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FlAsH LABELING OF A NUCLEAR RECEPTOR DOMAIN (D DOMAIN OF ULTRASPIRACLE) FUSED TO TETRACYSTEINE TAG

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Biarsenical fluorescein compounds feature unique fluorescence characteristics and special binding mechanism to tetracysteine tags with certain structures and these dyes offer a feasible method for site specific labeling of heterologously expressed proteins. We aimed FlAsH fluorescent labeling of tetracysteine fused hinge region of the ultraspiracle from *Drosophila melanogaster* (DmUSP-D domain) to facilitate functional studies of this receptor domain. A CCPGCC tetracysteine motif was integrated between His_6, Gateway attB1, and Flag tags and attached to the N-terminus of the DmUSP-D. The fusion protein was expressed in *Escherichia coli* and the FlAsH labeling was performed in bacterial extracts, under conditions which are compatible with receptor function. The dye was bound to the tetracysteine tag with high affinity and complex stability and the labeling proved to be specific for the target fusion protein. Results indicate that FlAsH labeling of the internal CCPGCC motif can be a valuable tool for the functional characterisation of any nuclear receptor domains.

Keywords: Fluorescent dyes – site specific labeling – recombinant fusion proteins

INTRODUCTION

Nuclear receptors are ligand-dependent transcription factors, which consist of several functional domains responsible for transactivation, DNA binding, ligand binding, and dimerization with other proteins. The D domains link the DNA binding C and the ligand binding E domains and have been considered as a simple hinge between the two regions. Recent studies revealed that the D domain was involved also in receptor function. Although no contact with ligand is possible, the D domain is indis-

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pensable for hormone binding [8, 14]. Participation in nuclear transport [15] and dimerization [20] is also described.

We are especially interested in the dimerization capacity of the D domains and we selected the D domain of the ultraspiracle (USP) for our studies. USP, the ortholog of the vertebrate nuclear receptor RXR in insects, is the most important dimerization partner of the ecdysone receptor (EcR). The heterodimer of the EcR and USP is generally considered as the functional ecdysteroid receptor which mediates the effect of molting hormone ecdysone and regulates molting, metamorphosis and development in insects [21, 23, 27]. Dimerization can be detected using fluorescent labeled recombinant receptors. Traditional green fluorescent protein (GFP) chimeras are, however, not suited for these studies due to the intrinsic dimerization or oligomerization properties of GFP. An alternative is the site specific fluorescent labeling of tetracysteine tagged recombinant proteins with biarsenical fluorescein dyes, which was introduced by R. Y Tsien's group (http://www.tsienlab.ucsd.edu) [1, 9] and was already successfully used for functional investigations *in vitro* and *in vivo* [4, 12, 13, 16, 17, 18, 22, 25, 26].

In this paper, we describe experiences in labeling of tetracysteine tagged D domain of USP from *Drosophila melanogaster* (Cys_4-DmUSP-D) with the biarsenical dye FlAsH (4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein). In the Cys_4-DmUSP-D fusion construct a CCPGCC tetracystein motif was inserted between His_6, Gateway attB1, and Flag tags and cloned to the N-terminus of the DmUSP-D. The fusion protein was expressed in bacteria and the labeling was performed in the crude bacterial extract.

MATERIALS AND METHODS

Design of the Cys_4-DmUSP-D fusion construct

The sequence encoding DmUSP-D was determined according to the record in Swiss-Prot Database (http://www.expasy.ch) and previous alignments in the literature [2, 7, 8, 19, 23] and was assigned as amino acids no. 168-229 of the full length USP of *Drosophila melanogaster*.

Tetracysteine tag was designed by the Consensus Secondary Structure Prediction software [3] available on-line (http://npsa-pbil.ibcp.fr). The best prediction for β-turn structure suitable for the labeling was achieved by the SOP and SOMPA approach [5, 6]. His_6 and Flag tags were additionally incorporated in the fusion construct for immunodetection, purification and protease cleavage. Flag sequence was introduced by polymerase chain reaction (PCR). His_6 is derived from the expression vector. The N-terminal Gateway cloning site, the attB1, results in the expression of additional nine amino acids. The complete map of the Cys_4-DmUSP-D construct is shown in Table 1.

