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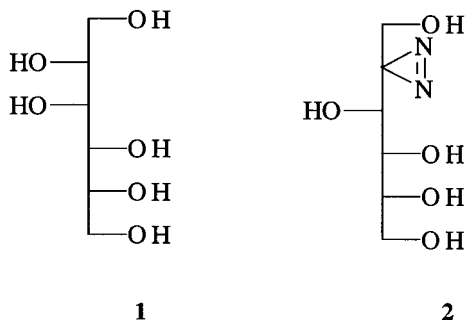
The Smallest Resonance Energy Transfer Acceptor for Tryptophan

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Diazirine compounds have found widespread use in biological sciences as photoactivatable reagents.^{1,2} Upon photoactivation; a highly reactive carbene intermediate is formed, which potentially can react with all functional groups within a protein. Introduction of the diazirinyl group in a ligand allows mapping of the ligand-binding site via identification of fragments of the photoactivated ligand-protein complex by analytical techniques such as mass spectrometry. Structural information about the diazirine ligand-protein interaction is limited to the characterization of binding site residues in very close contact with the carbene. In this communication, we report about the utility of the diazirinyl group as acceptor in resonance energy transfer (RET) distance measurements³ with tryptophan (Trp) or a Trp analogue as donor. The principle is demonstrated for a diazirine derivative of D-mannitol (**1**), 2-azi-2-deoxy-D-arabino-hexitol (**2**), and single-Trp-containing mutants of the mannitol transporter, EII^{mtl}, from *E. coli*.



EII^{mtl} is an integral cytoplasmic membrane protein consisting of the membrane-embedded C domain, covalently linked with the cytoplasmic B and A domains.⁴ EII^{mtl} is responsible for the transport of mannitol into the cytoplasm and its concomitant phosphorylation to mannitol-1-phosphate. Structural information on the 36 kD C domain is limited to a 5 Å projection map.⁵ The localization of the mannitol-binding site is not known for EII^{mtl} or any of the other EII-type carbohydrate translocators.⁴ To localize the mannitol-binding site, the EII^{mtl} substrate **2** was synthesized as a photoaffinity label.⁶ However, photoaffinity labeling was not successful due to an efficient intramolecular photolysis reaction, yielding (2S)-2-deoxy-D-arabino-hexose.⁶ **2** exhibits a weak absorption ($\epsilon = 53 \text{ mol}^{-1} \cdot \text{dm}^{-3} \cdot \text{cm}^{-1}$) at 335 nm.⁶ This absorption overlaps with the emission spectrum of Trp and therefore the diazirinyl group is a potential RET acceptor to measure distances with a Trp residue in a protein, an application of diazirines that has not been reported before.⁷ **2** is very weakly fluorescent upon direct excitation at 290 or 335 nm. In the presence of free indole or 5-hydroxyTrp, **2** exhibits sensitized fluorescence with an emission peak centered

around 363 nm.⁸ This experiment proves that RET takes place between these donors and **2**.

EII^{mtl} contains four Trps all located in the C domain at positions 30, 42, 109, and 117. Four functional single-Trp-containing mutants were constructed; the mutants were characterized by steady state and time-resolved fluorescence and phosphorescence spectroscopy.⁹ Mixing of the purified detergent-solubilized single-Trp mutant W30¹⁰ with 50 μM **1** resulted in a 16% increase of the emission signal.^{9a} A similar effect on emission was found upon mixing with perseitol, a mannitol analogue that specifically inhibits EII^{mtl}.¹¹ Thus upon carbohydrate binding, a protein conformation takes place resulting in enhanced emission of Trp at position 30. However, mixing of this mutant with 1 mM **2** resulted in a sharp decrease of the emission signal, which in turn increased to a level 16% higher than the initial value upon introducing 1 mM **1** to the same sample (Figure 1A). This experiment demonstrates that **2** is bound at the mannitol-binding site of EII^{mtl} and that it can be completely replaced by **1**. Compared to the 1-EII^{mtl} complex, the emission signal of the 2-EII^{mtl} complex is 29% lower. A distance (R) between Trp at position 30 and **2** of 11 Å was calculated, using a Förster distance (R_0) of 9.8 Å for this donor-acceptor couple.¹²

