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## Biosynthetic incorporation of the azulene moiety in proteins with high efficiency

Jinfeng Shao · Ivan V. Korendovych · Jaap Broos

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**Abstract** Biosynthetic incorporation of  $\beta$ -(1-azulenyl)-L-alanine, an isostere of tryptophan, is reported using a tryptophan auxotroph expression host. The azulene moiety introduced this way in proteins features many attractive spectroscopic properties, particularly suitable for in vivo studies.

**Keywords** Tryptophan analog · Spectroscopic probe · Tryptophanyl-tRNA synthetase · Fluorescence · *Lactococcus lactis*

Tryptophan (Trp) is one of the most popular fluorescent probes in the life sciences because it is an intrinsic fluorophore that is highly sensitive to structural changes in its microenvironment. Often a wild-type protein can be used for investigating protein conformational changes, folding or unfolding processes or its interactions with ligands or other proteins. However, obtaining site-specific information requires proteins that contain only a single Trp residue in most cases.

Trp has also some disadvantages as a spectroscopic probe; its photostability is limited and its photophysics are complex due to a relatively low ionization potential (IP) and spectral overlap of the two lowest excited states,  $^1L_a$  and  $^1L_b$  (Callis 1997, 2009). Trp needs to be excited

by UV light with wavelengths less than 300 nm, which interferes with other chromophores often present in a biological sample.

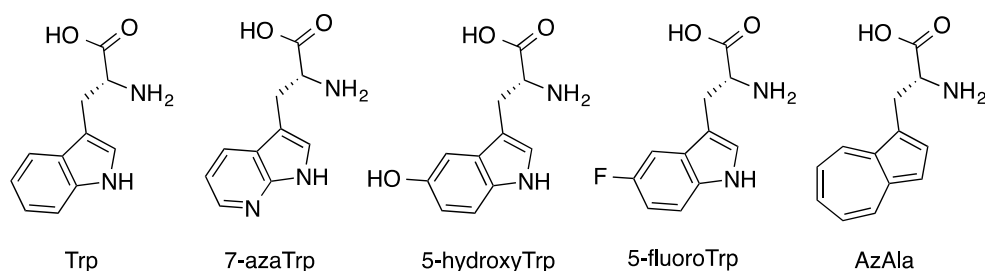
Replacing Trp by a Trp analog, which can be biosynthetically introduced in a protein, offers a number of practical advantages for biochemical and biophysical studies (Ross et al. 1997; Twine and Szabo 2003b; Broos 2014) (Scheme 1). For example, replacing Trp by 5-fluoroTrp (5-FTrp) creates in most cases a protein showing a 50 % higher quantum yield and a monoexponential fluorescence decay (Broos et al. 2004). The higher IP of 5-FTrp, compared to Trp, efficiently suppresses electron transfer of the excited indole moiety to nearby amide groups in the protein. As a result, the decay kinetics become independent of the rotameric states of the probe, explaining the simple decay kinetics of 5-FTrp embedded in a protein (Liu et al. 2005). Several azaTrp derivatives and 5-hydroxyTrp (5-OHTrp), with red-shifted absorption spectra that allow for selective excitation in the presence of multiple native Trp residues, have also been introduced in proteins using Trp auxotrophic expression hosts (Ross et al. 1992; Hogue et al. 1992; Soumillion et al. 1995; Twine et al. 2003a; Lepthien et al. 2008). These analogs can be excited using wavelengths of up to ~315 nm, enabling their specific excitation under most in vitro conditions (Ross et al. 1997) and sometimes also using in vivo conditions (Broos et al. 1999). A protein containing a Trp analog that allows for excitation at higher wavelengths is expected to give significant less autofluorescence when analyzed under in vivo conditions; however, production of such a protein has not been reported to date.