PCR amplification of DmUSP-D sequence

DNA plasmid for bacterial protein expression was prepared with the PCR Cloning System with Gateway Technology (Invitrogen). First, the DmUSP-D coding region was amplified by PCR using pZ7-I-dUSP plasmid as template. (The plasmid of full length USP from *Drosophila melanogaster* was kindly donated by F. C. Kafatos, EMBL, Heidelberg, Germany.) The specific attB1 and attB2 cloning sites as well as the DNA sequences of the tetracysteine and Flag tags were introduced by elongated primers (Table 1). PCR was performed with Pfx polymerase according to the protocol of the manufacturer Invitrogen.

Cloning of the expression plasmid of Cys_4-DmUSP-D by Gateway technology

The target gene was inserted into pDONR201 donor vector by BP Clonase and then directly into pDEST17 destination vector by LR Clonase [11] according to the "one-tube" protocol provided by the manufacturer. After the LR recombination arabinose inducible *Esherichia coli* BL21-AI (Invitrogen) cells were transformed by electroporation. Appropriate expression clones were selected by ampicillin resistance and then by monitoring expression of the fusion protein in core cultures (see details in the next section). pDEST17-Cys_4-DmUSP-D expression plasmid was isolated with an NA0200 Midiprep kit (Sigma), and then further propagated in *Esherichia coli* DH5α cells. Sequence of the target gene in the expression plasmid was verified by sequencing (SequiServe, Vaterstetten, Germany).

Expression of Cys_4-DmUSP-D in Esherichia coli

Transformants of *Esherichia coli* BL21-AI cells were plated and next day core cultures were inoculated from ampicillin resistant colonies. Expression cultures were prepared in 250 ml Luria-Bertani (LB) medium using 500 ml flasks, and pregrown at 37 °C with shaking speed of 250 min^{-1}. At $OD_{600} = 0.4$, the cultures were cooled to 15 °C and the shaking speed was reduced to 200 min^{-1}. Expression of the recombinant protein was induced by 0.2% arabinose. Culturing was continued for 60 hours and terminated at $OD_{600} = 2.0$.

Bacterial proteins were analysed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) by Coomassie staining as well as by Western blot with His_6-specific antibody (Dianova, Hamburg, Germany) and Amersham ECL detection system (Amersham Biosciences).

FlAsH labeling in bacterial homogenate

Bacteria of 500 µl culture were sedimented by centrifugation. Cells were resuspended in 50 µl SDS-PAGE probe buffer without 2-mercaptoethanol (2 w/v% SDS, 4 v/v% glycerol, 0.01 w/v% bromophenolblue in 0.04 M Tris-HCl pH = 6.8 buffer) and homogenised in ultrasonic bath for 5 min. Labeling was accomplished following the protocol of Adams et al [1]. Homogenate was pretreated with 50 mM (final concentration) of the reducing agent triscarboxyethylphosphine (TCEP, from Pierce) for 30 min. Then 0.3 µl 2 mM stock solution in dimethylsulphoxide of FlAsH-(1,2-ethanedithiol)$_2$ (FlAsH-EDT$_2$, Fig. 1) from Panvera (San Diego, USA) was added to the homogenate. The labeling reaction was run on room temperature for 1 hour. Then the sample was heated on 95 °C for 3 min, cooled and centrifuged on 2000 g for 5 min. A 15% polyacrylamide gel was loaded with the sample and run as usual. Fluorescent band of the labeled fusion protein was visualised in ultraviolet light and the gel was scanned by a Fluor-S Multiimager (BioRad).

