Biological Macromolecules: UV-visible Spectrophotometry

Introductory article

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Biological macromolecules such as proteins and nucleic acids absorb light in the UV-visible region of the spectrum. Absorbance measurements are used for measuring concentrations, for the detection of conformational changes and of ligand binding, and for following enzyme reactions.

Introduction

Spectroscopy is a technique that measures the interaction of molecules with electromagnetic radiation. Light in the near-ultraviolet (UV) and visible (vis) range of the electromagnetic spectrum has an energy of about 150–400 kJ mol^{-1}. The energy of the light is used to promote electrons from the ground state to an excited state. A spectrum is obtained when the absorption of light is measured as a function of its frequency or wavelength. Molecules with electrons in delocalized aromatic systems often absorb light in the near-UV (150–400 nm) or the visible (400–800 nm) region.

Absorption spectroscopy is usually performed with molecules dissolved in a transparent solvent, such as in aqueous buffers. The absorbance of a solute depends linearly on its concentration and therefore absorption spectroscopy is ideally suited for quantitative measurements. The wavelength of absorption and the strength of absorbance of a molecule depend not only on the chemical nature but also on the molecular environment of its chromophores. Absorption spectroscopy is therefore an excellent technique for following ligand-binding reactions, enzyme catalysis and conformational transitions in proteins and nucleic acids. Spectroscopic measurements are very sensitive and nondestructive, and require only small amounts of material for analysis.

Spectrophotometers

Spectrophotometers are standard laboratory equipment. They usually contain two light sources: a deuterium lamp, which emits light in the UV region and a tungsten–halogen lamp for the visible region. After passing through a monochromator (or through optical filters) the light is focused into the cuvette and the amount of light that passes through the sample is detected by a photomultiplier or a photodiode. In double-beam instruments a cuvette with buffer is placed in the reference beam, and its absorbance is subtracted from the absorbance measured for the sample. A spectrum is obtained when the wavelength of the incident light is changed continuously. In diode-array spectrophotometers the sample is illuminated by the full lamp light. After passage through the cuvette the transmitted light is spectrally decomposed by a prism into the individual components and quantitated by an array of diodes, often in intervals of 2 nm. In diode-array spectrophotometers the entire spectrum is recorded at the same time and not by a time-dependent scan as in conventional instruments. Thus spectral changes can be followed simultaneously in a wide range of wavelengths.

The buffers used for absorbance measurements should not absorb light in the wavelength range of the experiment. For work in the near-UV, buffer absorbance should be small above 220 nm, and indeed most of the solvents commonly used in biochemical experiments do not absorb in this spectral region. Buffers that contain carboxyl and/or amino groups absorb light below 220 nm, and therefore should not be used when working in this wavelength range. Buffers with very low absorbance in the far-UV include phosphate, cacodylate and borate.

Protein and Nucleic Acids Concentrations

Lambert–Beer law

The concentrations of proteins or nucleic acids in solution can be easily and accurately determined by absorbance measurements. The absorbance (A) is related to the intensity of the light before (I_0) and after (I) passage through the protein solution by eqn [1], and the absorbance depends linearly on concentration, according to the Lambert–Beer law (eqn [2]).

$$A = -\log_{10}(I/I_0) \qquad [1]$$

$$A = \varepsilon c l \qquad [2]$$

In eqn [2], c is the molar concentration, l is the pathlength in cm, and ε ($L\,mol^{-1}\,cm^{-1}$) is the molar absorption coefficient. The concentration of a substance in solution can thus be determined directly from its absorbance using eqn [2]. The measurement of absorbances higher than 2 should be avoided, because only 1% of the incident light is transmitted through a solution with an absorbance of 2 (and is quantitated by the photomultiplier).

Absorption coefficients of proteins

Proteins usually show absorption maxima between 275 and 280 nm (**Figure 1**), which are caused by the absorbance of the two aromatic amino acids tryptophan (Trp) and tyrosine (Tyr) and, to a small extent, by the absorbance of cystine (i.e. of disulfide bonds). The absorbances of Trp and Tyr depend on the microenvironment of their chromophores, and they are slightly red-shifted when transferred from a polar to a nonpolar environment, such as in the interior of a globular protein (see below). As a consequence, in native proteins, the residues that are exposed to solvent and those that are buried will contribute differently to the absorption coefficient. These differences are small, however, typically smaller than 5%.