Almost 30 years ago, Hudson et al. speculated that replacing the indole moiety of Trp by the pseudo-isosteric azulene moiety could result in a very attractive fluorophore for incorporation in proteins (Hudson

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**Scheme 1** Structures of Trp and some Trp analogs

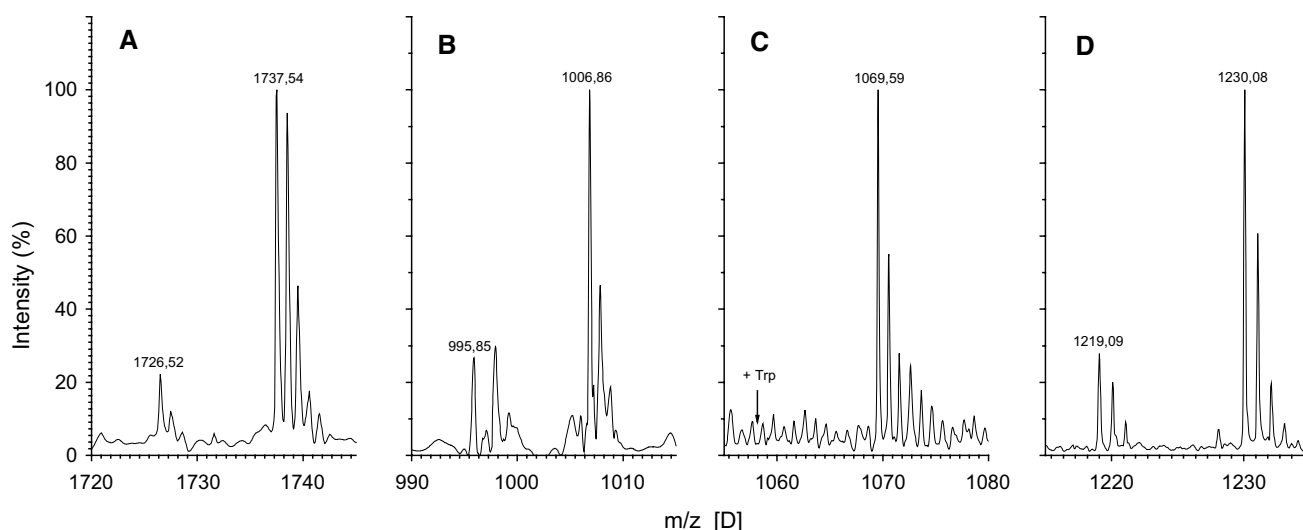
et al. 1986). Azulene shows a quite strong  $^1L_a$  absorption band centered at 320–380 nm ( $\epsilon \sim 4200 \text{ cm}^{-1} \text{ M}^{-1}$ ) (Moroz et al. 2013) and a weak  $^1L_b$  absorbance at 600 nm ( $\epsilon \sim 400 \text{ cm}^{-1} \text{ M}^{-1}$ ). The latter absorbance band gives azulene its characteristic dark blue color. Excitation at this wavelength does not result in measurable fluorescence signal while excitation in the  $^1L_a$  band yields an emission band centered around 380 nm with a quantum yield comparable to Trp. Thus in azulene the  $^1L_a$  and  $^1L_b$  states do not overlap, the IP is relatively high, and the decay kinetics are simple (Hudson et al. 1986; Moroz et al. 2013). Moreover, a high photostability of azulene was reported (Muller-Werkmeister and Bredenbeck 2014) and we have recently also shown that the azulene moiety exhibits weak environmental dependence and thus allows for using weak intrinsic quenchers, such as methionines, to monitor protein–protein interactions while not perturbing them (Moroz et al. 2013). Synthesis of an amino acid with an azulene moiety has been described but its biosynthetic incorporation in proteins could not be achieved (Hudson et al. 1986; Ross et al. 1997; Loidl et al. 2000; Moroz et al. 2013).

The enzyme tryptophanyl-tRNA synthetase (TrpRS) gives the Trp translation pathway its high fidelity and the most important molecular determinant for its specificity is realizing a H-bond between N-1 of indole and a conserved Asp residue in the active site (Doublet et al. 1995; Antonczak et al. 2011). Azulene cannot provide such H-bond when bound in the active site and this might offer an explanation why AzAla so far could not be biosynthetically incorporated using an amino acid tRNA synthetase evolved by directed evolution (Xie and Schultz 2006).

We created a Trp auxotroph (PA1002) of the gram-positive bacterium *Lactococcus lactis* (*L. lactis*) (Khattabi et al. 2008), because *L. lactis* is an attractive host for recombinant production of proteins including membrane proteins (Kunji et al. 2003). A more relaxed substrate specificity towards Trp analogs was observed compared to *E. coli* Trp auxotrophic strains (Khattabi et al. 2008). To explore the potential of *L. lactis* for the incorporation of Trp analogs further, the endogenous tryptophanyl-tRNA synthetase

(lacTrpRS) was cloned and co-expressed together with a target protein (Petrovic et al. 2013). This expression system translates Trp analogs, labeled with bulky substituents such as bromine or chlorine, with very high efficiencies (>89 %), making it the most versatile Trp analog expression system known.