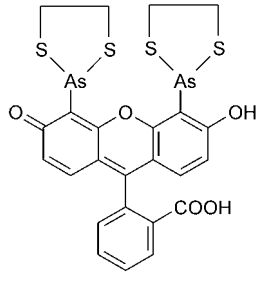

Fig. 1. Structural formula
of 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein-(1,2-ethanedithiol)$_2$ (FlAsH-EDT$_2$)

Preparation of crude bacterial extract

Bacteria were collected by centrifugation, resuspended in 50 mM sodium acetate buffer (pH = 7.9) and disrupted by sonification (Sonifier B-12, Branson, Danbury, USA). Homogenates were centrifuged (36,000 g, 20 min) to remove cell debris and inclusion bodies.

FlAsH labeling in crude bacterial extract

Cell extract was preincubated with 1 mM (final concentration) TCEP for 15 min at room temperature and then 10 µM (final concentration) FlAsH-EDT$_2$ was added. Fluorescence was measured by a Hitachi F-2000 Spectrofluorometer (excitation: 495 nm, emission: 530 nm). Emission spectra were scanned between 475 and 600 nm.

RESULTS

We aimed to clone and express a His_6–attB1–Tetracysteine tag–Flag–DmUSP-D fusion protein named Cys_4-DmUSP-D for fluorescent labeling with FlAsH. The tetracysteine CCPGCC sequence has strong tendency to form β-turn structure essential for the labeling [1] therefore we inserted this motif into the fusion construct as the FlAsH binding site. We enhanced prediction for the β-turn structure of the CCPGCC motif by two modifications: amino acid at the end of the attB1 was changed from the usual F to L and an additional A was inserted between the attB1 cloning site and the CCPGCC motif (Table 1).

Table 1
PCR primers and amino acid sequence of the tetracysteine-tagged *Drosophila melanogaster* ultraspiracle D domain fusion protein, Cys_4-DmUSP-D

PCR primer 5'

ggggacaagtttgtacaaaaaagcaggc**ttggcttgctgcccgggttgctgc***gactacaaggacgacgatgacaag*ggcatgaagcgcgaagcggt

PCR primer 3'

ggggaccactttgtacaagaaagctgggtcctattagaagtcatcagaaccgttgccgct

Amino acid sequence of Cys_4-DmUSP-D (M_w = 9.93 kDa)

MSYYHHHHHHLESTSLYKKAG**LACCPGCC***DYKDDDDK*GMKREAVQEERQRGARNAAGRLSASGG GSSGPGSVGGSSSQGGGGGGGVSGGMGSGNGSDDF

(Underlined: Gateway attB1 and attB2 cloning sites, bold: tetracysteine tag, italics: Flag tag)

The pDEST17-Cys_4-DmUSP-D expression plasmid of the construct was successfully cloned by the Gateway methodology and then the recombinant protein was expressed in *Esherichia coli* BL21-AI cells. Sequence check certified a proper expression plasmid and SDS-PAGE analysis of the bacterial homogenate with Coomassie staining and His_6 Western blot detected the band of the expressed Cys_4-DmUSP-D at the expected molecular weight (9.93 kDa).

FlAsH binding of Cys_4-DmUSP-D was first checked with labeling in bacterial homogenate (under denaturing conditions). Fluorescent image of the SDS-PAGE analysis showed a single and bright green band of the Cys_4-DmUSP-D (Fig. 2). Endogenous bacterial proteins were not stained to a significant extent demonstrating the specificity of the labeling reaction.

Labeling of the Cys_4-DmUSP-D was then performed in bacterial extract without any previous or subsequent isolation procedure. Following the addition of FlAsH a rapid increase in fluorescence with the highest intensity at 530 nm was measured in the extract (Fig. 3, data of a representative experiment are shown). The fluorescence reached its maximum in 10–15 min time, signaling a rapidly completed binding reaction. The emitted green light was intense and the fluorescence was stable for several days.

