**Course** : PG Pathshala-Biophysics

**Paper 10** : TECHNIQUES USED IN MOLECULAR BIOPHYSICS II (Based on Spectroscopy)

**Module 07** : Absorption spectroscopy of Proteins: peptide bond, aromatic amino acids and prosthetic groups

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**Introduction**

Absorption spectroscopy is a technique that measures absorption of photons in samples dissolved in the transparent medium, in ultraviolet, visible or near infra red range of electromagnetic radiation (EMR) with the energy 150-400 kJmol\(^{-1}\). Molecules with electrons in delocalized aromatic system such as: proteins, nucleic acids and their constituents absorb in near UV (150-400 nm) or visible (400-800 nm) region. Absorbance depends linearly on the concentration of the solute and is suitable for quantitative measurements. This makes protein spectroscopy a very important tool which has many applications in biochemistry, medicinal chemistry, biophysics, industry etc.

**Objectives**

The objective of the present module is to:

a) Describe basic features of absorption spectroscopy of proteins,

b) Discuss UV-Vis absorption by: peptide bond, non-aromatic and aromatic amino acids, di-sulfide linkages and proteins,

c) Enumerate application of the technique for study of prosthetic groups in proteins,

d) Explain use of UV-Vis absorption spectroscopy for determination of pKa values of amino acids,

e) Introduce to use of UV-Vis spectroscopy for conformational changes in proteins, protein folding and unfolding,

f) Introduce to application of UV-Vis spectroscopy for study of protein-ligand interaction and enzyme kinetics.

**7.1 Basic features of absorption spectroscopy of proteins**

Proteins are the most important bio-molecules as these control body’s vital functions. Proteins are either linear polymers or the aggregates of linear polymers. The building blocks of proteins are amino acids (figure 7.1) which have a backbone of an amine (-NH2) and a carboxylic (-COOH) functional groups attached to a central asymmetric carbon atom ‘C\(\alpha\)’ with sp\(^3\) valence electron configuration, with four bonds in tetrahedral symmetry. With the exception of \(\alpha\) - amino proline, these have a hydrogen atom attached to the C\(\alpha\) atom. The amino acids differ from each other in side chain ‘R’ that is attached to the fourth valence of C\(\alpha\) atom. Latter can be either aliphatic, aromatic or heterocyclic in nature. All amino acids except ‘glycine’ are chiral.
in nature and exist in two different forms (handedness) ‘L’ and ‘D’ type (usually we use smaller size of capital letters).

The peptide bonds are formed by the release of a water molecule between α-carboxyl and α-amino groups of amino acids, when these combine. During polymerization C=O and N-H groups of two adjacent amino acids form a peptide bond (figure 7.2a). Latter has a partial double bonded character. The π-bond resonates between C=O and N-H groups. This leads to planarity of C=O N-H group. (Hoppe et al 1983; Darnell et al 1986) (figure 7.2b).

Figure 7.1 IUPAC-IUB nomenclature for twenty standard amino acids

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7.2 UV-Vis absorption by peptide bond, amino acids and proteins

Protein backbone as well as aromatic amino acids such as: Tyr, Trp and Phe can absorb in the UV region because of the presence of π electrons.
7.2.1 Absorption by peptide backbone

The n-π* transition in the peptide bond is usually observed between 210-220 nm \((\varepsilon_{\text{max}}=100 \ \text{L mol}^{-1} \ \text{cm}^{-1})\) while the main π–π* transition is around 190 nm with \(\varepsilon_{\text{max}}=7000 \ \text{L mol}^{-1} \ \text{cm}^{-1}\).

7.2.2 Absorption by non aromatic side chains

Side chains of His, Arg, Glu, Gln, Asn and Asp have a transition around 210 nm which is not usually observed. This is because it is masked from the absorption from a more intense amide bond of the backbone which absorb at 230 nm and 290 nm. It consists of at least three unresolved electronic transitions.

The haem group and copper containing cofactors absorb in visible region. The porphyrin ring in heme shows Soret band absorption at 429.5 nm and series of partially forbidden Q-bands between 500-600 nm. Both these bands are due to electronic transitions singlet to singlet, mostly n-π*, while plastocyanin absorption at 597 nm is due to Cu-S1 bond where S_pz-Cu_{dx2-y2} charge transfer occurs. This is the reason why haemoglobin is red and plastocyanin is blue.