The above experiments indicate that the Trp at position 30 is sensitive to changes in microenvironment induced by ligand binding. To prove that the decrease in emission upon introduction of **2** is indeed due to RET and not to a different protein-ligand complex of 2-EII^{mtl} compared to 1-EII^{mtl}, a Trp analogue with an emission less sensitive for changes in microenvironment was biosynthetically incorporated into the protein.^{13,9c} 5-FluoroTrp, an isosteric analogue of Trp, was introduced with very high efficiency at position 30.¹⁴ Mixing of 5-fluoroTrp W30 with **1** resulted in only a 3% lower emission intensity. When this protein was mixed with 1 mM **2**, a large decrease in emission was found. This intensity returned to a value 3% lower than found for 5-FluoroTrp W30 upon mixing with 1 mM **1** (Figure 1B).¹⁵ These experiments confirm that **2** is an energy acceptor for both the Trp and 5-FluoroTrp containing W30 mutant. Results of experiments with 3 other single-Trp mutants are presented in Figure 1C,D. In the case of W42 a net decrease of 11% was observed corresponding to a donor-acceptor distance of 15 Å. Although the W109 and W117 mutants bind radiochemically labeled **1** with similar affinity as W30 and W42,^{9a} no spectral changes were observed upon introducing **2** and/or **1**,^{9a} indicative for a donor-acceptor distance $> 17 \text{ Å}$ (Figure 1D,E). Due to the low ϵ value of the diazirinyl group, a relatively high concentration of 1 mM can be used without interference of an inner filter effect (see Figure 1D,E). The lack of knowledge about the actual donor-acceptor orientation (value of κ^2) introduces an uncertainty in R . However, the mixed polarization of Trp, resulting in a relative low intrinsic anisotropy value of < 0.3 and the fact that the single-Trp EII^{mtl} mutants show a sub nanosecond anisotropy decay component^{9b} reduces the uncertainty in R caused by κ^2 to 10% or less.^{3c} Another

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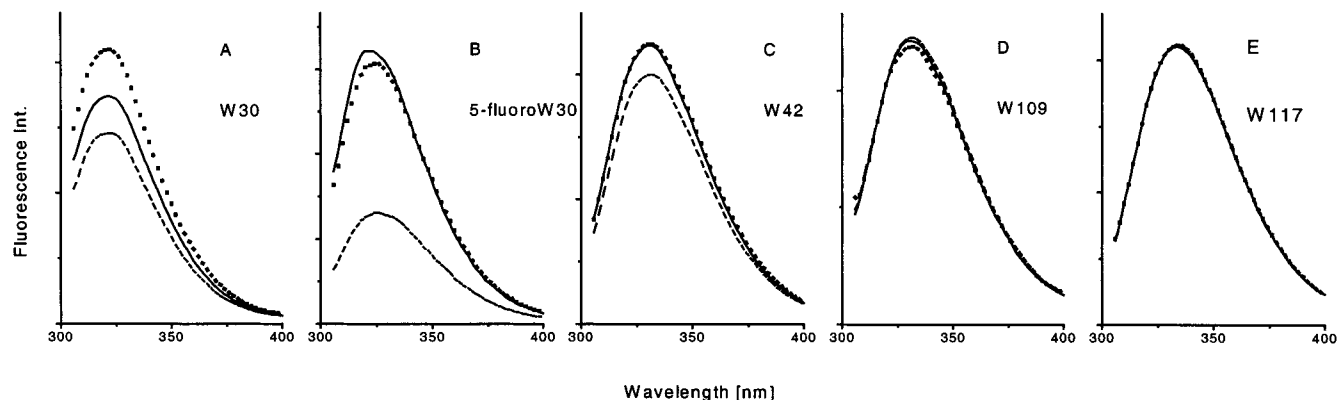