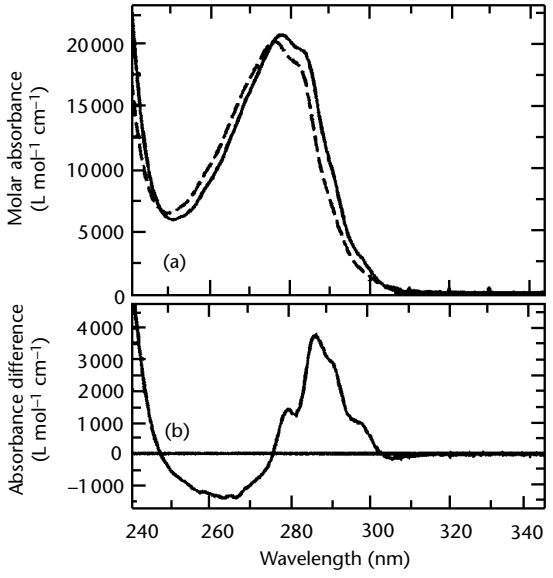

Figure 1 (a) Ultraviolet absorption spectra of the protein ribonuclease T1. The spectrum of the native protein (in 0.1 mol L^{-1} sodium acetate, pH 5.0) is shown by the continous line, the spectrum of the unfolded protein (in 6.0 mol L^{-1} guanidinium chloride in the same buffer) is shown by the broken line. Ribonuclease T1 contains nine Tyr residues, which give rise to the maximum at 278 nm, the single Trp residue leads to the shoulders between 280 and 300 nm. The small contributions of the four Phe residues near 260 nm are barely detectable. (b) Difference spectra between the native and the unfolded protein. The major difference at 287 nm arises from the exposure of the Tyr residues in the unfolded protein, the shoulders between 290 and 300 nm originate from exposure of the Trp residue. Spectra of 15 μmol L^{-1} protein were measured at 25°C in 1-cm cuvettes.

Accordingly, the absorption coefficient ε of a protein can be calculated in a simple fashion. First the numbers of its Trp, Tyr and Cys disulfide bonds (n_{Trp}, n_{Tyr} and n_{SS}, respectively) are counted, and then ε is calculated by use of eqn [3] as the linear combination of the individual contributions of these amino acid residues.

$$\varepsilon_{280}\,(L\,mol^{-1}\,cm^{-1}) = 5500 \times n_{Trp} + 1490 \times n_{Tyr} + 125 \times n_{SS} \qquad [3]$$

The molar absorbances of Trp, Tyr and Cys disulfide bonds (5500, 1490, and 125 $L\,mol^{-1}\,cm^{-1}$ respectively) represent average values for the chromophores in folded proteins. ε_{280} values calculated by this simple procedure show an accuracy of about $\pm$ 5%. A paper by Pace *et al.* (see Further Reading) compares calculated and experimental ε_{280} values for many proteins.

If more accurate ε_{280} values are required, two solutions with identical protein concentrations must be analysed: one containing just buffer and one containing buffer plus 6 mol L^{-1} guanidinium chloride. The absorbance of the unfolded protein with solvent-exposed chromophores can then be modelled by using reference ε_{280} values for Trp, Tyr and the disulfide chromophore determined in 6 mol L^{-1} guanidinium chloride.

Concentrations of nucleic acids

The concentrations of nucleic acids in solution are routinely determined from their strong absorbance at 260 nm. In fact, amounts of nucleic acid are often given as 'A_{260} units'. For double-stranded deoxyribonucleic acid (DNA) one A_{260} unit is equivalent to 50 μg DNA; for single-stranded DNA it is equivalent to 33 μg DNA; and for single-stranded ribonucleic acid (RNA) it is equivalent to 40 μg RNA. All these amounts would cause an A_{260} of 1 when dissolved in 1 ml and measured in a 1-cm cuvette. Proteins absorb much more weakly than nucleic acids. Contaminating proteins therefore hardly affect the concentrations of nucleic acids, as measured by A_{260}. In a 1:1 mixture of nucleic acids and proteins, the proteins contribute only about 2% to the total absorbance at 260 nm.

