

Alexander P. Demchenko

# ***Ultraviolet Spectroscopy of Proteins***



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Professor Dr. Alexander P. Demchenko

Palladin Institute of Biochemistry  
Academy of Sciences of the Ukraine  
Leontovichstr. 9  
Kiev 252030, USSR

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## Preface

The aim of this book is to give a comprehensive description of the basic methods used in the ultraviolet spectroscopy of proteins, to discuss new trends and development of these methods, and to analyze their different applications in the study of various aspects of protein structure and dynamics.

Ultraviolet spectroscopy is one of the oldest and most popular methods in the field of biochemistry and molecular biophysics. At present, it is difficult to imagine the biochemical laboratory without a recording spectrophotometer or spectrofluorimeter. There are several hundreds of publications directly devoted to protein ultraviolet spectroscopy and in a great number of studies UV spectroscopic methods are used for the structural analysis of different proteins. Meanwhile a unified description of the theoretical basis of the methods, experimental techniques, data analysis, and generalization of results obtained in solving the specific problems of protein structure are lacking.

There are three reasons for which a monograph on ultraviolet spectroscopy is needed today. Firstly, there has been significant growth in facilities of experimental technique, its precision, and versatility associated with computer data analysis. This new technique is available to a wide circle of scientists engaged in the field of protein research. Most of them are not spectroscopists and, thus, there is a need for a conceivable and precise source of information on how to use this method and what kind of data it should provide. Secondly, the recent development of electronic spectroscopy of liquids, especially the molecular relaxation and molecular interaction spectroscopy has laid the foundations of the analysis of the origin of correlation between the structure of proteins and their spectroscopic properties. A very important step can be made now from the empirical treatment of data to their understanding within the framework of general theory. And finally, the methods of UV spectroscopy aimed at studies of protein conformation and conformational dynamics at the level of interacting groups of atoms, provide scientists with ample opportunities in elucidating the fundamental principles of protein submolecular structure and its relation with functional properties of proteins. It is time to draw the attention of biochemists and biophysicists to the opportunities now available.

This volume is intended to serve both as a laboratory manual presenting different spectroscopic procedures which are currently of interest in various fields of protein studies and as a scientific monograph in which spectroscopic information is analyzed in terms of protein structure and dynamics and aimed at solving fundamental problems. This work was not designed as a comprehensive

source of detailed reference material. It is rather an attempt to integrate the ideas and current understanding in this field of study.

An introduction to electronic absorption and emission spectroscopy and to the spectroscopy of molecular interactions in condensed media with special reference to the chromophore groups that occur in proteins will be presented in this book. The methods of difference, solvent, and temperature perturbation spectroscopy are also described in detail. The theory and application of the recently developed method of derivative spectroscopy are discussed.

The correct interpretation of the protein emission spectra depends on the analysis of the excited state processes, the most important of which are quenching, structural relaxation, and excitation energy transfer. The application of methods of steady state and time-resolved emission spectroscopy to the investigation of protein structure and dynamics is discussed. The unified approach based on inhomogeneous broadening of spectra and molecular relaxation spectroscopy results in new experimental techniques for studies of the dynamic behavior of proteins.

On the basis of the different spectroscopic methods presented in this book, along with the other physical methods aimed at studies of proteins, the general "molecular probe" approach to the problem of protein structure is formulated and its advantages are discussed.

The monograph describes the analytical application of ultraviolet spectroscopy: protein assay, determination of tyrosine, tryptophan, and phenylalanine, etc. Special attention is paid to experimental technique.

The author in his development as a researcher in the field of protein spectroscopy is much obliged to prominent works of G. Weber and S. V. Konev showing how the unambiguity of physical analysis may be in harmonic unity with the complexity of the subject of research, the logic and beauty of the problem. The author expresses his sincere thanks to his teacher in protein research V. A. Belitser for helpful discussions and advice and to G. V. Troitsky for encouragement and criticism. He also wishes to thank many colleagues for participation in the experimental work, reading of the manuscript as well as for discussion and critical advice.

