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The dityrosine cross-link as an intrinsic donor for assembling FRET pairs in the study of protein structure

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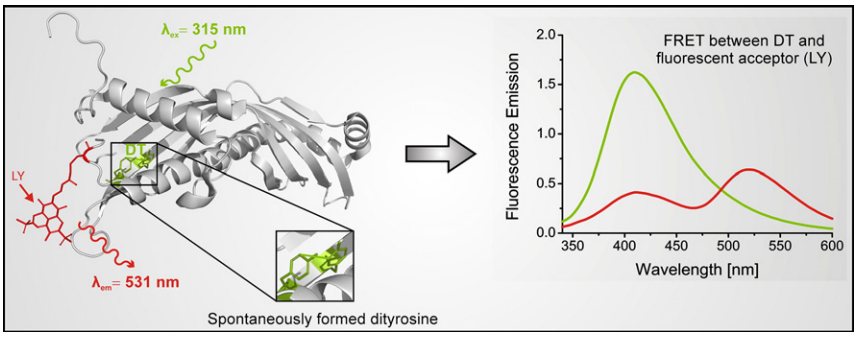
HIGHLIGHTS

- Dityrosine and Lucifer yellow (LY) were applied for analysis of JHBP structure.
- The separation distances between the fluorophores were compared with X-ray data.
- LY moiety in position 171 is embedded in the protein molecule structure.

GRAPHICAL ABSTRACT



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ABSTRACT

Dityrosine-Lucifer yellow (DT-LY) was used as the donor–acceptor pair in studies of the topography of juvenile hormone-binding protein (JHBP). The Förster distance, $R_0 = 30.5$ Å for DT-LY was determined. Separation distances (R) between DT and the fluorescent probes placed at the 219 and 224 position were 26 and 28 Å and correspond to that found from X-ray analysis (23 and 24 Å, respectively). Higher than expected efficiency of energy transfer between DT and LY probe placed in position 171 was observed, indicating that this probe is immobilized in the protein structure ($\kappa^2 = 3.25$). Red-edge excitation shift (REES) analysis supported this assumption. Slight changes in Förster resonance energy transfer (FRET) efficiency were observed after incubating the labeled proteins with juvenile hormone III (JH III). This is the first report showing the application of DT fluorescence for the analysis of protein conformation.

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1. Introduction

Förster resonance energy transfer (FRET) stands out as a technique with the potential to monitor structural transitions in protein molecules and to evaluate distances between relatively remote (10–100 Å) pairs of fluorophores with an angstrom resolution [1]. The transfer occurs between two molecules, a donor and an acceptor [2,3]. W and Y residues provide intrinsic fluorescence probes [4] in most proteins. The fluorescence of W residues is almost always dominant; therefore, the vast majority of studies of intrinsic protein fluorescence focus on this residue. In the course of studies which investigated the fluorescence properties of various proteins, an additional emission appeared which could not be attributed to any of the expected chromophores [5,6]. The observed fluorescence was associated with the formation of dityrosine (DT) residues [7,8]. DT is a Y-Y dimer derived from tyrosyl radicals, which can be formed by reactive oxygen species [7], ultraviolet irradiation [8], γ-irradiation [9], metal-catalyzed oxidation [10] and the action of peroxidases [11,12]. Oxidation of proteins

Abbreviations: D-A, donor–acceptor; DT, dityrosine; FRET, Förster resonance energy transfer; JH, juvenile hormone; REES, red-edge excitation shift; rJHBP, recombinant juvenile hormone-binding protein.

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containing adjacent Y residues is often accompanied by an increase in violet/blue fluorescence at $\lambda_{em} = 420$ nm ($\lambda_{ex} = 315$ nm) observed in pH > 7.2. DT fluorescence is pH dependent and decreases in acidic solutions [7,13,14].

In a previous study, the spectral properties of *Galleria mellonella* juvenile hormone-binding protein (JHBP) were investigated [15]. It was shown that between residues Y128 and Y130, located in the juvenile hormone-binding pocket, the intramolecular formation of DT may occur. Since DT in JHBP molecules forms spontaneously, the question arose as to whether it may serve as a valuable intrinsic fluorescent probe which could be used to examine the topography and internal motion of protein molecules. No studies on the application of DT as a FRET donor to monitor proteins structure have been reported until now.

G. mellonella hemolymph JHBP (hJHBP) is a low molecular weight glycoprotein [16,17]. The role of this carrier protein is to transport lipophilic juvenile hormone (JH) molecule in the aqueous environment of the hemolymph reducing nonspecific binding [18], and protecting the hormone molecules from enzymatic degradation by nonspecific hydrolases [19]. JH binding to JHBP in *G. mellonella* induces a profound conformational transition in the protein molecule and enhances protein stability against proteolysis. This is reflected in the changes in the sedimentation coefficient, electrophoretic mobility, and perturbation of Y residues and disulfide bridges. [20,21]. A solution of the 3D structure of *G. mellonella* hJHBP showed that this molecule has a cylindrical form with two hydrophobic binding sites (named *W* and *E*) that are located at opposite poles of the molecule with a flexible N terminus region situated at the opening of the *W* cavity [22]. Recently, an X-ray structure of the apo- and JH-bound JHBP from the silkworm *Bombyx mori* has been reported [23]. Since the 3D structure of the two apo proteins appeared to be very similar, it became possible to correlate our fluorescence investigations with crystallographic data.