Absorbance of Proteins

Molecular origin of protein absorbance

The peptide groups of the protein main chain absorb light in the 'far-UV' range (180–230 nm). The aromatic side-chains of Tyr, Trp and Phe also absorb light in this region and, in addition, they absorb in the 240–300 nm region (**Table 1**). This region is called the 'near-UV' or the

Table 1 Absorbance of the aromatic amino acids

Compound	λ_{max} (nm)	ε^{a}_{max} ($L\,mol^{-1}\,cm^{-1}$)	ε^{b}_{280} ($L\,mol^{-1}\,cm^{-1}$)
Tryptophan	280	5600	5500
Tyrosine	275	1400	1490
Phenylalanine	258	200	

[a]Absorption coefficient at λ_{max} in water at neutral pH; data are from Eftink MR (1991) In: Suelter CH (ed.) *Methods of Biochemical Analysis*, vol. 35, p. 127. New York: Wiley.

[b]Absorption coefficients at 280 nm; average values, as found for folded proteins; data are from Pace CN and Schmid FX (1997) How to determine the molar absorption coefficient of a protein. In: Creighton TE (ed.) *Protein Structure: A Practical Approach*. p. 253. Oxford: IRL Press.

'aromatic' region. Disulfide bonds that form between two cysteine residues also show an absorbance band near 260 nm. Many cofactors of proteins absorb light in the UV-vis region. Reduced nicotinamide–adenine dinucleotide (NADH) and reduced flavin–adenine dinucleotide ($FADH_2$) show spectra in the near-UV; haem groups and copper-containing cofactors absorb in the visible region. Therefore haemoglobin is red and plastocyanin is blue. When the peptide groups and the aromatic residues are part of an asymmetric structure, or when they are immobilized within an asymmetric environment (as in folded proteins), left-handed and right-handed circularly polarized light are absorbed to different extents. This phenomenon is called circular dichroism.

The absorbance properties of the aromatic amino acids are shown in **Table 1**. 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 1**) between 240 and 300 nm is therefore dominated by the contributions from the Tyr and Trp side-chains. 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. Proteins without Trp residues do not absorb above 300 nm.

Dependence on environment

The absorption spectra of the aromatic amino acids are sensitive to changes in their environment. In general, shifts in the wavelength of maximal absorption predominate: a blue-shift of the spectra is observed when the polarity of the solvent increases. For example, the maximum of the absorption of tyrosine is shifted by about 3 nm, from 277 nm to 274 nm, when the solvent is changed from carbon tetrachloride to water. This spectral shift, combined with minor changes in the strength of absorbance and in the fine structure of the spectrum, leads to maxima in the difference spectra in the descending slope of the original spectrum, that is in the 285–288 nm region for tyrosine and around 290–300 nm for tryptophan.

In folded native proteins, the aromatic residues that are buried in the hydrophobic core of the molecule also show a small red shift in their absorbance, which is reversed when they become exposed to the aqueous solvent upon unfolding. This is illustrated by the spectra obtained for the protein ribonuclease T1 (**Figure 1**). The maximal differences in absorbance occur in the 285–295 nm region. The difference spectrum shows several bands between 280 nm and 300 nm, which originate from the nine tyrosines and the single tryptophan residue. Generally, the absorbance differences between the native and the unfolded forms of a protein are small, but extremely useful for monitoring conformational changes of a protein.

Protein unfolding

Native proteins can be unfolded by heat or by denaturants such as urea or guanidinium chloride (GdmCl). Protein unfolding transitions can thus be measured by following the absorbance changes at 287–292 nm as a function of temperature or denaturant concentration. The absorbances of native and of unfolded protein molecules can also depend on temperature. The refractive index decreases slightly with temperature, as well as the protein concentration (due to the thermal expansion of the solution), and the ionization of dissociable groups can change. Together, 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. When denaturants are added, the absorbances of tyrosine and tryptophan at 287 nm and at 291 nm, respectively, increase 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. Similar effects are also observed when other denaturants are employed.

Absorbance of Nucleic Acids

Molecular origin of nucleic acid absorbance

Nucleic acids show a strong absorbance in the region of 240–275 nm. It originates from the $\pi \rightarrow \pi^*$ transitions of the pyrimidine and purine ring systems of the nucleobases. The bases can be protonated and therefore the spectra of DNA and RNA are sensitive to pH. At neutral pH the absorption maxima range from 253 nm (for guanosine) to 271 nm (for cytidine), and, as a consequence, polymeric DNA and RNA show a broad and strong absorbance near 260 nm. Spectra for the individual bases and for nucleic acids are

found in the text book by Cantor and Schimmel (see Further Reading).