Here we report our results about the biosynthetic incorporation of  $\beta$ -(1-azulenyl)-L-alanine (AzAla) in recombinant proteins using this expression system. A transcriptional regulatory protein from *L. lactis* (LmrR) was used as model protein; this protein controls the expression of a major multidrug transporter LmrCD (Agustiandari et al. 2008; Madoori et al. 2009). LmrR has two Trp residues at positions 67 and 96, as well as a Trp in the engineered C-terminal strep-tag at position 120. The gene was introduced in a nisin-inducible expression vector (pNSC8048-*lmrR*) (Agustiandari et al. 2008) and this plasmid, as well as the pMG36e-*trpRS* plasmid harboring the lacTrpRS gene (Petrovic et al. 2013), was electroporated in *L. lactis* P1002. Incorporation of azulene in LmrR proceeded essentially as described before for the incorporating of Trp analogs bearing bulky substituents such as 5-bromo-tryptophan (Petrovic et al. 2013). In brief, an overnight culture was prepared at 30 °C by transferring a single colony in GM17 medium, containing two antibiotic markers, chloroamphenicol (5  $\mu\text{g/ml}$ ) and erythromycin (75  $\mu\text{g/ml}$ ); the overnight culture was used to inoculate (1:50) a fresh culture of the same medium at 30 °C. When an  $\text{OD}^{600} = 0.8$  was reached, cells were pelleted down, washed with PBS, and resuspended in the same volume of a chemically defined medium (CDM) containing 1 mM AzAla but no Trp. After 4 h at 30 °C, LmrR expression was induced by nisin as previously described (El Khattabi et al. 2008) and induction was continued overnight at 30 °C. LmrR was isolated using a heparine column (Madoori et al. 2009) and the yield of the purified protein was 2–3 times lower compared to the CDM medium supplemented with Trp. A tryptic digest was analyzed by MALDI-TOF (Petrovic et al. 2013) and the MALDI spectra for the three tryptic peptides of LmrR,



**Fig. 1** MALDI-TOF spectra of tryptic digests of LmrR (**a–c**) and LysM tandem protein (**d**), labeled with AzAla at Trp positions. **a** Peptide DGISSYW<sup>67</sup>GDESQGGR (theoretical  $m/z$  1726.8 D + Trp, 1737.9 D + AzAla), **b** peptide LAFESW<sup>96</sup>SR (theoretical  $m/z$  995.1

D + Trp, 1006.2 D + AzAla), **c** peptide W<sup>120</sup>SHPQFEK (theoretical  $m/z$  1058.2 D + Trp, 1069.3 D + AzAla), **d** peptide SGDTLW<sup>14</sup>GISQR (theoretical  $m/z$  1219.3 D + Trp, 1230.4 D + AzAla)

containing the Trp positions, are shown in Fig. 1. High incorporation efficiency of AzAla was observed in each position: >80 % for position 67, >75 % for position 96, and >90 % for position 120. Variation of Trp analog incorporation efficiency for different positions in the same protein is not uncommon (Abbott et al. 2004).

As second model protein, double Trp lysM tandem protein (Petrovic et al. 2012) was expressed using the same protocol. This protein consists of two identical lysin motifs (LysM); each domain has a Trp residue at position 14. Expression of this protein in the presence of AzAla was also 2–3 times lower than when the CDM medium was supplemented with Trp. MALDI analysis of a tryptic digest of the expressed protein revealed an incorporation efficiency of >75 % (Fig. 1d).

In conclusion, the availability of an efficient synthetic route for AzAla (Loidl et al. 2000; Moroz et al. 2013) and its biosynthetic incorporation using a *L. lactus* Trp auxotroph, co-expressing lacTrpRS (Petrovic et al. 2013), enables the straightforward production of proteins containing AzAla at Trp positions. These proteins, structurally and functionally expected to be essentially the same as the wild-type proteins (Speight et al. 2014), can be characterized with various spectroscopic techniques such as fluorescence, absorbance, and Raman spectroscopy at uniquely long wavelengths that could not be used before for any other pseudo-isosteric analogs of Trp. Significant advantages in monitoring expression, localization, and biophysical characterization of proteins under various conditions (including in vivo) with AzAla as probe are foreseen.

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**Conflict of interest** The authors declare no conflict of interest.

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