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PRACA POGLĄDOWA
REVIEW

Spectroscopic study of proteins

Spektroskopowe badanie białek

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ABSTRACT

Proteins are macromolecular compounds made up of amino acids linked by peptide bonds. They are the basic structural component of all living organisms. Their diversity has to do with their structure, and therefore with the way the amino acid residues are arranged in the molecule. Albumin shape is determined by the sequence of amino acids that make it up. The primary amino acid in its composition is cysteine and in smaller amounts glycine, methionine and one tryptophan residue, thanks to which it exhibits fluorescent properties.

For structural studies of proteins, many physical methods are used. The most commonly chosen are spectroscopic methods, which deal with the interaction between electromagnetic radiation and matter.

The article provides an overview of spectroscopic methods employed in the structural studies of proteins. It also serves as a prelude to further research that aims to utilise one of the spectroscopic methods to study the structural changes of albumin after exposure to physicochemical agents. The described spectroscopic methods are characterized by different sensitivity, specificity and procedures to be performed during sample preparation for the study. In the spectroscopic study of proteins, with particular attention to changes in their structure, the method of fluorescence spectroscopy deserves attention. It is the best choice for studying structural changes in proteins that occur under the influence of physicochemical factors. Capturing these changes and relating them to the functions of proteins enriches the knowledge of the normal functioning of the body, as well as the basis of some diseases.

KEYWORDS

albumin, fluorescence spectroscopy, spectroscopic methods, proteins, protein structure study

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STRESZCZENIE

Białka są związkami wielkocząsteczkowymi zbudowanymi z aminokwasów połączonych wiązaniami peptydowymi. Stanowią podstawowy składnik strukturalny wszystkich żywych organizmów. Ich różnorodność ma związek z budową, a co za tym idzie ze sposobem uszeregowania reszt aminokwasów w cząsteczce. Albumina stanowi około 60% białek osocza, jej kształt determinuje sekwencja tworzących ją aminokwasów. Podstawowym aminokwasem wchodzącym w skład albuminy jest cysteina oraz w mniejszych ilościach glicyna, metionina i jedna reszta tryptofanowa, dzięki której wykazuje właściwości fluorescencyjne.

Do badań strukturalnych białek stosowanych jest wiele metod fizycznych. Najczęściej wybierane są metody spektroskopowe, opierające się na generacji widm powstałych wskutek oddziaływania promieniowania elektromagnetycznego z materią. Najpowszechniej stosowaną metodą spektroskopii jest spektroskopia optyczna, wykorzystująca promieniowanie elektromagnetyczne od podczerwieni po ultrafiolet.

Praca stanowi przegląd metod spektroskopowych wykorzystywanych w badaniach strukturalnych białek. Stanowi także wstęp do dalszych badań, które mają na celu wykorzystanie jednej z metod spektroskopowych do badań nad zmianami strukturalnymi albuminy po ekspozycji na czynniki fizykochemiczne. Opisane metody spektroskopowe cechują się różną czułością, specyficznością oraz procedurami, jakie należy wykonać podczas przygotowania próbki do badania. W spektroskopowym badaniu białek, ze szczególnym uwzględnieniem zmian zachodzących w ich strukturze, na uwagę zasługuje metoda spektroskopii fluorescencyjnej. Metoda ta jest niezwykle czuła i precyzyjna. Jest najlepszym wyborem do badania zmian strukturalnych białek, jakie zachodzą pod wpływem działania czynników fizykochemicznych. Nowoczesna aparatura pozwala na badanie zmian zachodzących w strukturze białek na poziomie molekularnym. Uchwycenie tych zmian i powiązanie ich z funkcjami białek wzbogaca wiedzę dotyczącą prawidłowości funkcjonowania organizmu, a także podłoża niektórych chorób.