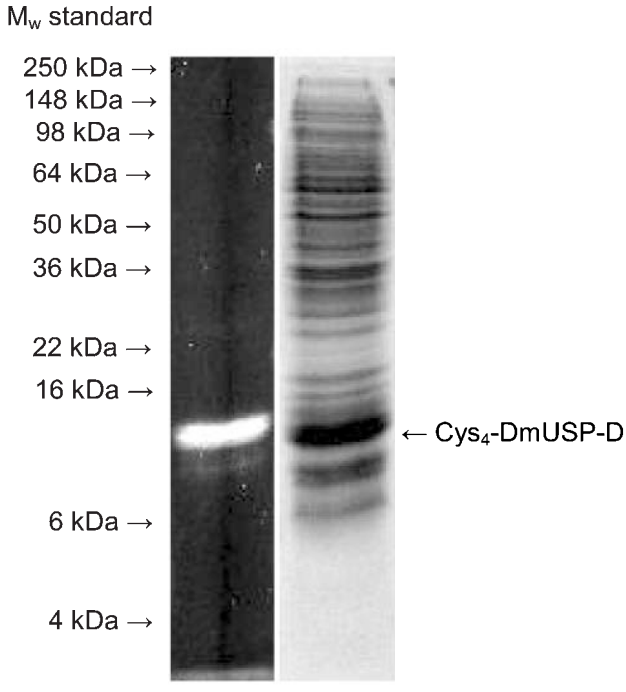

Fig. 2. Fluorescent image (left) and Coomassie stained picture (right) of the SDS-PAGE analysis of the FlAsH labeled bacterial homogenate containing the recombinant Cys_4-DmUSP-D

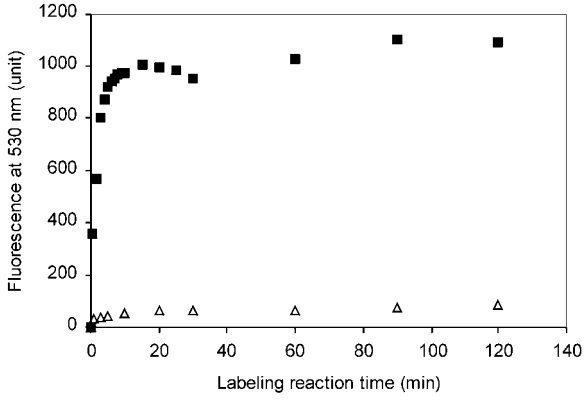

Fig. 3. Time course of fluorescence (excitation 495 nm, emission 530 nm) following FlAsH labeling of Cys_4-DmUSP-D (■) in crude bacterial extract. Non-specific fluorescence was measured by the labeling of extract of non-transformed bacteria (blank, △)

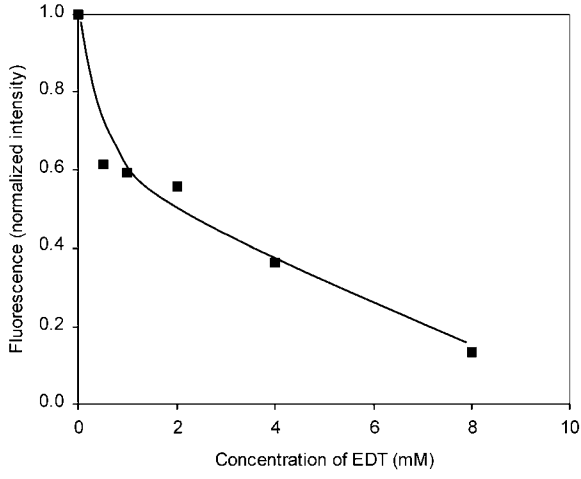

Fig. 4. Fluorescence quench caused by 1,2-ethanedithiol (EDT) induced dissociation of the FlAsH complex of Cys_4-DmUSP-D

Stability of the protein-dye complex can be determined with EDT induced dissociation [1]. We added EDT in increasing concentrations to the labeled cell extract and the reversed binding equilibrium of FlAsH and the Cys_4-DmUSP-D was detected by the decreasing fluorescence at 530 nm (Fig. 4). Fifty% displacement was observed at 2.0–2.5 mM EDT concentration what reveals a considerably high complex stability.

Non-specific fluorescence was determined by adding FlAsH into the extract of non-transformed bacteria. This fluorescence was first negligible but increased after a longer (12–24 h) time. It was characterised with a broader spectrum (Fig. 5) with the emission maximum at 517 nm.

