Molecular Environment Sensitivity of Tryptophan

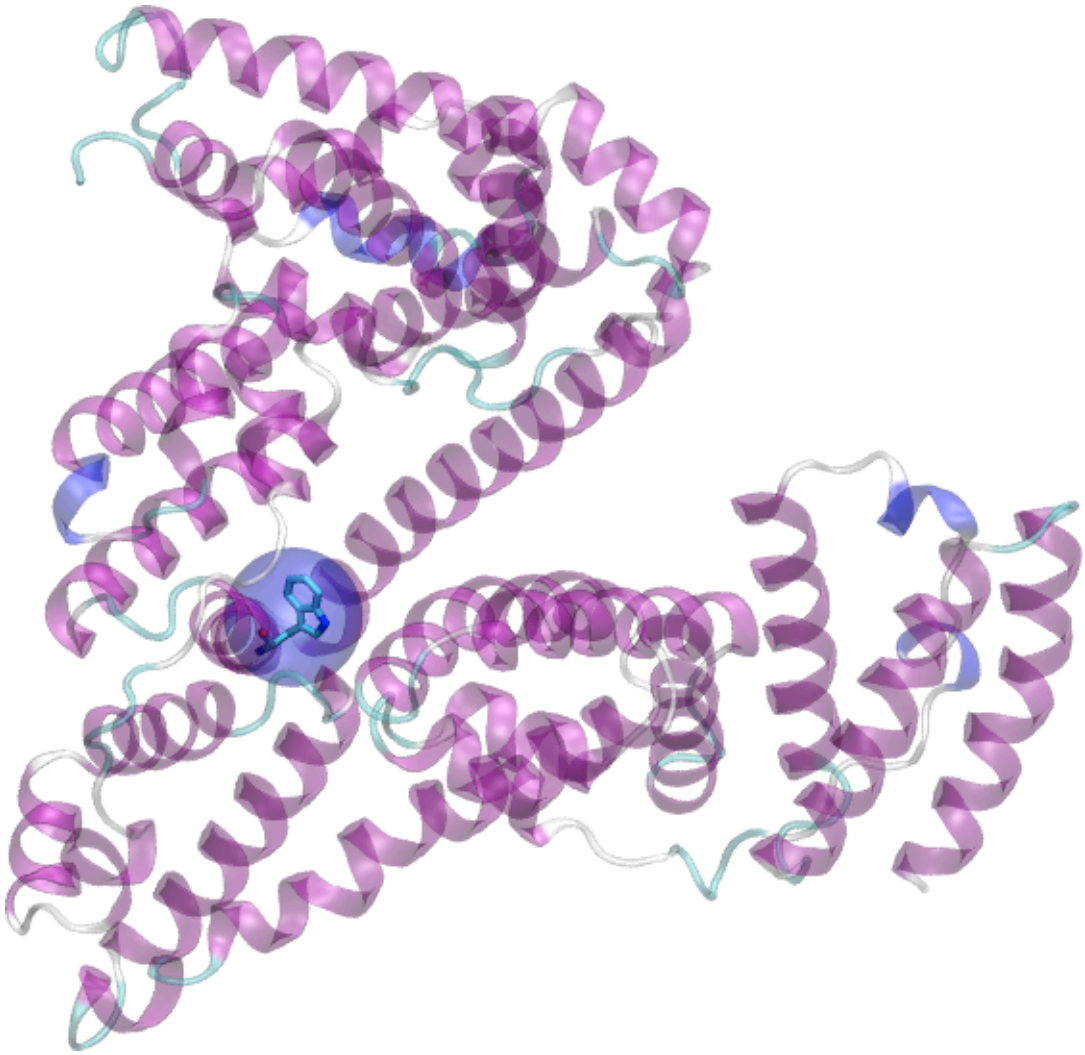

Figure. Crystal Structure of Human Serum Albumin (2XSI). This protein contains only a single trypthophan residue (here as a licorice representation) which is located in one of the two major drug binding sites on this macromolecule.

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Background

In proteins the dominant fluorophores is the indole group of tryptophan, Fig 1.[1] Indole absorbs near 280 nm and emits near 340 nm in aqueous solution at pH 7. The emission spectrum of indole is highly sensitive to solvent polarity and/or local environment. The emission of indole may be blue-shifted if the group is buried within a native and protein and its emission may be shifted to longer wavelengths when the protein is unfolded. Tryptophan is sensitive to general solvent effects and also display substantial spectral shift upon formation of a hydrogen bond to the imino nitrogen on the indole group which can be regarded as a specific solvent interaction.

Fluorophores that are sensitive to solvent environment are typically those that display a large charge separation in the excited state. Although indole is uniquely sensitive to solvent polarity, its spectral properties cannot be fully explained by the change in the dipole moment in the excited state. It is found that the complex spectral properties of tryptophan is due to the presence of two overlapping transitions in the long wavelength absorption band, transitions to the ^1L_a and ^1L_b-states of indole.

For most fluorophores the long wavelength absorption band represents a single electronic transition to the first excited state (S_1). In the case of tryptophan, the long-wavelength absorption band consits of two overlapping transitions to the ^1L_a and ^1L_b-states. These states have similar energies and depending on their environment either state can have the lower energy. Moreover, each state has a different dipole moment and they respond differently to solvent polarity.