Figure 1. Fluorescence emission spectra (solid) of W30 (A), 5-fluoroTrp-containing W30 (B), W42 (C), W109 (D), and W117 (E), and the effect of 1 mM **2** (dashed) and 1 mM **2** + 1 mM **1** (dotted) on these spectra. Fluorescence spectra were recorded at 5 °C on a SLM-Aminco SPF-500 fluorometer adjusted to 2 nm excitation band-pass and 5 nm emission band-pass. The excitation was at 295 nm. The buffer used was 20 mM Tris-HCl pH 8.4, 250 mM NaCl, 0.5 mM reduced glutathione, and 0.25% decylpentaethyleneglycol detergent. The EII^{ml} concentration was 1 μM. Spectra were corrected for tryptless EII^{ml} and for instrument response. Variation in emission spectra integrals was <2%. Mutants were purified as described.^{9a}

concern in RET distance measurements is the uncertainty about the exact position of the chromophoric group relative to the ligand due to the presence of a (flexible) linker. This uncertainty is absent in **2** since the chromophoric group is an integral part of the substrate. The small size of the diazirinyl group allows its introduction into biological ligands, e.g. amino acids and peptides, sugars, steroids, and lipids with a relative small effect on the biological activity.^{1,2,16} Moreover, the diazirinyl group is known to be very stable under physiological conditions and harsh conditions used in organic synthesis.¹⁷ In conclusion, the versatility of Trp as an intrinsic spectroscopic probe in protein chemistry and the small size of the diazirinyl group make this a very attractive donor–acceptor couple for accurate RET distance information in protein chemistry. Especially for membrane-bound receptor and transport proteins, for which high resolution structures are not readily obtained, this distance information can contribute significantly to the understanding of the mechanism of these systems. We are currently extending our research on the localization of the substrate-binding site in EII^{ml} by screening other single-Trp mutants.

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- (7) Photoinstability of **2** is not limiting its use in RET experiments since only 1% decrease in absorbance was observed upon illumination at 335 nm and 25 °C during 30 min in a spectrophotometer.
- (8) Conditions: 0.1 M **2**, 5 μM indole or 10 μM 5-hydroxyTrp in 20 mM Tris-HCl pH 8.4 in a 3 × 3 mm² cuvette with 290 nm excitation at 25 °C. By comparing the net decrease in donor emission (>90%) with the net increase at 363 nm, a quantum yield (*Q*) of **2** of <0.02 was calculated. The lower quenching of Trp by **2** in the EII^{ml} mutants investigated and the lower enzyme concentrations used compared to free indole explain why the sensitized fluorescence contribution is not clearly visible in the spectra presented in Figure 1.
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- (12) The *R*₀ was calculated by using values 2/3 for κ^2 , *n* = 1.4, an overlap integral of $4.18 \times 10^{-17} \text{ M}^{-1} \text{ cm}^3$, and a quantum yield^{9b} of W30 of 0.14 ($4.13 \times 10^{-17} \text{ M}^{-1} \text{ cm}^3$ and 0.21, respectively, for W42). At 10 mM **2** similar distances were found, indicative that the EII^{ml} mutants are saturated with **2** at 1 mM.
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- (14) No 0–0 triplet band typical for the Trp containing W30 protein at 411.4 nm^{9d} was observed in this sample but at 413.0 nm and no spectral differences were found when excited at 290 or at 300 nm,^{13b} indicative of a very high 5-fluoroTrp incorporation efficiency (J. Broos, G. B. Strambini, to be submitted for publication). The long and almost monoexponential fluorescence lifetime ($\tau = 4.0 \text{ ns}$, $\alpha = 0.92$) supports this conclusion.
- (15) The net decrease is 55%, corresponding to a distance of 10 Å. *R*₀ = 10.4 Å for the 5-FluoroTrp W30–**2** couple ($J = 4.25 \times 10^{-17} \text{ M}^{-1} \text{ cm}^3$, *Q* = 0.20).
- (16) The very high substrate specificity of EII^{ml}⁴ is also expressed in its binding affinity toward **2**. Titration experiments of wild-type and single-trp EII^{ml} mutants revealed a *K*_D of 50–100 μM for **2**, 2–3 orders of magnitude higher than that for **1**^{9a} (E. P. P. Vos, J. Broos, to be submitted for publication).
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