The structure of proteins containing W and DT is difficult to analyze via FRET because solvatochromic fluorescence emission of the W residue, ranging ca. 300 to 350 nm [24], overlaps with the excitation spectra of both DT ($\lambda_{ex} = 315$ nm) and commercially available probes. This makes it difficult to estimate the portion of the energy transfer from W to the extrinsic fluorescence probe, which is required for calculate the donor–acceptor distance. A protein molecule containing two strong intrinsic fluorophores (W, DT) labeled with external fluorescent dye creates a complicated system for analyzing protein molecule topography using FRET. The goal of this study is to develop the conditions in which DT cross-links can be used as intrinsic donors for FRET pairs in the presence of a W residue. We think that the JHBP molecule containing the N4W mutation labeled with an external fluorophore resembles such a system. In this report, the FRET between DT residues and the single engineered and labeled C residues of recombinant JHBP (rJHBP) is described. Three recombinant JHBP mutants (N4W/L171C, N4W/S219C and N4W/N224C) were expressed, purified, and labeled with the extrinsic fluorescent probe, Lucifer yellow. The energy transfers within these constructs were calculated from FRET efficiencies that were measured between an individual donor–acceptor pair. Our results provide the first FRET-based distance measurements in which DT was used as a fluorescent probe.

2. Materials and methods

2.1. Organisms and culture media

The *E. coli* TOP10 (Invitrogen, USA), grown at 37 °C in low-salt LB broth [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl (pH 7.5)] was used for cloning and propagation of all plasmids. The *P. pastoris* SMD 1168 (Invitrogen), cultured at 29 °C, was utilized for protein expression. Yeast growth media were as follows. YPDS (used for selection after cell transformation): 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1% (w/v) sorbitol. BMGY (used for cell propagation): 1% (w/v) yeast extract, 2% (w/v) peptone, 0.1 M potassium phosphate pH 6.0, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, 1% (v/v) glycerol. BMMY (used to induce of protein expression): 1% (w/v) yeast extract, 2% (w/v) peptone, 0.1 M potassium phosphate, pH 6.0, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, and 0.5% (v/v) methanol.

2.2. Materials

The restriction enzymes, T4 DNA ligase and *Vent* polymerase were purchased from Fermentas and used according to the manufacturer's instructions. Zeocin™ was from Invitrogen. Juvenile hormone III (10*R*,*S*-JH III) and dithiothreitol were purchased from Sigma. Dityrosine was synthesized at BioCentrum Ltd. (Kraków, Poland) from L-tyrosine as described by [25] using Mn(III) as an oxidizing agent. Lucifer yellow iodoacetamide, dipotassium salt was purchased from Molecular Probes®. All other chemicals were of analytical grade and commercially available.

2.3. Construction of expression vectors

The full-length *G. mellonella* JHBP cDNA (GenBank accession no. AF410772) was mutated by site-directed mutagenesis using the "megaprimer" mutagenesis method [26,27]. Primers that were used in pairs to generate the mutations are given in Table 1. The resulting PCR products were analyzed with 0.8% agarose gel electrophoresis, and extracted with Gel-Out kit (A&A Biotechnology, Poland). Purified DNA was double-digested using EcoRI and SalI, and ligated into the identically treated and dephosphorylated pPICZαA expression vector (Invitrogen). Cloning into the EcoRI and SalI sites introduced four extra amino acids, EF and VD at the N terminus and the C terminus, respectively, of each of the recombinant proteins. To facilitate purification, six H residues (6×His tag) were also added at the C terminus. Each of the new plasmids, named pPICZαA/rJHBP and encoded N4W recombinant protein, are designated as rJHBP. Additionally, three double mutated recombinant proteins were prepared (N4W/L171C, N4W/S219C, and N4W/N224C mutant). After being transformed into the chemically competent cells of *E. coli* TOP10, the positive recombinants were selected from an LB agar low-salt medium containing Zeocin™. Plasmids isolated from Zeocin™-resistant colonies were first evaluated

Table 1
Nucleotide sequences of the primers used.

Primer name	Sequence	Application
Forward_1	CCGCGAATTCAGTAAACTCTGGTTATCGACAGA	Change of asparagine 4 to tryptophan
Forward_2	GCCTTGAAATGCGATTCAGATT	Change of lysine 171 to cysteine
Reverse_1	CCGCGTCGACAACATTTTTGAAGTATGCACTCTT	Construction of L171C megaprimer
Reverse_2	CCGCGTCGACAACATTTTTGAAGTATGCGCACTTCGGT	Change of serine 219 to cysteine
Reverse_3	CCGCGTCGACAACGCATTTGAAGTATGCACTC	Change of asparagine 224 to cysteine
αFactor	TACTATTGCCAGCATTGCTGC	DNA sequencing and colony PCR to test the integration of inserts into the pPICZαA vector
AOX1-3'	GCAAATGGCATTCTGACATCC	

Underlined fragments indicate mutation sites.

by restriction enzyme digestion analysis and then confirmed by DNA sequencing.