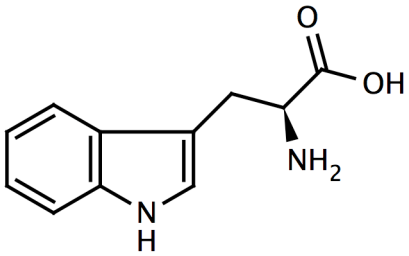

Figure 1. *The amino acid Tryptophan with its fluorescent indole ring.*

Aim of this Exercise

Investigate the molecular environment sensitivity of the amino acid tryptophan by recording a series of UV- and fluorescence spectra in buffer (Tris-HCl, 0.1 M, pH 7.0) and organic solvent mixtures thereof.

Material and Methods

Materials & Apparatus

- L-Tryptophan purchased from Sigma-Aldrich (Molecular weight, M_w= 204.23 g mol^{-1})
- UV cuvettes from Hellma (quartz, total volume of 3 mL)
- Fluorescence cuvettes (plastic, total volume of 3 mL)
- *Solvents:* Buffer (Tris-HCl, 0.1 M, pH 7.0), ethanol and acetonitrile
- Organic waste (where you should deposite your used acetonitrile)
- 250 mL volumetric flasks with stoppers (for stock solution preparation)
- 10 mL volumetric flasks with stoppers (for final sample solutions)
- UV-spectrophotometer (Hitachi-2000)
- Fluorimeter (Fluoromax-3 from Jobin-Yvon Horiba)
- Pasteur-pipettes (plastic)
- Voll-pipettes (1 mL, glass)

+ Standard lab equipment such as spoons, protective glasses, lab-coat…

Methods

Sample Preparation & UV-spectroscopy

1. Get safety instructions from the lab-supervisor!
2. Initially, mix buffer, ethanol and acetonitrile to end up with three flasks, each containing either pure buffer, buffer/ethanol (50:50 v/v) or buffer/acetonitrile (50:50 v/v). *Two groups can do this for all groups!*
3. Weigh up the amount needed of tryptophan in three separate volumetric flasks (250 mL, each) to prepare 100 µM solution of tryptophan! *Two groups can do this for all groups!*
4. Thereafter fill each 250 mL volumetric flask with either pure buffer, buffer-ethanol or buffer-acetonitrile mixtures. You should now have three different 100 µM solutions of tryptophan! ***Be sure to mark these solutions accordingly!***
5. Every group should then transfer 1 mL (Voll-pipette) from each of these 250 mL stock solutions to a new 10 mL volumetric flask and fill up to the mark with solvent. These solutions (three 10 mL volumetric flask with 10 uM tryptophan in either pure buffer, buffer-ethanol and buffer-acetonitrile mitures will be the solutions measured with UV and fluorescence spectroscopy! ***So take much care of these solutions!***
6. Start to blank the UV-spectrophotometer from background absorption from the solvent by recording in the wavelength interval 200-600 nm after filling both the reference cuvette (which should be placed in the back sample holder on the machine) and the sample cuvette (placed in the front) which just pure solvent). Approximately 75% of the total cuvette volume (with a plastic Pasteur pipette).

7. After this absorption scanning procedure, remove the sample cuvette in the front sample holder and fill it up with the tryptophan solution of choice.
8. Take up an UV absorption spectrum of tryptophan in the solvent investigated by scanning for absorption between 200-600 nm.
9. Repeat steps 6-8 for all the solvents investigated.
10. Save your output data after every experiment, import it in Graphpad or Excel and analyze it!

Fluorescence Steady-State Emission Spectroscopy

1. To remove background fluorescence (scattered light) from the solvent itself, initially take up a fluorescence emission spectrum for the solvent itself using a excitation wavelength (λ_{exc}) of 295 nm and record a spectrum between 250-650 nm.
2. Repeat this procedure for a cuvette that has been filled with your tryptophan solution in the same solvent.
3. Repeat this procedure for every solvent system
4. Download all spectra on your USB-stick after exporting your data to the .csv format.
5. Analyze your recorded spectra
6. Make graphs where you have inserted UV-absorbance and the emitted fluorescence in the same graphs for each solvent system.
7. These graphs should then be inserted into a word-document where specific questions regarding this laboratory exercise (see below) should be addressed and answered!

The Hand-in Assignment

Questions for You to Answer:

1. Which other amino acids than tryptohan display fluorescence emission?
2. Why does an absorption spectrum of a molecule very often display several peaks whereas the fluorescence emission spectrum only contains one single peak?
3. Why was 295 nm chosen as excitation wavelength during the recording of tryptophan fluorescence emission spectra?
4. Fluorescence spectroscopy is a much more sensitive technique when investigating local molecular environments compared to UV spectroscopy. Why is that?
5. What is the definition of the quantum yield and what are typical fluorescence lifetimes of biological fluorophores?
6. What is fluorescence quenching?
7. How can one use fluorescence quenching as a tool in the investigation on molecular interactions in biochemical and biophysical related problems?
8. Why is typically fluorescence spectra recorded in polar environments more red-shifted compared to non-polar environments?

9. What is the underlying principles for using reporter molecules such as tryptophan as a molecular reporter on conformational changes in proteins?
10. Please present models and physical properties for the solvents studied: Create the molecules (exchange buffer for water) and present them as 3D models (e.g. through PRODRG-server+VMD). Report also solvent polarities in terms of dielectric constants at 20 degrees Celsius.

References

(1) Lakowicz, J. R. *Principles of fluorescence spectroscopy*; Second edition.; Kluwer academic/Plenum publishers: New York, Boston Dordrecht, London, Moscow, 1999.