Amino Acids (2015) 47:213–216
DOI 10.1007/s00726-014-1870-4

SHORT COMMUNICATION

Biosynthetic incorporation of the azulene moiety in proteins with high efficiency

Jinfeng Shao · Ivan V. Korendovych · Jaap Broos

Received: 3 November 2014 / Accepted: 4 November 2014 / Published online: 16 November 2014

Abstract Biosynthetic incorporation of β-(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.

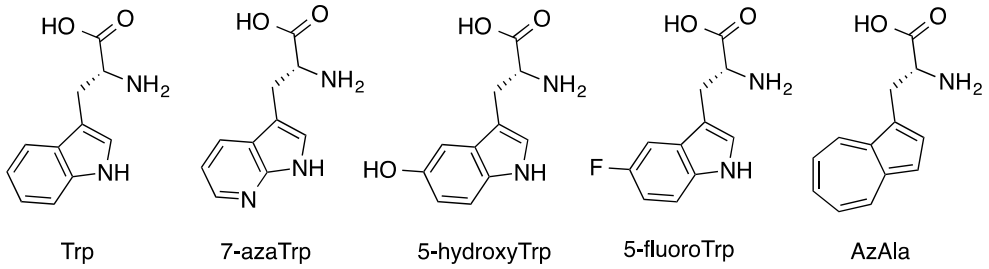
Trp has also some disadvantages as a spectroscopic probe; its photostability is limited and its photophysics are complex due to a relatively low ionization potential (IP) and spectral overlap of the two lowest excited states, ^1L_a and ^1L_b (Callis 1997, 2009). Trp needs to be excited by UV light with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

J. Shao · J. Broos (✉)
Laboratory of Biophysical Chemistry and GBB (Groningen Biomolecular Science and Biotechnology Institute), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands
e-mail: j.broos@rug.nl

I. V. Korendovych
Department of Chemistry, Syracuse University, Syracuse, NY 13244, USA



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 (ε~4200 cm^{-1} M^{-1}) (Moroz et al. 2013) and a weak ^1L_b absorbance at 600 nm (ε~400 cm^{-1} 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 (Doublie 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 systems translates Trp analogs, labeled with bulky substituents such as bromime 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 β-(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 µg/ml) and erythromycin (75 µg/ml); the overnight culture was used to inoculate (1:50) a fresh culture of the same medium at 30 °C. When an 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,

