35 °C. All spectroscopic measurements and irradiations were performed in 3 mL quartz fluorescence cuvettes (1 cm optical path) at 21 °C. Absorption spectra were recorded on a Varian Cary Bio 100 UV-Visible spectrophotometer. Fluorescence measurements of aerated solutions were performed on a Horiba-Jobin-Yvon SPEX Fluorolog 3.22 spectrofluorimeter. All emission spectra were collected with 1.5 nm slit bandwidth for excitation and emission, with correction files.

β-Cyclodextrin was chosen due to the proximity between its internal cavity diameter (7.8 Å internal diameter; 8 Å height [12,13]) and the dimensions of the coumarin (5.0 Å short axis, 9.2 Å long axis), allowing the formation of a 1:1 complex (Fig. 1).

2.2. DEACM derivatives: synthesis and purification

P3-[7-(diethylamino)coumarin-4-yl]methyl adenosine 5′-triphosphate trisodium salt (DEACM-ATP) was synthesized as described by Geiβler et al., method B [7]. 7-Diethylamino-4-hydroxymethylcoumarin (DEACM-OH) was synthesized and purified as described by Schönleber et al. [14]. A Hitachi-Merck HPLC L6200A Pump with an L-4500 Diode Array Detector using a Polystyrene-Divinylbenzene (PLRP-S, Polymer Labs, Germany) semi-preparative column (7.4 mm × 15 mm, 8 μm, 300 Å) was employed for separation and purification of DEACM-ATP. Eluent A was triethylammonium acetate buffer in water, 5 mM, pH 6.9; eluent B was methanol. Gradient started with 20 min at 30% of B in A; with an increase to 100% B after 21 min, and finished after 26 min at 100% of B. Separations were run at a flow rate of 3 mL/min and the column temperature was 35 °C. After peak separation and collection, samples were lyophilized, re-suspended in water and stored in the dark at −20 °C. A purity of >95% was determined by HPLC. All solutions were protected from light and DEACM-ATP manipulations were made in a dark chamber under red-light illumination.

2.3. Association constant determinations

The absorption and emission spectra of a 30 μM DEACM-OH solution in transcription buffer (50 mM Tris–HCl, 6 mM $MgCl_2$, 10 mM Dithiothreitol (DTT), 30 mM NaCl and 2 mM spermidine) were measured after the addition of increasing β-cyclodextrin quantity at 37 °C. A 10 min equilibration period was used between each cyclodextrin addition and measurement cycle. Emission at 570 nm was corrected for absorption/dilution variation and plotted as function of β-cyclodextrin concentration. Emission at 570 nm was chosen to minimize interference from the DEACM-OH/cyclodextrin complex, i.e. at this wavelength emission is almost only due to the free DEACM-OH. Using Valeur's model [15] and a non-linear least square fit, the association constant was determined (see Section 3 for details).

2.4. In vitro transcription reactions

In vitro RNA synthesis was performed using 400 ng of a 130 bp DNA template and 10 U of T7 RNA Polymerase (Fermentas) according to the manufacturer's protocol. In brief, reactions were carried out in a volume of 25 μl containing *in vitro* transcription buffer (50 mM Tris–HCl, 6 mM $MgCl_2$, 10 mM DTT, 30 mM NaCl and 2 mM spermidine), 50 μM of each NTP, template DNA and T7 RNA polymerase. As DNA template a T7 promoter coupled to the human p53—exon 7 was used [5]. The reaction mixtures were incubated at 37 °C for 60 min, followed by heat inactivation of the enzyme for 15 min, at 75 °C. For DEACM-OH inhibition of transcription, increasing concentrations of DEACM-OH (water solution) were used to induce decrease of transcription. For DEACM-OH inhibition suppression, *in vitro* transcription reactions were performed with increasing amounts of DEACM-OH, in the presence of 500 μM of β-cyclodextrin.

All transcription reaction products were analyzed in 3% agarose gels (TBE) with GelRed™ (Biotium, Hayward, CA, USA) staining. Determination of the 130 bp transcription product quantity was performed by pixel intensity/counting using Adobe Photoshop™ imaging software.