2.4. Pichia strain transformation

Transformations of *P. pastoris* were done by the electrotransformation method [28]. Approximately 10 μg of the above constructed plasmid DNA was linearized within the *AOX1* promoter with the restriction enzyme SacI, purified with the Clean-Out PCR purification kit (A&A Biotechnology), and used to transform competent cells of the SMD1168 strain with a Gene Pulser II apparatus (Bio-Rad). The transformed cells were spread on YPDS agar plates containing Zeocin™ in order to select positive clones. Integration into the yeast genome was confirmed by performing colony PCR [29].

2.5. Purification of recombinant JHBPs

Harvested yeast cells were sedimented at 1 500 g for 10 min and the polyhistidine-tagged rJHBP mutants were purified at 4 °C from the culture medium using nickel-based metal affinity resins, according to procedures described previously [30]. Following the elution of protein-containing fractions, the elution buffer was exchanged to 0.1 M sodium phosphate, pH 7.2, using PD-10 desalting columns (GE Healthcare) and proteins were concentrated with an Amicon Ultra centrifugal filter device (Millipore) to 20-60 μM. To estimate the concentration of rJHBPs, the Bradford protein assay [31] was performed and bovine serum albumin was applied as a standard. Protein visualization was done by separating proteins on 10% SDS-PAGE gels [32] and either staining them with Coomassie Brilliant blue R-250 [33] or transferring them to a nitrocellulose membrane for Western blot [34]. In agreement with our previous work, two isoforms of recombinant JHBP were present in the analyzed protein sample in similar quantities [35]. One form of the N4W mutants was non-glycosylated, whereas the second form was glycosylated at position N94. Purified rJHBP and its mutated forms (N4W) had the same JH-binding activity similar to that of hJHBP ($K_d = 0.48 \times 10^{-6}$ M). The JH-binding activity was determined as previously described [36].

2.6. Labeling with Lucifer yellow fluorescent dye

Prior to labeling, C/W rJHBP mutants were pre-incubated with a DTT in 0.1 M of a sodium phosphate buffer, pH 7.2, using a twofold molar excess of the reducing agent over the free sulfhydryl groups. Reactions were carried out for 1 h at room temperature. The DTT was then removed by rapidly desalting the protein samples on a PD-10 column, equilibrated with a 0.1 M of a sodium phosphate buffer, pH 7.2. The labeling reaction was performed shortly after the elimination of the reducing agents to prevent reoxidation of the target thiols. This was done in the same buffer at a 20–40 μM protein concentration using a molar ratio of 5:1 of the thiol-reactive probe to the protein. The reaction was allowed to proceed for 12 h at 4 °C in the dark. The labeled derivatives were separated from the unreacted free fluorophores by passing them through the PD-10 gel-filtration column equilibrated with a 0.1 M sodium phosphate buffer, pH 7.2.

2.7. Steady-state fluorescence assays

Steady-state fluorescence measurements were carried out using the Fluorolog-3 fluorescence spectrophotometer (Spex, Jobin Yvon Inc.). Spectra were recorded at room temperature using a 315-nm excitation wavelength while monitoring the emission in the range 340–600 nm. Measurements were performed in a 0.3-cm path length quartz cuvette at a 2 μM protein concentration in a 0.1 M sodium phosphate buffer, pH 7.2. Control baselines were subtracted from each spectrum for all fluorescence measurements. The ratio between the intensities of the dityrosine emission in the labeled (F_{DA}) and unlabeled (F_D) protein was used to calculate the efficiency of the energy transfer (E):

$$E = 1 - F_{DA}/F_D \tag{1}$$

The average distance between the dityrosine and the fluorophore, R, corresponding to the measured E value was calculated using the equation:

$$R = R_0 \, (1/E - 1)^{1/6} \tag{2}$$

where R_0 is the distance at which the efficiency of the energy transfer is 50%. Obtained R values were compared with the respective DT-C distances in the crystal structure of JHBP [22].

The steady-state fluorescence measurements of the red-edge excitation shift (REES) [37,38] were performed under the same conditions as FRET. The emission spectra were collected using different excitation wavelengths from 315 to 342 nm for DT and from 426 to 453 nm for Lucifer yellow at 3 nm intervals.

2.8. Effect of JH binding on FRET

To analyze the influence of JH on rJHBP structure a defined amount of the hexane solution of JH III (10 μl of 6.75×10^{-4} M) was applied into glass tubes coated with polyethylene glycol. With this method there is fast desorption of the hydrophobic hormone from the test tubes after evaporation of the solvent and the addition of a water solution The amount of added JH was calculated from JHBP $K_d = 4.7 \times 10^{-7}$ M [36] to ensure 99% saturation of the protein molecules with the ligand molecules. After the solvent had evaporated, 2 μM rJHBP solutions (100 μl) in a 0.1 M sodium phosphate buffer, pH 7.2, were added and incubated for 2 h at 4 °C. Steady-state fluorescence measurements were performed as described above to calculate the FRET efficiency.

3. Results

Preliminary experiments showed that excitation of the W residue in N4W rJHBP ($\lambda_{ex} = 295$ nm) resulted in an energy transfer from the W residue to DT (not shown), whereas excitation of the DT residue in this protein sample ($\lambda_{ex} = 315$ nm) did not particularly affect the spectral properties of the protein (Fig. 1A).