Alexander P. Demchenko

## Abbreviations

CD	Circular Dichroism
CT	Charge Transfer
DS	Difference Spectra
ESR	Electron Spin Resonance
IR	Infrared
NMR	Nuclear Magnetic Resonance
ODMR	Optically Detected Magnetic Resonance
SPDS	Solvent Perturbation Difference Spectra
TPDS	Thermal Perturbation Difference Spectra
UV	Ultraviolet

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## Introduction

The analysis of the structure-function relationships at the molecular level is one of the most important fields of research in biophysics, biochemistry, and molecular biology. The specific properties of proteins are to be understood on the basis of their submolecular structure, structural requirements, and elementary stages of their interactions.

In studies of proteins a number of questions arise which should be considered: which bonds are responsible for the stabilization of the unique three-dimensional structure of the protein molecule? Which of them and in what way do they allow rearrangements necessary for protein functioning? What is the pathway of molecular assemblance? And what are the physical principles of the arrangement of supermolecular structures, membranes, organelles, and cells? Why are the protein molecules so large that the functional centers occupy only a small part of them? And why are they so small that the thermodynamic fluctuations of structure are significant? Why do the native proteins, being resistant to external perturbations, change their properties significantly under the influence of specific internal factors (substitution or modification of single residues or ligand binding)? What is the structural basis of the high rate and selectivity of enzymatic catalysis and molecular recognition?

Many other questions may appear in the analysis of the structural basis of any specific reaction in which the protein molecules participate. The important finding of the past is that proteins are flexible structures and that there is a necessity to consider not only their space and time-averaged native conformation, but also divergences from the mean, the intramolecular motions in space and time.

By analogy with any other field of research, the study of protein conformation and conformational dynamics is directly related to the means of experiment. The achievements of X-ray diffraction analysis have led to the establishment of the three-dimensional protein structure and determination of the position of polypeptide chains and specific groups of atoms. Limitations of this method requiring protein crystallization and low sensitivity to small local changes in conformation necessitate the use of other methods of protein structural analysis. Development and application of the physical methods of study in solutions (optical rotary dispersion and circular dichroism, infrared and Raman spectroscopy, high resolution NMR, fluorescence, and spin probes) allowed us to approach the analysis of conformational states and transitions in proteins and their dependence on different factors, including those occurring under physiological conditions. The latter methods are most essential in the studies of membrane proteins and protein-phospholipid complexes.

In comparing different physical methods of analysis of protein conformation, it was found that they are aimed at studies of protein conformation at different levels. Properties of protein molecules exist which require thorough examination of conformation as a whole by means of X-ray crystallography. Besides, there are the methods of circular dichroism, infrared and Raman spectroscopy, etc. The latter methods produce incomplete information on a great number of groups within the molecule that may be expressed by such integral parameters as the  $\alpha$ -helical content. Giving a general description of protein molecule conformation, these methods are of low sensitivity to the local conformational change.

Furthermore, there are a number of problems which do not require knowledge of the protein conformation as a whole. There is a necessity to observe the changes in exposure of different local regions of the molecular surface, the environment of groups, stereochemistry, and energetics of their interactions. The methods providing such information may be called the "molecular probe" methods. These are the ultraviolet light absorption and fluorescence spectroscopy, near ultraviolet circular dichroism, nuclear magnetic resonance (NMR) of aromatic protons as well as application of extrinsic labels or probes which are bound to proteins and allow investigation of their binding center topography and dynamics by fluorescence, circular dichroism, electron spin resonance (ESR), or  $\gamma$ -resonance spectroscopy.