A typical absorption spectra of hemoglobin and plastocyanin is shown in figure 7.3

![Absorption spectra of oxy and deoxy hemoglobin](https://en.wikipedia.org/wiki/Near-infrared_window_in_biological_tissue)

![Absorption spectrum by plastocyanin](https://en.wikipedia.org/wiki/Plastocyanin)

7.2.3 UV-Vis Absorption by aromatic amino acids

Molecular extinction coefficient of aromatic amino acids: Phe, Tyr and Trp at \(\lambda_{\text{max}}\) 257, 274 and 280 nm are respectively 195, 1490, and 5500 \(\text{L mol}^{-1} \ \text{cm}^{-1}\). The transition in Phe at 257.5 nm is weak as it arises from a forbidden π–π* transition. There is couple of minor peaks between 240-270 nm (Schmid) (figure 7.4).
7.2.4 Absorption by di-sulfide linkage

The Cys side chains in the protein can form a di-sulfide (S-S) bonds. Latter is usually the end product of oxidation according to reaction

\[2\text{CH}_2\text{SH}+\frac{1}{2}\text{O}_2 \leftrightarrow \text{-CH}_2\text{-S-S-CH}_2+\text{H}_2\text{O}\]

SH groups in different parts of the protein structure are oxidized and form S-S linkage or di-sulfide linkage. Latter also possess \(\pi\) electrons and can give absorption in the UV region with a small extinction coefficient 125 L mol\(^{-1}\) cm\(^{-1}\).

![Ultraviolet absorption spectra of Trp, Tyr and Phe at pH 6](image)

Figure 7.4 Ultraviolet absorption spectra of Trp, Tyr and Phe at pH 6, Wetlaufer D B 1962 Figure from Advances in protein chemistry 17,303.

We list in table 7.1 some important transitions of proteins and amino acids in UV-Vis region.

Table 7.1 UV-vis transitions in proteins (table from Hoppe et al 1983).

<table>
<thead>
<tr>
<th>Bond</th>
<th>(\lambda_{\text{max}})</th>
<th>(\varepsilon)</th>
<th>transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>-COO-R</td>
<td>205</td>
<td>50</td>
<td>n-(\pi^*)</td>
</tr>
<tr>
<td></td>
<td>165</td>
<td>4x10(^3)</td>
<td>(\pi-\pi^*)</td>
</tr>
<tr>
<td>&gt;C=O</td>
<td>280</td>
<td>20</td>
<td>n-(\pi^*)</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>2x10(^3)</td>
<td>n-(\sigma^*)</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td></td>
<td>(\pi-\pi^*)</td>
</tr>
<tr>
<td>&gt;C-S</td>
<td>500</td>
<td>10</td>
<td>n-(\pi^*)</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>9x10(^3)</td>
<td>(\pi-\pi^*)</td>
</tr>
<tr>
<td>S-S</td>
<td>250-230</td>
<td>10</td>
<td>(\pi-\sigma^*)</td>
</tr>
<tr>
<td>&gt;C=C&lt;</td>
<td>190</td>
<td>9x10(^3)</td>
<td>(\pi-\pi^*)</td>
</tr>
<tr>
<td>N-N</td>
<td>175</td>
<td>8x10(^3)</td>
<td>(\pi-\pi^*)</td>
</tr>
<tr>
<td></td>
<td>220</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrimidine</td>
<td>300</td>
<td>325</td>
<td>n-(\pi^*)</td>
</tr>
</tbody>
</table>
When peptide group and aromatic amino acids are part of asymmetric environment, as in folded proteins, left handed and right handed circularly polarized light have different absorption coefficients. This leads to circular dichroism (CD) and optical rotator dispersion (ORD) (discussed elsewhere).

The absorbance of Trp and Tyr depend on the micro environment of the chromophore and shows a slight red shift (bathochromic effect) in shifting from non polar to polar environment. Example can be of interior of globular proteins which has different environment compared to surface of the protein.

<table>
<thead>
<tr>
<th></th>
<th>245</th>
<th>2x10^3</th>
<th>n-π*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>165</td>
<td>8x10^3</td>
<td>π-π*</td>
</tr>
<tr>
<td>Porphyrin</td>
<td>&gt;400</td>
<td></td>
<td>Extended π electron system</td>
</tr>
<tr>
<td>Peptide bond</td>
<td>190</td>
<td></td>
<td>Small π electron system</td>
</tr>
</tbody>
</table>

In native proteins the residues that are buried in the centre and those which are exposed will contribute differently to UV-Vis spectrum. A typical example of wild type RNAse in .1M sodium acetate and unfolded protein in 6M GdmCl is shown in figure 7.5a. The differences (shown by dotted and continuous lines for wild type and in unfolded protein in Guanudium chloride in the same buffer) are usually small. The difference spectrum (shown in figure 7.5b) is helpful for analysis.