3.1. Spectral overlap calculation

A major criterion for choosing donor–acceptor pairs for FRET studies is the existence of an essential overlap between the donor fluorescence emission spectrum and the absorption spectrum of an acceptor. The normalized DT emission spectrum recorded in 0.1 M of sodium phosphate, pH 7.2, observed upon excitation at 315 nm strongly overlaps the absorption spectrum of free Lucifer yellow, as illustrated in Fig. 1B and C. The spectral overlap integral (J) of the recorded spectra was calculated from the Eq. (3):

$$J = \int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \Big/ \int F_D(\lambda) d\lambda \tag{3}$$

where $F_D(\lambda)$ is the fluorescence intensity of the donor in the absence of the acceptor at wavelength λ, and $\varepsilon_A(\lambda)$ is the molar absorption coefficient of the acceptor at λ. According to this equation, J for the dityrosine-Lucifer yellow pair was determined and found to be 2.28×10^{14} M^{-1} cm^{-1} nm^4.

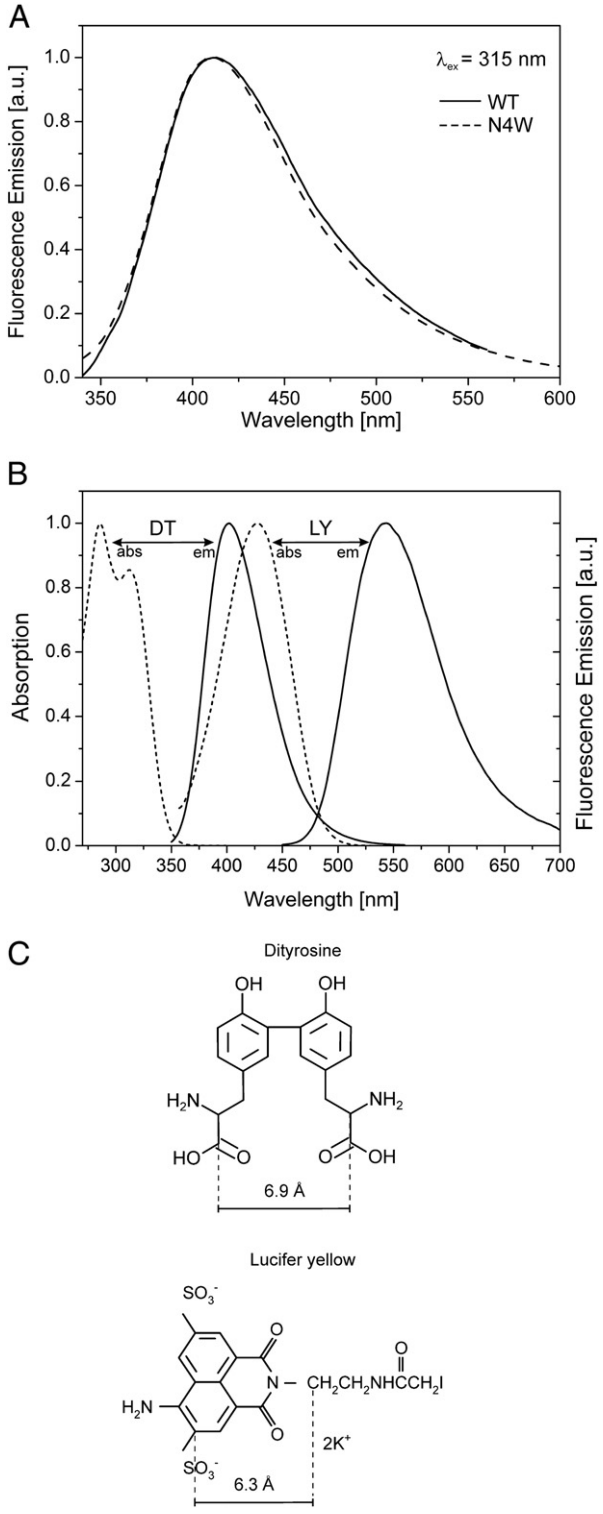

Fig. 1. The spectroscopic properties of rJHBP, DT and Lucifer yellow. (A) Normalized emission spectra of 2 μM wild-type rJHBP (solid line) and N4W mutant (dashed line) recorded in the presence of 0.1 M of sodium phosphate, pH 7.2. Spectra were monitored at λ_{ex} = 315 nm (slit widths: 5 nm). (B) Comparison of the absorption (dashed line) and fluorescence emission spectra (solid line) of the DT and Lucifer yellow iodoacetamide. Absorption spectrum (abs) of 40 μM DT and emission spectrum (em) of 0.5 μM DT was recorded in the presence of 0.1 M of sodium phosphate, pH 7.2. The emission spectrum was monitored at a fixed λ_{ex} = 315 nm (slit widths: 5 nm). Data for Lucifer yellow spectra are taken from http://www.invitrogen.com. Spectra have been normalized to the same intensity for comparison purposes. (C) The molecular structures of DT and Lucifer yellow iodoacetamide. Black horizontal lines schematically show the distances between indicated atoms. DT: dityrosine; LY: Lucifer yellow; WT: wild-type.