The capability of high resolution techniques, like X-ray crystallography or NMR, of enumerating the finest details of protein structure produces significant problems in operation of these parameters for practical purposes. The protein molecule becomes the "large system", i.e., the one in which a number of distinctions, which the observer can note by the use of his methods, is too large to be subjected to the analysis. The situation has been discussed earlier by Weber (1975). The dynamic fluctuations of structure increase the size of the system enormously. The advantage of molecular probe methods is that they operate with a small number of effective parameters. Their relation to the structure may not be direct and unambiguous. But they are preferred for comparative studies, the evaluation of kinetic and thermodynamic parameters of reactions, as well as for the evaluation of structural dynamics of local sites of large molecules.

The ultraviolet spectroscopic approach to protein conformation is definitely such a method. The studies performed by UV light absorption and emission spectroscopy are not aimed at describing the protein conformation as a whole. They are applied to analyze the fine properties of the conformational state of the molecule, to reveal and describe the transitions, as well as to study the conformational dynamics at the level of interacting groups of atoms. The advantages of these methods are the simplicity of performance and nondestructiveness. The studies may be conducted in the concentration ranges and under conditions of the medium under which the molecules exist in living organisms. Many reactions may be reproduced and followed in solution by these methods. The selectivity of information is determined by the specific properties of chromophores absorbing or emitting light quanta. It may be increased by the application of the external factors that permit studying

the solvent and temperature perturbation of absorption spectra and fluorescence quenching. The internal dynamics can be followed in the important time ranges of nanoseconds by fluorescence and milliseconds using the phosphorescence methods.

The ultraviolet spectroscopic methods of protein research are methods with a long history. More than 60 years ago Stenstrom and Reinhardt (1925) demonstrated that the absorption spectrum of blood serum proteins were longwave shifted from the spectrum of the amino acids contained in them. Subsequent studies showed that among amino acids, tryptophan and tyrosine and, to a lesser extent, phenylalanine, contribute most significantly to the near ultraviolet protein spectrum. Furthermore methods appeared for determining the amount of these amino acids, thus, increasing the sensitivity of analysis of shifts in their spectra as well as for relating the spectroscopic information to the structure of the immediate environment of the chromophore in protein.

Studies on protein fluorescence began in the early 1960's (Teale 1960; Weber 1960b; Vladimirov and Burstein 1960; Konev 1967). It was found that only fluorescence of tryptophan is usually observed in proteins containing this amino acid and its emission is sensitive to the protein conformational state. The empirical correlations between the emission spectra and the structure of chromophore environment in proteins were suggested, and a number of attempts have been made to simulate the chromophore environments using simple chromophore-solvent model systems. The latest developments of experimental technique brought resolution in time: one can directly observe the spectra with nanosecond resolution.

There are a number of reviews and monographs covering the field of light-absorption spectroscopy (Beaven and Holiday 1952; Wetlaufer 1962; Herskovits 1967; Donovan 1969; Demchenko 1981b) and fluorescence spectroscopy of proteins (Konev 1967; Chernitsky 1972; Burstein 1977; Lakowicz 1980). Still there is a gap between the knowledge of physical principles of spectroscopy of molecular interactions and relaxations in solutions and the level at which they are applied in the field of protein spectroscopy. In most cases the UV difference spectra are used solely to indicate "conformational changes" and very important structural information is lost. This is also the case of shifts in the fluorescence spectra. The knowledge of the mechanisms of spectroscopic phenomena and simple experiments may distinguish whether these shifts are due to the changes in polarity or to relaxational properties of the chromophore environment.

One facet of the complexity of protein molecules from the standpoint of spectroscopy is the chromophore group heterogeneity. One may consider three levels of such heterogeneity. The first is the level of different structure of chromophores participating in absorption or emission. The second is the level of the same type of chromophores, for instance, tryptophans, which are in different structural positions within one protein molecule. The third is the level of population of microstates for one chromophore in the same structural position, but whose interactions with the environmental groups fluctuate. Each level of heterogeneity contributes to the observed electronic spectra. And it is the goal

of a scientist to develop experimental conditions in such a way that at least some heterogeneity is excluded or can be accounted for. However, another possibility has been suggested by recent developments of spectroscopy of inhomogeneous broadening and molecular relaxation spectroscopy, i.e., to obtain new information from microstate heterogeneity. New ideas and experimental achievements in this field together with the description of traditional methods are the subject of this book.