In native DNA the bases are stacked in the hydrophobic core of the double helix and accordingly their absorbance is considerably decreased relative to the absorbance of single-stranded DNA and even more so relative to short oligonucleotides in which the aromatic bases are exposed to the aqueous solvent. The decrease in absorbance upon base stacking in the interior of DNA or RNA double helices is called hypochromism. It provides a very sensitive and convenient probe for monitoring strand dissociation and unfolding ('melting') of DNA double helices.

Nucleic acid denaturation

The two strands in a double-helical DNA or RNA (a duplex) are held together by noncovalent forces, primarily by hydrogen bonds between the bases and by hydrophobic and van der Waals interactions between the aromatic rings (the so-called stacking interactions) of the bases. These weak interactions can be broken by heating. The concomitant denaturation is usually referred to as 'melting' of the duplex structure. It can be followed conveniently by the increase in absorbance near 260 nm. A plot of the absorbance as a function of temperature is called a melting curve, and the temperature at which the increase in absorbance is 50% complete is defined as the melting temperature of the double-helical nucleic acid that is studied.

The melting temperature of a double-helical DNA molecule depends on the nature of the solvent and on its relative content of G + C versus A + T. In double-helical DNA the negatively charged phosphate groups of the backbone repel each other and thus lower the stability of the duplex. This unfavourable electrostatic repulsion is progressively screened when counter-ions (typically in the form of NaCl) are added in increasing concentrations. Thus, duplex stability increases strongly with the concentration of salt in the solvent. GC base pairs form three hydrogen bonds and are thus more stable than AT base pairs, which form only two hydrogen bonds. Therefore, the melting temperature of DNA duplexes increases with G + C content; in fact, the G + C content of a DNA can be determined from its melting temperature. Long DNA duplexes contain regions with different G + C contents, which can melt independently of one another. Their melting curves are complex and encompass several melting points; they can be identified as maxima in the first derivative of the melting curve.

Applications in Enzyme Kinetics

An enzyme catalyses the conversion of one or several substrates to one or several products. The rate of the catalysed reaction or the activity of the enzyme can be determined by measuring either the decrease in substrate concentration or the increase in product concentration as a function of the reaction time. When the substrate (S) and the product (P) differ in absorbance, the progress of an enzymatic reaction can be followed directly by monitoring the change in absorbance as a function of time. The absorbance changes are linearly related with the changes in concentration (via the Lambert–Beer relation, see eqn [2]) and therefore the reaction rates ($d[P]/dt$ or $-d[S]/dt$) can be calculated directly from the absorbance data when the absorption coefficients of the reacting species are known. NADH-linked enzyme reactions, such as those catalysed by the lactate, malate or alcohol dehydrogenases provide excellent examples for absorbance-based enzyme assays. The reduced nicotinamide ring in NADH shows an absorbance maximum near 340 nm ($\varepsilon_{340} = 6220\,\mathrm{L\,mol^{-1}\,cm^{-1}}$), which is lost upon oxidation to NAD^+. Thus the activities of these dehydrogenases can be measured directly by following the decrease in A_{340} as a function of time.

When no absorbance changes occur during the reaction of an enzyme with its natural substrate, often coloured derivatives can be synthesized with chromophoric reporter groups. A commonly used reporter is 4-nitrophenolate, which absorbs near 400 nm. This group can be esterified with acetic acid in 4-nitrophenyl acetate (to serve as a substrate for proteases), with phosphate (as a substrate for phosphatases) or with sugars (to probe amylases or glycosidases).

In favourable cases a 'silent' enzyme reaction with a colourless product can be coupled with another enzyme reaction that uses the product of the first enzymatic reaction for a conversion that leads to a change in absorbance. Often such silent reactions are coupled to an NADH-consuming or NADH-producing step. As outlined above, changes in NADH concentration are easily followed by the strong change in absorbance at 340 nm. It is, of course, essential that in such coupled enzyme assays the rate of the indicator reaction is much higher than the rate of the primary reaction. This is usually achieved by using the indicator enzyme in a high concentration.

Further Reading

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