3.2. Förster distance calculation

The value of the Förster distance R_0, required for the estimation of the energy transfer, was calculated using Eq. (4):

$$R_0 = 0.211\left(J\kappa^2 Q_D n^{-4}\right)^{1/6}(\text{Å}) \quad (4)$$

where J is the spectral overlap integral (in $M^{-1}\,cm^{-1}\,nm^4$) and Q_D represents the quantum yield of the fluorescence of the donor in the absence of an acceptor. In this equation, Q_D was taken to be 0.33, which is the quantum yield of DT estimated by Sakura and Fujimoto [39] in reference to the value of Y residue (quantum yield = 0.14) in a neutral aqueous solution [40]. The refractive index n was taken to be 1.4 [41] and the orientation factor κ^2 was assumed to be 0.476 [42,43]. The Förster distance, R_0, calculated on the basis of these parameters and calculations was 30.5 Å.

3.3. Determination of the intramolecular distances in rJHBP molecules

Native JHBP contains four cysteine residues [44] paired to form two disulfide bridges [45] which are required for JHBP activity and thus not available for labeling. To investigate the application of DT as an intrinsic fluorescence donor for the study of protein structures, three constructs of rJHBP containing additional C residues were designed and expressed. One of these mutants had a C residue adjacent to the *E* cavity (N4W/L171C), and the other two contained C residues on the flexible C-terminal arm, adjacent to the JH-binding pocket (N4W/S219C and N4W/N224C). All constructs, which contained the spontaneously formed DT residue, were labeled with the fluorescence acceptor Lucifer yellow. The position of the mutated residues and the distances investigated are schematically shown in Fig. 2.

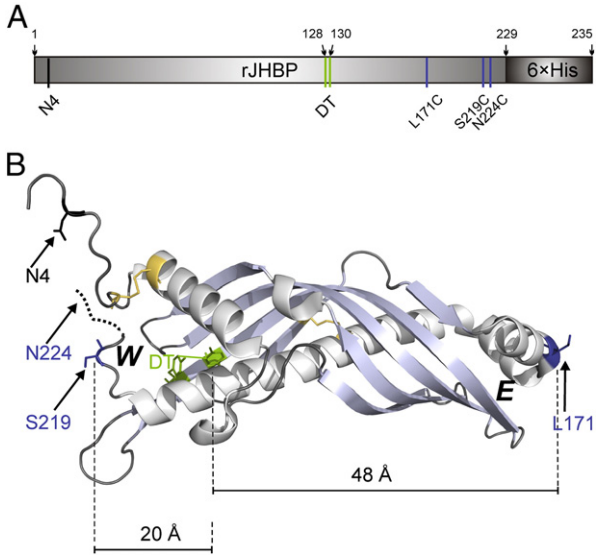

Fig. 2. Location of DT and mutated residues in the schematic representation and three-dimensional structure of JHBP. (A) Schematic representation of rJHBP mutants consisting of 235 amino acid residues and containing the C-terminal affinity tag (6×His; black box). Single black vertical line indicates the position of N4W mutation and single blue vertical lines indicates the positions of L171, S219 and N224 mutations. Double green vertical line symbolizes the dityrosine residue that is derived from Y128 and Y130. (B) The PyMOL-generated cartoon of the JHBP structure (Protein Data Bank ID: 2rck). Two cavities (*W* and *E*) located at the opposite poles of the molecule are indicated. The two disulfide bridges are marked in yellow. Tyrosine residues involved in the formation of dityrosine are in green. Mutated residues are shown in black/blue and indicated by black arrows (position of the N224 residue is approximated by the dotted line because the 3D model includes only residues 1–221). Distances (in Å) between Cα of the Y128 and L171 or S219 residue are indicated by black horizontal lines.

The summarized DT emission spectra of N4W and the Lucifer-labeled rJHBP double mutants recorded either in the presence or in the absence of JH III molecules are shown in Fig. 3. The fluorescence of all the mutants had an emission peak at approximately 415 nm after excitation at 315 nm, whereas the fluorescence acceptor emission peak was observed near 525 nm. After JH-binding the fluorescence intensity of N4W rJHBP increased by 11–14%. However, as a result of FRET, the emission of DT (at about 415 nm) for apo-rJHBP double mutants was quenched by 60–75% and for holo-rJHBP double mutants by 64–77% compared to the emission of the N4W. The acceptor emission either slightly decreased (for the N4W/S219C mutant) or remained at a similar level (for N4W/L171C or N4W/N224C mutant), indicating that almost no energy transfer changes were observed in the presence of JH for N4W/L171C and N4W/N224C mutants. The efficiency of FRET, determined using the changes in the intensity of the DT fluorescence in the absence and presence of Lucifer yellow, was calculated using the Eq. (1). The obtained *E* values yielded intramolecular distances between DT and 171C, 219C and 224C residues of 28 Å, 26 Å and 28 Å, respectively. Although the N4W mutant showed an apparent increase in the intrinsic fluorescence intensity of DT (415 nm) in the presence of JH, the calculated distances did not change significantly, yielding 28, 25 and 27 Å for N4W/L171C, N4W/S219C and N4W/N224C mutants, respectively. Table 2 summarizes data from the steady-state fluorescence spectra for all tested mutants and the data obtained from X-ray analysis of the JHBP crystals.