**7.3 Study of prosthetic groups of proteins**

Prosthetic groups in proteins very often have a strong electronic absorption bands that depend on oxidation, ligation and conformational state of chromophores. These are sensitive to the conformational changes in the polypeptide chain into which these are embedded. Steady-state absorption spectroscopy
provides information on ligand binding equilibria, from which the Gibbs free energy (differences between the ligated and unligated states) can be computed. Time-resolved absorption spectroscopy allows one to detect short-lived intermediate states that may not get populated significantly under equilibrium conditions, but may nevertheless be of crucial importance for biological function. Moreover, the energy barriers that have to be surmounted in the reaction can be determined.

A typical example can be of heme proteins. Presence of heme prosthetic group in these proteins gives rise to UV-Vis absorbance spectra that vary with the state of heme group. Compact spark spectral sensor can be used to measure spectral changes in hemoglobin, myoglobin and cytochrome C. Spectra obtained in water 1µg/mL is shown in figure 7.6.

![Absorbance Spectra of Heme Proteins](image)

Figure 7.6 The spark Vis absorption sensor and its attached holder to cuvette with white led module measured absorbance spectra of heme proteins. Figure from http://oceanoptics.com/small-footprint-absorbance-system-for-characterizing-heme-proteins/

7.4 Use of UV-Vis spectroscopy for determination of pK values of amino acids

Free amino acids are present in neutral aqueous solvent in a dipolar zwitterions form which makes them *amphoteric* electrolytes (*ampholytes*). This can be described by equations:

\[
H_3N^+ - CHR - COOH + H_2O \rightleftharpoons H_3N^+ - CHR - COO^- + H_3^+ O_{pH<2}
\]

\[
H_3N^+ - CHR - COO^- + H_2O \rightleftharpoons H_2N - CHR - COO^- + H_3^+ O_{pH>8}
\]

The acid base properties of such *ampholytes* are covered by Brönstead_Lowry theory (Hoppe et al 1983). The pK values are defined as the actual midpoints of the curve (figure 7.7) at which half of the protons of conjugated acid are dissociated after addition of either half or one and half
base equivalent. One can differentiate between $pK_1'$ value for dissociation of the carboxylic group in the acidic pH range and $pK_2'$ value for the dissociation of amino group in basic pH range. The bifunctional amino acids have an additional $pK_a$ value for their side chains. Values of $pK'$ for some important amino acids are given below in the table 7.2.

Table 7.2 Values of $pK'$ for some important amino acids

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>$pK_1'$</th>
<th>$pK_2'$</th>
<th>$pK_a$ Side chain R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>2.34</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>2.34</td>
<td>9.69</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>2.36</td>
<td>9.60</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>2.21</td>
<td>9.15</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>2.63</td>
<td>10.43</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>2.17</td>
<td>9.13</td>
<td></td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>2.09</td>
<td>9.82</td>
<td>3.86</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2.19</td>
<td>9.67</td>
<td>4.24</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.82</td>
<td>9.17</td>
<td>6.0</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.71</td>
<td>10.78</td>
<td>8.33</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.20</td>
<td>9.11</td>
<td>10.07</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.18</td>
<td>8.95</td>
<td>10.53</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.17</td>
<td>9.04</td>
<td>12.48</td>
</tr>
</tbody>
</table>

(Table adapted from Hoppe et al 1983)

Figure 7.7 Titration curve for alanine. At the turning point predominant ionic forms are indicated.
Within hydrophobic amino acids, hydrophobicity character shows considerable variation Gly < Ala < Val < Ile. Latter can be estimated using $\Delta G$ -free energy for transfer of amino acid from water to organic solvent. $\Delta G_{\text{transfer}}$ can be calculated for different side chains by subtracting the contribution for backbone (value for Glycine) using UV-Vis spectroscopy because there are specific spectral changes associated with the same. Thus for example Tyr has absorbance maximum at 280 nm at pH 7.0. The pKa of phenolic group of Tyrosine is 10.1. At pH greater than 10, Tyrosinate absorbs at 295 nm. One can follow decrease in OD at 280 nm and increase in OD at 295 nm to determine the pK using the equation

$$pH = pK^' + \log \frac{[\text{Tyrosinate}]}{[\text{Tyrosine}]}$$

The pK is dependent on polar/nonpolar environment of the protein. The absorption maxima of tyrosine can reflect its ionization state.