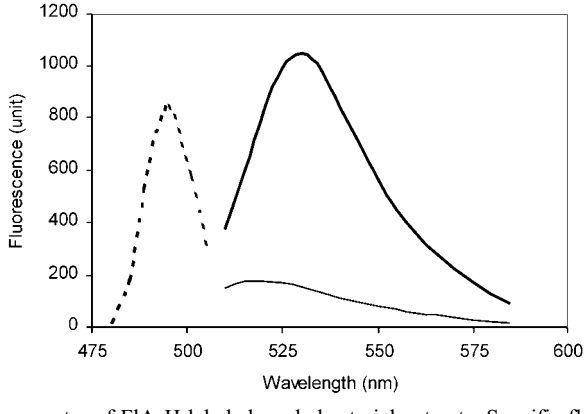

Fig. 5. Fluorescence spectra of FlAsH labeled crude bacterial extracts: Specific fluorescence of FlAsH labeled Cys_4-DmUSP-D after 1 h the labeling (——). Non-specific fluorescence of non-transformed bacteria extract after 24 h the labeling (blank, ——). Scattered excitation light after 24 h the labeling (– – –)

DISCUSSION

Dimerization experiments can be performed with soluble recombinant nuclear receptor protein present in bacterial extract. Biarsenical fluorescein labeling is highly site specific and the dye becomes fluorescent only after binding to the tetracystein tag [9]. These features allow to label target fusion proteins without isolation from the bacterial extract or separation from the excess dye and make this labeling particularly advantageous for the investigations of sensitive protein functions.

Fluorescent labeling of the Cys_4-DmUSP-D was accomplished in bacterial extract by the specific binding reaction between FlAsH and the internal CCPGCC tetracysteine tag in the fusion construct. The labeling reaction was rapid and resulted in an intense and stable green fluorescent signal. Complex stability estimated by the EDT induced dissociation could be compared to data of the original C-terminal CCPGCC tag, reported by Adams et al. [1]. In that case – under slightly different experimental conditions – 50% displacement was measured at 1.6 mM EDT concentration. The comparison with our result (50% displacement at 2.5 mM) indicates that internal CCPGCC tag in the Cys_4-DmUSP-D forms similar or even more stable complex than the original C-terminal variant. The high complex stability indicates that our modifications in the amino acid sequence as well as Gateway attB1 and Flag sequences in the surroundings of the CCPGCC motif promoted the β-turn structure suitable for the labeling.

In previous applications of the FlAsH labeling certain background or non-specific fluorescent signals occurred. Griffin et al. [10] observed that hydrophobic binding of the FlAsH to bovine serum albumin might result in fluorescence. Similar binding to hydrophobic sites piling up in dead or dying cells also caused bright background staining of cells [10]. Stroffekova et al. [24] detected substantial background FlAsH fluorescence in native living CHO-K and HEK239 cells which was attributed to the binding of CXXC, CX_5C, CXXH or $HX_{1-3}C$ motifs. Addition of low (micromolar) concentration of EDT or other dithiols was recommended to minimize the non-specific binding and fluorescence [9, 10].

Background fluorescence was also occured in FlAsH labeling of Cys_4-DmUSP-D in cell extract, although the labeling reaction itself was specific, as the SDS-PAGE analysis of the full homogenate proved it. This background fluorescence became significant only after longer (12–24 h) time and had different emission maximum than the specific signal. (Adding EDT, even in millimolar concentration, did not diminish this background.) This background fluorescence is, however, not likely to disturb detection in 530 nm and the experiments executed in shorter (a few hours) time, but its possible interference should be considered in other applications.

The biarsenical dye and the other reagents (TCEP, EDT) used for the labeling, as well as the further reaction conditions (i.e. pH, ionic strength) are compatible to the experimental conditions for nuclear receptors. The short labeling reaction time is also a great advantage in retaining receptor functionality.

In conclusion, biarsenical fluorescent labeling of recombinant receptor fusions with internal CCPGCC tetracysteine tag provides a feasible method for the functional investigations of ecdysteroid or other nuclear receptors.

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