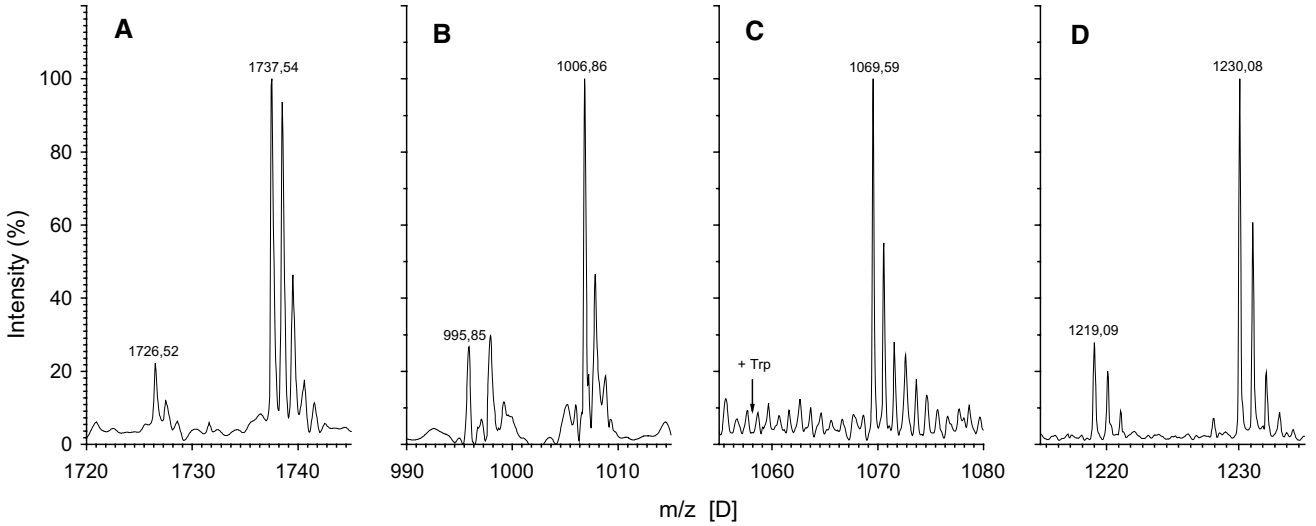


Fig. 1 MALDI-TOF spectra of tryptic digests of LmrR (**a**–**c**) and LysM tandem protein (**d**), labeled with AzAla at Trp positions. **a** Peptide DGIISSYW^{67}GDESQGGR (theoretical *m/z* 1726.8 D + Trp, 1737.9 D + AzAla), **b** peptide LAFESW^{96}SR (theoretical m/z 995.1 D + Trp, 1006.2 D + AzAla), **c** peptide W^{120}SHPQFEK (theoretical *m/z* 1058.2 D + Trp, 1069.3 D + AzAla), **d** peptide SGDTLW-^{14}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.

Acknowledgments We thank the China Scholar Council for financial support. I.V.K. is supported in part by a grant number 1332349 from NSF-EFRI, ORAU Ralph E. Powe Junior Faculty Enhancement award and a Humboldt Research Fellowship.

Conflict of interest The authors declare no conflict of interest.

References

Abbott GL, Blouse GE, Perron MJ, Shore JD, Luck LA, Szabo AG (2004) F-19 NMR studies of plasminogen activator inhibitor-1. Biochemistry 43:1507–1519

Agustiandari H, Lubelski J, van Saparoea HBV, Kuipers OP, Driessen AJM (2008) LmrR is a transcriptional repressor of expression of the multidrug ABC transporter lmrCD in Lactococcus lactis. J Bacteriol 190:759–763

Antonczak AK, Simova Z, Yonemoto IT, Bochtler M, Piasecka A, Czapinska H, Brancale A, Tippmann EM (2011) Importance of single molecular determinants in the fidelity of expanded genetic codes. Proc Natl Acad Sci USA 108:1320–1325

Broos J (2014) Biosynthetic incorporation of tryptophan analogs in proteins. Methods Mol Biol 1076:359–370

Broos J, ter Veld F, Robillard GT (1999) Membrane protein-ligand interactions in Escherichia coli vesicles and living cells monitored via a biosynthetically incorporated tryptophan analogue. Biochemistry 38:9798–9803

Broos J, Maddalena F, Hesp BH (2004) In vivo synthesized proteins with monoexponential fluorescence decay kinetics. J Am Chem Soc 126:22–23

Callis PR (1997) L-1(a) and L-1(b) transitions of tryptophan: applications of theory and experimental observations to fluorescence of proteins. Methods Enzymol 278:113–150

Callis PR (2009) Exploring the electrostatic landscape of proteins with tryptophan fluorescence. In: Geddes CD (ed) Reviews in fluorescence 2007. Springer Science + Business Media, New York, pp 199–248

Doublie S, Bricogne G, Gilmore C, Carter CW (1995) Tryptophanyl-Transfer-Rna synthetase crystal-structure reveals an unexpected homology to Tyrosyl-Transfer-Rna synthetase. Structure 3:17–31

El Khattabi M, van Roosmalen ML, Jager D, Metselaar H, Permentier H, Leenhouts K, Broos J (2008) Lactococcus lactis as expression host for the biosynthetic incorporation of tryptophan analogues into recombinant proteins. Biochem J 409:193–198