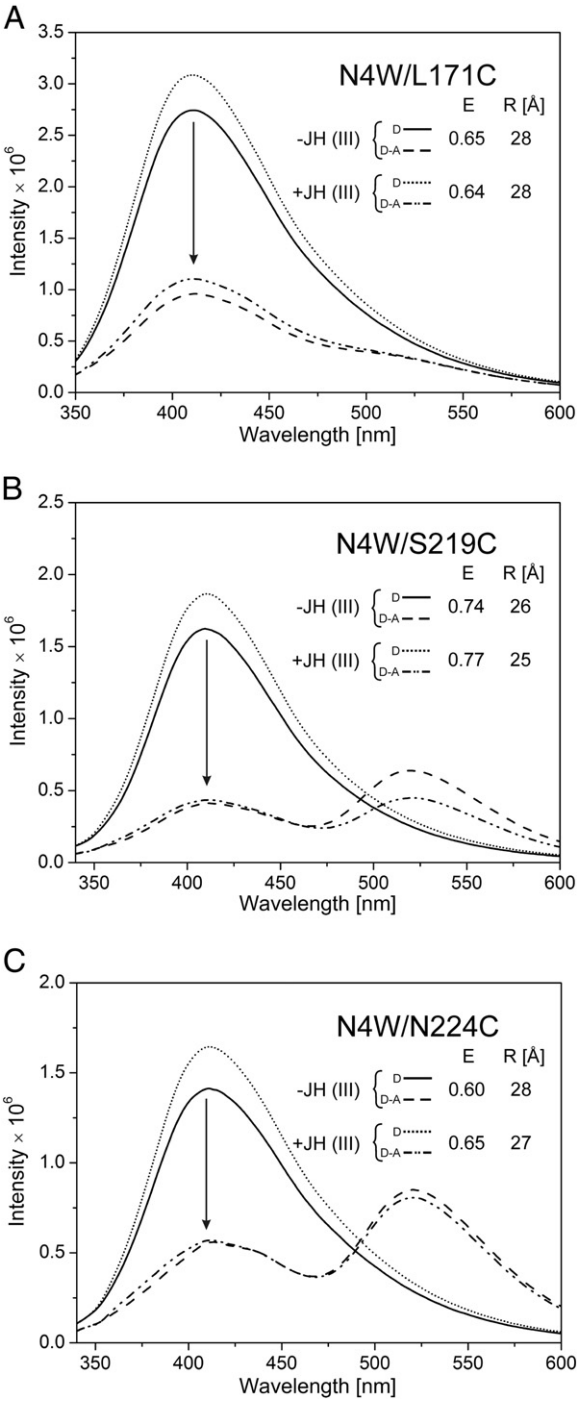

Fig. 3. Steady-state fluorescence emission spectra of rJHBP mutants. Spectra for (A) N4W/L171C mutant, (B) N4W/S129C mutant and (C) N4W/N224C mutant. All spectra were recorded in 0.1 M of sodium phosphate, pH 7.2, at a fixed λ_{ex} = 315 nm. Slit widths: 5 nm. The protein concentration was 2 μM. The decrease in the intensity at ~415 nm due to FRET is indicated by arrows. Solid lines represent donor spectra (D) in the absence of JH, whereas dotted lines represent donor spectra in the presence of JH. Dashed lines represent donor–acceptor spectra (D-A) recorded in the absence of JH, whereas dashed-dotted lines represent donor–acceptor spectra recorded in the presence of JH. Calculated efficiencies of FRET (*E*) and donor–acceptor distances (*R*) (Å) are indicated.

3.4. Probing the DT and Lucifer yellow microenvironment

To investigate the microenvironment surrounding the intrinsic DT residue and extrinsic Lucifer yellow moiety the red-edge excitation shift (REES) was analyzed. We detected a pronounced shift in the DT emission maximum (from 412 to 419 nm) as a function of change in the excitation wavelength (Fig. 5A). In contrast to this, no change in the emission maximum was observed for free Lucifer yellow (Fig. 5B). A moderate 4 nm shift in the Lucifer yellow emission for the N4W/L171C mutant was observed. The emission maxima of the fluorophore residue attached at position 219C (Fig. 5B) shifted by only 2 nm.

4. Discussion

The native JHBP molecule does not contain W residues. Since proteins with a single W residue are good subjects for designing FRET experiments, we obtained rJHBP mutants containing single W residue as a potential fluorescence donor and labeled the -SH groups with AEDANS (not shown). Unexpectedly, when the W mutant of rJHBP was illuminated at 295 nm to excite the W residue, we were faced with a problem caused by the interference from the spontaneously formed DT fluorescence with the fluorescence of the acceptor molecule. Since the position of the DT residue in the JHBP molecule had been previously determined, we decided to examine the application of DT as a fluorescence probe in protein structure studies. To our knowledge DT has not been used in FRET experiments.

It is important to note that all the distance estimates derived from the FRET measurements were based on the simplification of some variables. The efficiency of the energy transfer depends on the orientation and distance of the two dipoles of the donor and acceptor molecules. The accuracy of the distance measurements by FRET depends on the assumption of an orientation factor, κ^2. As shown by [46], the κ^2 value is within the range $0<\kappa^2<4$. This variation contributes to differences in the value of R_0 (Eq. (4)). Despite these ambiguities, in most experiments the value of $\kappa^2 = 2/3$ was assumed, even though using an average value of κ^2 is only valid if the fluorophores rapidly diffuse through all possible orientations over the timespan of the energy transfer [47]. In many cases these conditions do not exist. For the static but still random orientation of dyes $\kappa^2 = 0.476$ [42,48]. R_0 values for both the values of κ^2 are shown in Table 2. Consequently, the difference in the value of the determined D-A separation distance, *R*, was not greater than 6% when the two values of κ^2 were used (Table 2). This implies that the error associated with assuming the value of $\kappa^2 = 0.476$ is rather small.