SŁOWA KLUCZOWE

albumina, spektroskopia fluorescencyjna, metody spektroskopowe, białka, badanie struktury białka

INTRODUCTION

Proteins are the basic structural components of all living organisms, both animal and plant. They are macromolecular compounds made up of amino acids linked by a peptide bond. The diversity of proteins is determined by their structure, which is in turn influenced by the arrangement of amino acid residues within the molecules [1]. Proteins perform a number of important functions: they are elements of the structure of tissues (elastin, collagen) and biologically active compounds (enzymes, toxins); they are regulators of metabolic processes (insulin, glucagon); they are involved in immune processes (antibodies) and also in the transport of substances (albumin, hemoglobin, lipoprotein) [2].

The shape of albumin is determined by the sequence of amino acids that make it up. Albumin is formed by 585 amino acids of 67 kDa. Its basic component is cysteine and in smaller amounts glycine, methionine and one tryptophan residue, thanks to which it exhibits fluorescent properties. The structure of albumin is 67% ά-helix stabilized by disulfide bridges. In this structure, 3 ά-helical domains can be distinguished, each representing 10 helixes. The domains are numbered as I, II, III. The domains are further distinguished by subdomains A and B [3]. This classification is both structural and functional because the various domains are responsible for different binding properties. For example, a specific drug-binding site could be located in subdomain II A and another in subdomain III A. The binding of albumin to fatty acids causes changes in the polarity and volume of the binding site in subdomain I A [4]. These are specific binding sites, but in addition, albumin also has non-specific binding sites. In these, the hydrophobic nature of the ligand is largely responsible for its attachment. Thus, there is a certain relationship between the binding site and its affinity. Specific binding sites in albumin are characterized by high affinity but low binding capacity as opposed to non-specific sites, which are characterized by weak affinity but virtually complete ligand binding capacity. Ligand binding capacity is largely dependent on the concentration of the ligand [5].

Albumin is believed to be the most essential plasma protein and accounts for about 60% of the mass of all proteins in plasma. It determines the level of blood oncotic pressure, which is the force that is responsible for the distribution of water between the plasma and other fluids in extracellular spaces. It maintains the constant hemodynamics of the blood, preventing the formation of edema. In addition to this very important function, albumin binds and transports endogenous and exogenous substances. Some of these endogenous substances include hormones, such as tyrosine, triiodothyronine, cortisol, fatty acids, lipids, bilirubin, vitamins, and metal cations. Exogenous substances, on the other hand, mainly include drugs such as antibiotics, anticancer drugs, anti-inflammatory drugs, etc. [6]. Albumin is an ideal material for the synthesis of therapeutic nanoparticles. It is used especially for transporting poorly permeable and low-permeability substances across biological membranes. Owing to its low cytotoxicity and high efficiency, albumin has become an excellent tool for both therapy and

diagnostic imaging [7]. In addition to the functions described above, the protein is also part of the buffering system of the blood, which helps to regulate and maintain the constant pH of 7.35–7.45; it participates in the immune response to inflammatory processes and it also has the ability to bind free radicals. Its concentration in the blood reflects liver function because it is synthesized mainly in hepatocytes [8].

DISCUSSION

Spectroscopy

Many physical methods are employed to study the structure of proteins. The most commonly chosen are spectroscopic methods, which deal with the interaction between electromagnetic radiation and matter. This interaction involves matter absorbing some of the energy in the form of radiation (absorption) or giving up some of the energy (emission) [9]. One criterion for dividing spectroscopy is based on the wavelength of electromagnetic radiation. Here we distinguish between microwave, optical, X-ray and gamma-ray spectroscopy. The most widely involves the interaction of electromagnetic radiation ranging from infrared to ultraviolet with matter [10]. This technique measures the interaction of molecules with electromagnetic radiation. The resulting spectrum is a representation of the measured factor as a function of frequency or wavelength. Molecules absorb energy from specific wavelengths and then become excited, i.e. move to a higher energy level. Spectra are measured using spectrometers equipped with appropriate detectors. Their task is to determine the frequency and wavelength of the radiation. The choice of the appropriate technique is closely dependent on the type of sample and the parameter that is being investigated. For example, a commonly used type of spectroscopy is UV-Vis spectroscopy, which covers the wavelength range from 250 nm to 700 nm. Its range makes it an excellent choice as an extremely sensitive method for studying protein chromophores and their electron states. Another range of electromagnetic wavelengths is infrared (IR). This radiation is also related to photons, but has a lower frequency and therefore lower energy. On the other hand, it is equally important in its interaction with molecules and the changes that occur in them. IR spectroscopy makes it possible to obtain vibrational information that is helpful, for example, in deciphering the chemical composition of a molecule [9].