# Chapter 1. Spectroscopic Properties of Protein Chromophores

The protein molecule, with respect to its spectroscopic properties, may be considered as a complex system of chromophore groups which differ in structure and position of spectra. The protein absorption spectrum is at first approximation, the superposition of spectra of chromophores composing the protein molecule. The most extensive research effort has been focused on the 190–220 nm spectral range in which the peptide group absorption and the absorption of many amino acid residues are observed, as well as on the range of approx. 280 nm in which tyrosine and tryptophan absorption occurs. Both ranges are used for conducting experiments in protein spectroscopy. In emission spectra the contribution of the tryptophan chromophore is the most important one. A detailed description of spectroscopic properties of chromophores composing the protein molecule and their analogs is given below.

## 1.1 Basic Principles and Definitions of Light Absorption and Emission Spectroscopy

The aim of this section is to provide an introduction to the language of the spectroscopist, to explain the principles on which the interaction of light and matter is based, as well as to describe the ordinary ways by which spectroscopic information is obtained and presented.

### 1.1.1 Absorption

The optical spectrum is the dependence of a parameter describing the distribution of intensity (probability) of absorption or emission on wavelength or wave number. The wave number  $\nu$  is expressed in reciprocal centimeters ( $\text{cm}^{-1}$ ). The wave number is widely used in physics: the energy of a quantum is proportional to its frequency in wave numbers. In chemistry, biochemistry, and some other fields, it is more common to use the wavelength  $\lambda$ , expressed in nanometers (nm). Their relationship is as follows:

$$\nu (\text{cm}^{-1}) = 10^7 / \lambda (\text{nm}) \quad (1.1)$$

The transmittance  $T$  of a solution, expressed as a percentage, is defined as the ratio of intensity ( $I$ ) of the light emerging from the solution to the intensity ( $I_0$ ) of the light which is incident on the solution, or

$$T = (I/I_0) \cdot 100\% \quad (1.2)$$

According to the Beer-Lambert law, the dependence of  $T$  on the thickness of solution (light path)  $l$  and solute concentration  $C$  is exponential. The logarithm of  $T$  is linearly dependent on solute concentration within the range in which the Beer-Lambert law is applicable. Its extinction (or optical density)  $E$  (some authors denote it as  $D$ ,  $A$ , or  $OD$ ),

$$E = (1/l) \lg (I/I_0), \quad (1.3)$$

where  $l$  is expressed in centimeters,  $E$  is a function of the concentration of the light-absorbing substance. To express quantitatively the ability of any substance to absorb light, the specific extinction ( $E^{1\%}$ ) or  $E^{1\text{ mg ml}^{-1}}$  and the molar extinction ( $\epsilon^M$ ) are introduced. These are the extinction coefficients reduced to 1%, 1 mg ml<sup>-1</sup>, or 1 mol l<sup>-1</sup> solute concentrations  $C$ , respectively.

$$E = C_{(\text{mg ml}^{-1})} E^{1\text{ mg ml}^{-1}} = C_{\%} E^{1\%} = C_M \epsilon^M \quad (1.4)$$

The absorption of light by a molecule results in conversion of radiant energy into the energy of rotation and vibration, as well as in altered electronic configuration of this molecule. The energy of the light quantum  $h\nu$  ( $h = 6.6252 \times 10^{-34} \text{ J} \cdot \text{s}$  is the Planck constant and  $\nu$  is expressed in s<sup>-1</sup>) is proportional to the frequency of light. On absorbing the quantum by the molecule, its internal energy increases from the ground state (in which the energy is minimal) to the higher energy state. These states are called excited ones, and the process of transition of a ground state molecule to an excited state is called excitation. The energy of a quantum  $h\nu$ , which the molecule is able to absorb, is the difference in energy between the excited state  $E_e$  and the ground state  $E_g$ :