An essential question concerning the usage of DT fluorescence for determining distances is whether the values obtained experimentally

Table 2
Energy transfer and D-A separation between the DT residue (donor) and the Lucifer yellow (acceptor), determined in the presence and the absence of JH III.

	N4W/L171C			N4W/S219C			N4W/N224C		
		($\kappa^2 = 2/3$)	($\kappa^2 = 0.476$)		($\kappa^2 = 2/3$)	($\kappa^2 = 0.476$)		($\kappa^2 = 2/3$)	($\kappa^2 = 0.476$)
		(Å)	(Å)		(Å)	(Å)		(Å)	(Å)
Apo-rJHBP	0.65	29	28	0.74	27	26	0.60	30	28
Holo-rJHBP	0.64	29	28	0.77	26	25	0.65	29	27
Crystal structure									
$Y128^{C\alpha}$-$aa^{C\alpha}$[a]		48		20				(~24)[b]	
$S129^{C\alpha}$-LY^{C12}[c]		38		23				Not found	

Energy transfer efficiency (E) and estimated distances (R) were calculated as described in the text.
[a] Separation distances were taken from the crystallographic structure of JHBP between the Cα carbon atom of the Y128 residue and the Cα carbon atom of the amino acid residue labeled with Lucifer yellow (Kolodziejczyk et al., 2008).
[b] Separation distance for the 224C residue was approximated because the 3D model includes only residues 1–221. The value was determined assuming that the distance is the same between the Cα carbon atom of the Y128C residue and the Cα carbon atom of the both residues situated five amino acids from S219C (i.e. R214 and N224).
[c] Separation distances were taken from the crystallographic structure of JHBP between the Cα carbon atom of S129 which represents the position of the center of the DT residue and the carbon atom C12 of Lucifer yellow moiety.

correlate with those obtained from crystallographic studies. If we take into consideration the separation distance between Cα of Y128 of the DT residue and Cα of the cysteine residue labeled with Lucifer yellow, the values of R estimated by FRET (26 and 28 Å for N4W/S219C and N4W/N224C mutants, respectively) were larger by about 7 Å for both mutants (Fig. 2, Table 2). This difference seems less surprising if we consider the fact that covalently labeled residues reside at the flexible C-terminal region of the rJHBP molecule and may assume multiple conformations in solution (Fig. 2). It is known that the size of the probe and the length of the spacer coupled to the amino acid side chains are important factors in estimating inter-fluorophore distances. Errors may occur between ±5–10 Å [49]. If Cα of S129 present between Y128 and Y130 was assumed as the starting point of the distance determination and the atom C12 of the center of

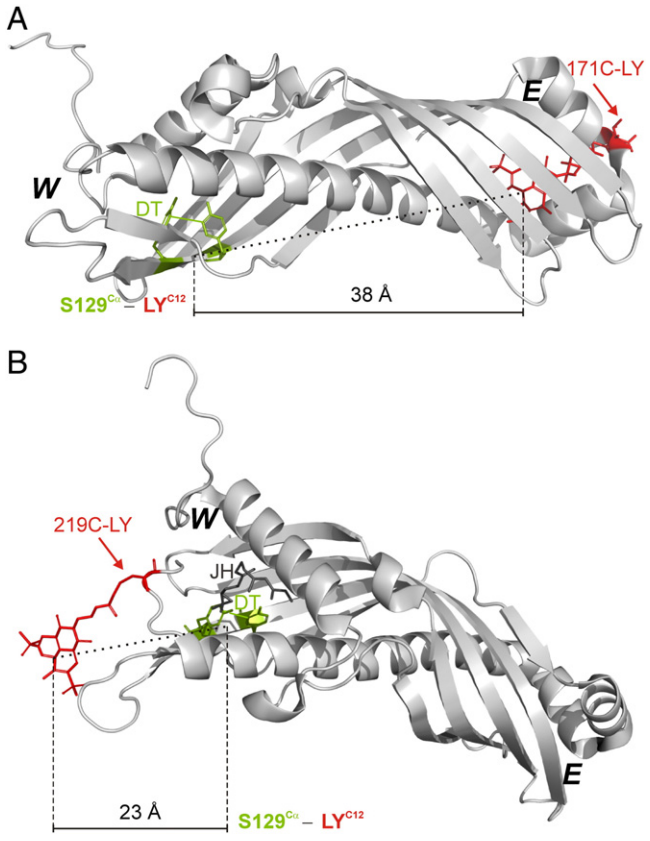

Fig. 4. Postulated localization of Lucifer yellow moiety in N4W/L171C and N4W/S219C mutants. The cartoons of the JHBP structure show two boundary arrangements of the Lucifer yellow (LY) residue in the protein molecule. (A) Shows LY adjacent to the *E* cavity (171C) with restricted freedom of rotation. (B) Shows (219C) LY protruding from the JHBP molecule with freedom of rotation. The location of the juvenile hormone molecule in the JH-binding pocket was assumed using (AutoDock Vina in PyRx 0.8 (Virtual Screening Tools) and the PDB file 2ASOS [51]. Two cavities located at the opposite poles (*W* and *E*) of the JHBP molecule are indicated. Tyrosine residues involved in the formation of DT are in green. JH is in black. Lucifer yellow-labeled 171C and 219C residues are shown in red. The distances (in Å) between the two carbon atoms: Cα of S129 and the C12 of LY moiety labeling cysteine residues (171C or 219C) are indicated.