Infrared spectroscopy

As early as the beginning of the 20th century, infrared spectroscopy was considered the most important method for identifying the structure of proteins. In this range we distinguish three regions: the NIR (near infrared), MIR (mid-infrared) and FIR (far infrared) regions. The FIR region is often best suited for analytical purposes. Here, the effect of excitation is the vibration of molecules, which are made up of atoms connected by chemical bonds. The total energy of a molecule can be described as the sum of the energies of the oscillatory and rotational levels, which determine both the elastic motion of the atoms around the equilibrium positions and the spinning of the molecule around its own axis. Thus, in order for a change in the energy of a molecule to occur, there must be absorption of electromagnetic radiation of the appropriate wavelength. This process is called excitation, which can be observed as a band in the oscillation-rotation spectrum [11]. In solids and liquids particularly, strong intermolecular interactions can sometimes inhibit the rotational motion of molecules, which can have an effect on the individual absorption bands.

The conventional infrared method uses an infrared radiation source that is focused on the sample. The beam of radiation is scattered by a grating or prism on the slit. This ensures that only narrow frequency ranges reach the detector. The resolution is strictly dependent on the width of the slit. To obtain the full spectrum, the angle of the grating must change continuously with respect to the incident beam of radiation. As a result, only one resolution can be measured at a time. The small distance of the radiation source from the sample and its overheating are other problems. The resulting spectrum is a low-energy spectrum. Obtaining this type of spectrum can be a slow process and is often dependent on several mechanical devices, which affect the accuracy of the result and the stability of the sample [12]. The undoubted disadvantage of this method is the low sensitivity of the spectrometers, which give spectra with a very low signal-to-noise ratio. This disadvantage can be minimized with the introduction of Fourier transform infrared spectroscopes. This development of spectroscopy has significantly affected the limits of detection and the precision of the conducted investigation [13]. The quality of the obtained spectrum is particularly influenced by the linearity of the utilized detector. Detectors used in the MIR region are usually pyroelectric DTGS (deuterated triglycine sulfate), which operates at room temperature, and photoconductive MCT (mercury cadmium telluride), which requires the use of very low temperatures. The MCT detector is a very sensitive detector but at higher irradiances it exhibits significant nonlinearity, which is a problem especially when analyzing weak and narrow absorption bands [14]. One of the most important advantages of the method is the ability to perform the analysis on a small amount of test material. The method of preparing the sample for the test strictly depends on its physical state and chemical properties.

Carrying out the measurement, together with the obtained spectrum and correct interpretation, gives us information about the chemical structure of the examined substance. Due to the high availability and versatility of the method, infrared spectroscopy is one of the most widely used spectroscopic techniques [15].