$$h\nu = E_e - E_g \quad (1.5)$$

When interacting with a molecule, the light induces excitation of states significantly differing in their energy. Depending on the amount of energy of the absorbed quantum, the absorption spectra may be divided into rotational, vibrational, and electronic. The rotational spectra are observed in the far-infrared region ( $\nu < 100 \text{ cm}^{-1}$ ), vibrational in the infrared region ( $100\text{--}10000 \text{ cm}^{-1}$ ) and the electronic spectra, having the highest energy, are located in the ultraviolet and visible regions. Since the electronic transition of the molecule is accompanied by rotational and vibrational transitions, in the ultraviolet and visible regions, complex vibronic (electronic-rotational-vibrational) transitions can be observed.

Due to the excitation of vibrational and rotational energy transitions, the fine structure of the electronic spectra becomes more or less apparent. The interaction



of vibrations with the system of electrons and with each other results in the loss of the vibrational structure. A number of other factors bring about the broadening of the spectra in solutions due to the solute-solvent molecular interactions. (These phenomena will be discussed in Chap. 2.) As a result, the observation of the vibrational structure of the electronic spectra in solutions is hardly possible, while the rotational structure is not visible at all.

The pure electronic or O—O transition is a transition between the ground and excited electronic levels with zero vibrational energy. The absorption band, corresponding to O—O transition of electronic origin, is normally located on the longwave edge of the vibronic absorption spectrum.

The probability of different electronic-vibrational transitions and corresponding band intensities could be estimated on the basis of the Franck-Condon principle for intramolecular phenomena. According to this principle, there is a possibility of separating two subsystems in the molecule. The first one is a subsystem of electrons with vibrational frequencies within the range of  $10^{14}$  to  $10^{15} \text{ s}^{-1}$  and of atomic nuclei with the frequency range of  $10^{12} - 10^{13} \text{ s}^{-1}$ . When the absorption of light quanta takes place, the electronic shell properties alter so rapidly that there is no time for nuclear positions and velocities to change. Under these conditions the most probable is not the O—O transition but, rather a “vertical” transition from the ground state with the lowest vibrational energy to the so-called Franck-Condon state which is characterized by the same relative positions of nuclei as the ground state, but having no energy equilibrium (Mataga and Cubata 1970; Terenin 1967). This state has a higher energy as compared to the equilibrium level of the excited state by the value of the so-called Franck-Condon stabilization energy, and the corresponding absorption band is shifted toward the shorter wavelengths.

The absorption band intensity depends on the probability of the electronic transition and, further on whether the quantum mechanical selection rules are obeyed, and if not, the transitions are referred to as forbidden transitions. The bands corresponding to the forbidden transitions may be revealed in the spectra, but they are of considerably lower intensity.

The electronic transitions are distinguished by the type of changes occurring in the electronic structure of the molecule when a light quantum is absorbed. The most longwave are the transitions in unsaturated compounds from the ground state to the excited  $\pi$ -state ( $\pi \rightarrow \pi^*$  transitions). The transitions between the  $\sigma$  levels ( $\sigma \rightarrow \sigma^*$ ) are located in the most shortwave (vacuum) region. If a chromophore group consists of heteroatoms with indivisible electron pairs ( $n$ -electrons), like nitrogen, oxygen, or sulfur, the electron transition from  $n$  to  $\pi^*$  and  $\sigma^*$  levels may take place ( $n \rightarrow \pi^*$  and  $n \rightarrow \sigma^*$  transitions). A redistribution of electronic density between the localized systems inside the molecules may also occur. Such transitions resulting in production of low frequency absorption bands are often a property of the inter- or intramolecular charge transfer complexes.

Thus, the absorption spectra in the ultraviolet and visible regions result from the electronic transitions in molecules from the ground to the excited state when light quanta are absorbed. The absorption occurs at certain wavelengths which depend on the electronic structure of a molecule in these states.