A typical example of use of UV-Vis spectroscopy for determination of pK values for amino acids Gly, Ala, Val, Arg, Lys and Cys is described by (Baran et al 1997). Authors used $(\text{Fe(CN)}_5\text{H}_2\text{O})^{-3}$ which has $\lambda_{\text{max}}$ at 440 which shifts to lower wavelength side (410 nm) in concentrated solution. The authors also used $(\text{Ru(CN)}_5\text{H}_2\text{O})^{-3}$ as it provides more labile coordination that allows for incorporation of amino acid. The reaction takes place as per equation:

$$\text{Ru(CN)}_5\text{H}_2\text{O}^{-3} + \text{AA} = \text{Ru(CN)}_5\text{AA}^{-3} + \text{H}_2\text{O}$$

Reaction of $[\text{Ru(CN)}_6]^{-4}$ with Br2 results primarily in producing oxidation state of Ru(II) in centre, which is rapid and has yellow color with $\lambda_{\text{max}}$ at 312 nm.

$$\text{Ru(CN)}_6^{-4} + \text{Br}_2 = \text{Ru(CN)}_5\text{H}_2\text{O}^{-3} + \text{BrCN} + \text{Br}^-$$

At higher concentration (greater than $10^{-4}$) the $[\text{Ru(CN)}_5\text{H}_2\text{O}]^{-3}$ ion is observed to undergo slow dimerization reaction presumably leading to cyanide-bridged $[\text{Ru2(CN)}_{10}]^{-6}$ ion which shifts $\lambda_{\text{max}}$ to 290 nm.
The pKa values can be determined by spectrophotometric titration. Absorbance is measured at fixed \( \lambda \) and pH. Absorbance is plotted with respect to [H⁺] and pK values determined by graphic and numerical methods. In these experiments NH₃ can be eliminated by bubbling. When strong acid HClO₄ is added to [Fe(CN)_5L]⁻³ and [Ru(CN)_5L]⁻³ there is a change in electronic spectrum as shown in figure 7.8. When the solution is titrated with HClO₄ the coordination of CN⁻ is protonated.

Amino acids Gly, Ala, Val, Lys, Arg Cys can be then added by slow stirring. Various amounts of HClO₄ were added. Figure 7.9 shows determination of pKa value for Cys complexes. The pKa values for Ala, Gly Val are respectively 2.53, 2.65 and 2.58, while those for Lys, Arg and Cys are respectively 2.50, 2.63 and 3.47. For more information refer to Baran et al.

7.5 Use of UV-Vis spectroscopy for conformational changes in proteins, protein folding and unfolding

Native proteins can be unfolded by denaturants as guanidium chloride (GdmCl) or urea. These changes lead to change in the micro-environment around chromophores Tyr, Trp and Phe and shift in absorption spectra. Protein uncoiling due to heating, change in pH of buffer etc would also lead to exposure of chromophores and change in absorbance. If the di-sulfide linkages are ruptured then it would lead to change in the absorption due to S-S linkage. There may be a small change in refractive index (it would decrease) with temperature as well as the protein concentration (due to the thermal expansion of the solution), and ionization of dissociable groups. However these effects influence protein absorbance only to a minor extent and therefore the dependence on temperature is usually small in the absence of structural transitions. Absorbance of native protein may change.

When denaturants are added, the absorbance of Tyr and Trp at 287 nm and at 291 nm, respectively, increases slightly, even in the absence of structural transitions. This originates from the change in refractive index (i.e. the polarity) of the solvent with the concentration of GdmCl or urea.

We show in table 7.3 absorbance properties of the aromatic amino acids (a-absorption coefficient at \( \lambda_{max} \) in water at neutral pH, and b-at 280 nm as found in folded proteins). In the near-UV the molar absorbance of phenylalanine is much smaller than that of tyrosine and tryptophan, and the spectrum of a protein (such as ribonuclease T1, figure 7.5) between 240 and 300 nm is therefore dominated by the
contributions from the Tyr and Trp side-chains only. Phe residues contribute fine structure (‘wiggles’) to the spectrum between 250 and 260 nm. The aromatic amino acids do not absorb above 310 nm, and therefore protein absorbance should be zero at wavelengths greater than 310 nm.