Raman spectroscopy

When studying the oscillatory-rotational structure of particles, Raman spectroscopy is a frequent choice of researchers. It is distinguished from infrared absorption spectroscopy primarily by the much higher energy of the incident radiation quantum compared to the energy of oscillatory transitions in the molecule [16]. The method is based on the excitation of rotations and oscillations of the molecule occurring under the influence of light from the ultraviolet, visible light and near-infrared regions [17]. The energy of the transition between oscillatory or oscillation-rotation levels is the difference between the energy of the scattered photon and the energy of the incident photon [18]. A huge influence on the development of the method was the invention of the laser in the 1960s, which, due to its characteristics (high intensity and beam coherence and also narrow spectral line), proved to be a very good light source for Raman spectroscopy. Using the Raman method, we can study samples in all states of matter, and the preparation of test samples does not require much processing. An important feature of this method is that it can be utilized to analyze samples in aqueous solutions. This is a very big advantage when studying biological samples. Raman spectroscopy also carries a number of disadvantages. One of them is the presence of a fluorescence background, which must be removed for correct interpretation of the result. Removal of the background requires a number of additional procedures [19]. Using Raman spectroscopy, it is possible to study first- and second-order proteins. With this method, the protein composition can be determined by detecting the presence of certain amino acids, but is not suitable for studying the protein chain sequence.

A variant of Raman spectroscopy, visible resonance Raman spectroscopy (VRRS), is a method employed to analyze heme proteins. VRRS is mainly used to analyze the ligand binding of macromolecules and also to study interactions between an endogenous ligand and a heme iron or an exogenous ligand and a close fragment of a protein [20]. Another variant of Raman spectroscopy is surface enhanced Raman spectroscopy (SERS). The analyzed spectra and the interpreter in this method exhibit specific bands that are not observed in classical Raman spectra. SERS is typically utilized for the qualitative analysis of drugs, glucose, DNA sequencing and proteins studied in their natural environment. It is a specific method for studying drug-protein interactions [21].

UV-Vis spectroscopy

UV-Vis spectroscopy is an absorption technique using electromagnetic radiation in the visible and ultraviolet light range [22]. Ultraviolet and visible radiation can interact with matter in various ways: transmission, reflection (in a point and diffuse manner), absorption, emission in a diffuse manner or as photoluminescence (fluorescence, phosphorescence), diffusion or the Raman effect can occur [23]. It is commonly employed to study biological molecules with analyses typically performed in the wavelength range above 190 nm. For this range, the spectrum has characteristic broad bands originating from groups containing multiple bonds. Such bands in the case of proteins are mainly due to the absorption of radiation by peptide bonds and also by the aromatic systems of side groups of amino acids such as phenylalanine, tyrosine and tryptophan. UV-Vis spectroscopy is mainly used to measure the concentration of proteins in a biological sample. It involves interpreting the spectra of aromatic chromophores taking into account their absorption coefficients. The observed changes in the spectra can indicate alterations in the environment of the chromophore under study, which can be caused by changes in the structure of the protein. Modifications in peak position can also be caused by variations in the polarity of the environment, which can lead to changes in the energy of the ground and excited states [24].

Fluorescence spectroscopy

Some chemical compounds have the ability to emit electromagnetic radiation. In order for emission to occur, a given compound must first absorb a certain portion of energy. Substances that exhibit this phenomenon are called fluorophores, that is they have the ability to fluoresce. The parameters that characterize fluorophores are primarily the quantum yield and fluorescence lifetime. If the rate of non--radiative transitions significantly exceeds the rate of radiative transitions, then the quantum yield is low and less than unity. On the other hand, the average lifetime of a molecule is determined by the average duration of the excited state before returning to the ground state. The occurrence of double bonds along with the corresponding substituents in the molecules of organic compounds has a significant effect on fluorescence. The phenomenon of fluorescence is often enhanced by corresponding substituents that delocalize electrons and thus increase the probability of transition between the lowest singlet state and the ground state [25]. Fluorescence is a characteristic of molecules with rigid structures. Substances that exhibit this phenomenon are mainly aromatic amino acids such as tyrosine, tryptophan, phenylalanine