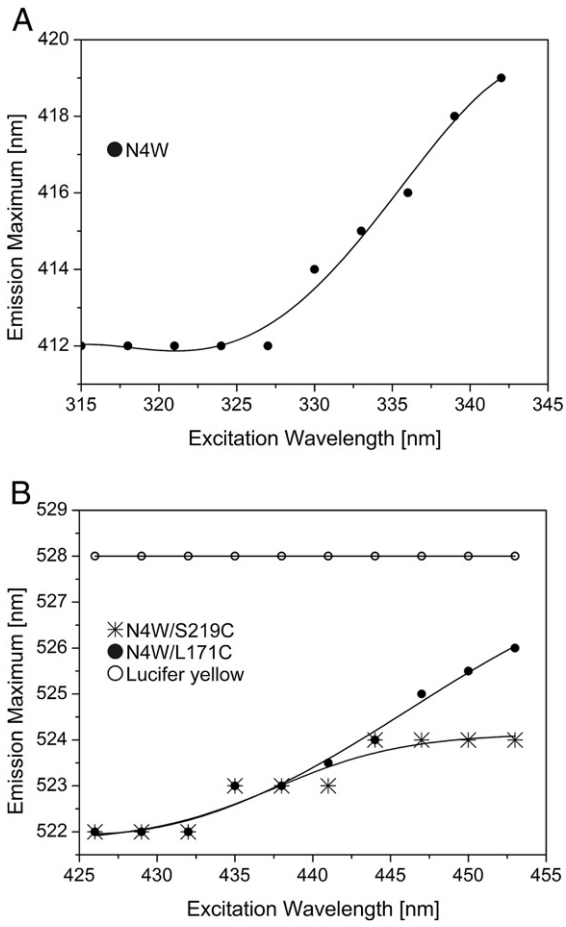

Fig. 5. Emission maxima of the steady-state fluorescence of DT and Lucifer yellow in relation to the excitation wavelength. REES displayed by (A) DT residue of N4W mutant, (B) free Lucifer yellow (open circle), Lucifer yellow placed in position 171C of rJHBP (filled circles) or Lucifer yellow placed in position 219C (stars) of rJHBP. All spectra were recorded in 0.1 M of sodium phosphate, pH 7.2. The protein concentration was 2 μM. Slit widths: 5 nm. The polynomial fit was used to draw the REES plots.

Lucifer yellow moiety was used as the second point, then the separation distance for the N4W/S219C mutant (23 Å) is close to that found in the crystal structure (20 Å) .

There is, however, a significant difference between the separation distance calculated from FRET (28 Å) and that derived from crystallographic studies (48 Å) for the N4W/L171C mutant (Table 2). It is possible that the Lucifer yellow residue attached to the 171C residue does not protrude from the β-turn structure but is immobilized and placed in the *E* cavity of the JHBP molecule (Fig. 4). This would shorten the distance to 38 Å, which is still too large in comparison to that found from X-ray analysis (Table 2, Fig. 4). Restriction of the freedom of rotation may lead to such a mutual orientation of the D-A dipoles which increases the energy transfer above the value expected from their random orientations. This could be reflected in the apparent shorter separation distance between the D-A pair. We recalculated that to obtain a separation distance close to that found from crystallographic studies (38 Å) an orientation factor of $\kappa^2 = 3.25$ should be assumed.

To test our supposition concerning the immobilization of LY in position 171, the dielectric relaxation of the hydration shells surrounding the D/A molecules was investigated using REES. REES represents a unique approach that can be used to monitor the environment and dynamics around a fluorophore in an organized molecular assembly. The effect of a shift in the excitation wavelength can be observed with polar fluorophores in motion restricted environments where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime [50]. DT residue immobilized in the protein structure showed the highest value of REES (Fig. 5). As expected, free LY did not exhibit any red-edge effect, but did show a REES when bound to the rJHBP. There was an almost twofold greater shift for the mutant labeled at position 171 in comparison to the mutant labeled at position 219. This indicates that the motion of the solvent molecules around the excited Lucifer yellow molecules around the 171C residue is more restricted than for the 219C residue.

We considered the possibility that JH may interact with the probe and effect the FRET. Based on Suzuki et al. (2011) we docked the hormone molecule in the *W* cavity of *G. mellonella* JHBP (Fig. 4). It seems that JH forms a hydrogen bond with the donor (DT) similar to the way in which it interacts with Y128 residue. The interaction of JH with Lucifer yellow is less likely, because it would require distortion of the C terminus of the JHBP molecule.

We found that in the regions where fluorescent probes were incorporated the recombinant W/DT JHBP molecules do not change their structure in the presence of hormone (Table 2). This is consistent with the findings of the latest studies by Suzuki et al., who have demonstrated that although amino acid residues surrounding the JH-binding site of the JHBP molecule assume multiple conformations in solution, the structure of the protein core remains unchanged [23]. Our findings may indicate, however, that the cross-linking of Y residues within the *W* cavity eliminates any potential conformational transitions within the protein molecule.

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