Hogue CW, Rasquinha I, Szabo AG, MacManus JP (1992) A new intrinsic fluorescent probe for proteins. Biosynthetic incorporation of 5-hydroxytryptophan into oncomodulin. FEBS Lett 310:269–272

Hudson BS, Harris DL, Ludescher RD, Cooney-Freed A, Cavalier S (1986) Fluorescence probe studies of proteins and membranes. In: Taylor DL et al (eds) Applications of fluorescence in biomedical sciences. A. R. Liss, New York, pp 159–202

Kunji ERS, Slotboom DJ, Poolman B (2003) Lactococcus lactis as host for overproduction of functional membrane proteins. Biochim Biophys Acta Biomem 1610:97–108

Lepthien S, Hoesl MG, Merkel L, Budisa N (2008) Azatryptophans endow proteins with intrinsic blue fluorescence. Proc Natl Acad Sci USA 105:16095–16100

Liu TQ, Callis PR, Hesp BH, de Groot M, Buma WJ, Broos J (2005) Ionization potentials of fluoroindoles and the origin of nonexponential tryptophan fluorescence decay in proteins. J Am Chem Soc 127:4104–4113

Loidl G, Musiol HJ, Budisa N, Huber R, Poirot S, Fourmy D, Moroder L (2000) Synthesis of beta-(1-azulenyl)-L-alanine as a potential blue-colored fluorescent tryptophan analog and its use in peptide synthesis. J Pept Sci 6:139–144

Madoori PK, Agustiandari H, Driessen AJM, Thunnissen AMWH (2009) Structure of the transcriptional regulator LmrR and its mechanism of multidrug recognition. EMBO J 28:156–166

Moroz YS, Binder W, Nygren P, Caputo GA, Korendovych IV (2013) Painting proteins blue: beta-(1-azulenyl)-L-alanine as a probe for studying protein-protein interactions. Chem Commun 49:490–492

Muller-Werkmeister HM, Bredenbeck J (2014) A donor-acceptor pair for the real time study of vibrational energy transfer in proteins. Phys Chem Chem Phys 16:3261–3266

Petrovic DM, Leenhouts K, van Roosmalen ML, KleinJan F, Broos J (2012) Monitoring lysin motif-ligand interactions via tryptophan analog fluorescence spectroscopy. Anal Biochem 428:111–118

Petrovic DM, Leenhouts K, van Roosmalen ML, Broos J (2013) An expression system for the efficient incorporation of an expanded set of tryptophan analogues. Amino Acids 44:1329–1336

Ross JBA, Senear DF, Waxman E, Kombo BB, Rusinova E, Huang YT, Laws WR, Hasselbacher CA (1992) Spectral enhancement of proteins: biological incorporation and fluorescence characterization of 5-hydroxytryptophan in bacteriophage lambda cI repressor. Proc Natl Acad Sci USA 89:12023–12027

Ross JBA, Szabo AG, Hogue CWV (1997) Enhancement of protein spectra with tryptophan analogs: fluorescence spectroscopy of protein-protein and protein-nucleic acid interactions. Methods Enzymol 278:151–190

Soumillion P, Jespers L, Vervoort J, Fastrez J (1995) Biosynthetic incorporation of 7-azatryptophan into the phage lambda lysozyme: estimation of tryptophan accessibility, effect on enzymatic activity and protein stability. Protein Eng 8:451–456

Speight LC, Samanta M, Petersson EJ (2014) Minimalist approaches to protein labelling: getting the most fluorescent bang for your steric buck. Aust J Chem 67:686–700

Twine SM, Szabo AG (2003) Fluorescent amino acid analogs. Methods Enzymol 360:104–127

Twine SM, Murphy L, Phillips RS, Callis P, Cash MT, Szabo AG (2003) The photophysical properties of 6-azaindole. J Phys Chem B 107:637–645

Xie JM, Schultz PG (2006) Innovation: a chemical toolkit for proteins—an expanded genetic code. Nat Rev Mol Cell Biol 7:775–782