(which are part of the structure of proteins), DNA and RNA bases, some plant dyes and also hormones and vitamins [26]. Owing to the presence of amino acids in proteins and their fluorescent properties, fluorescence spectroscopy is a highly effective technique for analyzing these macromolecules. By analyzing the fluorescence spectra of proteins and also knowing the effect of physicochemical factors, we can learn an abundance of important information about their spatial structure and also their interaction with other molecules. The fluorescence of proteins occurs in the ultraviolet range of electromagnetic radiation. It occurs for absorption starting from the wavelength of 260 nm for phenylalanine and for emission at wavelengths around 353 nm for tryptophan. Proteins containing a tryptophan residue are characterized by very good fluorescence; one such protein is albumin. Nonetheless, it should be remembered that tryptophan emission is very sensitive to environmental conditions [27]. Fluorescence spectroscopy has very high sensitivity and selectivity, making it an excellent choice for the investigation of conformational changes in proteins that may arise under the influence of physicochemical factors. Fluorescence spectroscopy is also an excellent tool in studies that simulate physiological processes occurring in the body. Thus, it is possible to determine protein-protein, protein-ligand interactions [28].

NMR spectroscopy

NMR spectroscopy can be used to evaluate the structure and properties of chemical compounds. This method makes it possible to analyze samples in both liquid and solid states. NMR spectroscopy uses nuclear magnetic resonance phenomenon to study samples in a magnetic field. The advantage of the NMR method is its conservative nature with respect to the examined structures. In addition to learning about the structure and properties of compounds in pharmacy, it is also often employed to study the dynamics and interactions of these compounds. Initially, the technique allowed the study of small proteins, but now, thanks to the use of 13C, 15N and 2H tracers, it enables the structures of proteins of up to 30 kDa to be determined. The experimental use of isotopic tracers combined with the TROSY (transverse relaxation optimized spectroscopy) effect makes it possible to analyze proteins of much larger masses. Most often, aqueous solutions of proteins are analyzed because they give better analytical results [29]. Initially, NMR spectroscopy utilized proton detection, but with the change to carbon detection, the resolution and sensitivity of the method was greatly improved. To further improve the quality of the spectra, sample spins were introduced at very high frequencies of the order of 65 kHz for carbon detection. The NMR spectroscopy of proteins is a rapidly developing technique that, as of yet, is not employed for routine protein structure studies. Currently, NMR spectrometers are not widely available for commercial research and are only available at a few institutions [30]. The examination of quantum phenomena in proteins with this physical method is within the scope of interest and cooperation of biologists, biophysicists, doctors, and medical physicists.

CONCLUSIONS

The selected spectroscopic techniques are primarily applicable in determining the elemental composition of the samples under study. Their varying levels of sensitivity and specificity, and the sample preparation procedures can also differ between them. The fluorescence spectroscopy method deserves special attention in the spectroscopic study of proteins, particularly in investigating changes in their structure. This method is extremely sensitive and precise. It is the best choice for studying the structural changes of proteins that occur under the influence of physicochemical factors. Gaining comprehensive information on the persistence of protein structures is important especially in terms of modern pharmaceutical research, where the biggest challenge is usually the short shelf life of pharmaceuticals that are based on proteins. In addition, the technological processes leading to the final form of a safe and effective (active) preparation, consist of procedures that can modify the protein in undesirable ways. For example, the starting material is subjected to ionizing radiation, UV (254 nm) or thermal sterilization to destroy bacteria and viruses. These actions are likely to be influenced by the structure and properties of macromolecules. Modern instruments enable the investigation of changes occurring in the structure of proteins at the molecular level. Capturing these changes and linking them to the functions of proteins enhances our knowledge of the normal functioning of the body, as well as the underlying mechanisms of some diseases. Protein conformational abnormalities are underying causes of neurodegenerative diseases amongst other conditions [31].

Author's contribution

Study design – K. Kierszniok, P. Dolibog
Data collection – K. Kierszniok, P. Dolibog, B. Pietrzyk, T. Pryzwan
Manuscript preparation – K. Kierszniok
Literature research – K. Kierszniok, P. Dolibog, B. Pietrzyk, T. Pryzwan
Final approval of the version to be published – P. Dolibog, K. Kierszniok

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