Table 7.3 (from Schmid)

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{max}}$</th>
<th>$\varepsilon_a_{\text{max}}$ L mol$^{-1}$ cm$^{-1}$</th>
<th>$\varepsilon_b_{\text{max}}$ L mol$^{-1}$ cm$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp</td>
<td>280</td>
<td>5600</td>
<td>5500</td>
</tr>
<tr>
<td>Tyr</td>
<td>275</td>
<td>1400</td>
<td>1490</td>
</tr>
<tr>
<td>Phe</td>
<td>258</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

Absorption coefficient of any protein at 280 nm can be calculated as a linear combination of absorbance of these groups. For these we calculate number of Trp, Tyr and di-sulfide linkages $n_{\text{Trp}}$, $n_{\text{Tyr}}$ and $n_{S-S}$ and use the relation given below

$$\varepsilon_{280} \text{ (L mol}^{-1} \text{ cm}^{-1} \text{)} = 5500 \times n_{\text{Trp}} + 1490 \times n_{\text{Tyr}} + 125 \times n_{S-S}$$

7.6 Application of UV-Vis spectroscopy for the study of protein-ligand interaction and enzyme kinetics

UV-Vis absorption spectroscopy is a powerful tool for steady-state and time resolved studies of protein-ligand interactions. Prosthetic groups in proteins frequently have strong electronic absorbance bands that depend on oxidation, ligation and conformational state of proteins.

A typical example of protein-ligand interaction can be of interaction of dye Coomassie Brilliant Blue G-50(CBB) used for Bradford protein assay or calorimetric protein assay with Bovine serum albumin BSA protein (Katrahalli et al 2010). Under acidic condition the red from dye is converted into its blue form by binding to the protein to be assayed. During the formation of the assay two types of interactions take place. First the red form of the Coomassie dye donates its free electron to the ionizable groups in proteins which causes disruption of the proteins native state, consequently exposing its hydrophobic pockets. In the next step these pockets bind non-covalently to the non-polar region of the dye through van Der Waal’s interaction and positioning positively charged amino acids in the proximity of negative charge of the dye which strengthens further due to electrostatic interaction. The binding stabilizes the blue form of the dye. The amount of complex formation can be measured by the absorbance in the blue region which has $\lambda_{\text{max}}$ at 595 nm (figure 7.10).
Enzymes are macromolecules speed up the chemical reaction without being used. The rate at which enzyme is being used depends on substrate concentration, pH, temperature and presence of inhibitors. The catalytic event that converts substrate to product takes place in two steps. In the first step an enzyme substrate complex (ES) is formed. The reaction product arises when the enzyme substrate complex breaks. The process is described by Michaelis- Menton equation. Series of events take place in between. Formation of (ES) is fast and decomposition is slow.

\[
[ES] = \frac{[E][S]}{[S]+K_m}
\]

The rate \(K_2[ES]\) is given by a similar equation

\[
[ES] = \frac{K_2[E][S]}{[S]+K_m}
\]

If \([S]\) is low, kinetics is of the first order in \([S]\). If \([S]\) is large the kinetics reaches the turn over number with respect to \([S]\) and the rate approaches a limiting value

\[
R = K_2[E]^*_T
\]

The plot of \(R\) vs \([S]\) is not linear but Lineweaver –Burk plot and Eadie-Hofstee plots give a linear relationship which can be used to obtain enzyme kinetics (figure 7.11) (Timoti et al).
A typical example is use of tyrosinase to catalyze conversion of DL-DOPA to halochrome- a red compound which absorbs visible radiation at 475 nm. Tyrosinase is a copper containing enzyme that catalyses production of melanin and other pigments from tyrosine by oxidation. It has hydrophobic pocket adjoining a binuclear copper active site. One of the copper atoms binds to ε-nitrogen of His38, His54 and His63. The other copper atom binds to His190, His194 and His216. One probable mechanism is to get O2 trapped between two copper atoms. The reaction is monitored by measuring increase in absorbance at 475 nm. Halochrome is not the initial product of the oxidation of DOPA. Oxidation of DOPA to DOPA quinine is the rate limiting process, which occur rapidly. As a result the rate of formation of halochrome is the same as the rate of formation of DOPA quinine. The rate constant can be calculated as shown in figure 7.11.

Summary

The module introduces reader to the basic features of absorption spectroscopy of proteins. We have discussed UV-Vis absorption by peptide bond, non-aromatic and aromatic amino acids, disulfide linkage and protein molecules. We described application of absorption in UV-Vis range to study prosthetic groups in proteins. We have elaborated on the application for measurement of pKa values of amino acids. A typical example of calculating pKa values of Gly, Val, Ala, Arg, Lys and Cys using (Fe(CN)₅H₂O)₃⁻ which has λ_max at 440 and (Ru(CN)₅H₂O)₃⁻ is given. Lastly we have introduced the reader to application of UV-Vis spectroscopy for study of conformational changes in proteins, protein folding and unfolding, ligand protein interaction and enzyme kinetics.