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Studying the denaturation of bovine serum albumin by a novel approach of difference-UV analysis

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ABSTRACT

A novel approach in the analysis of difference-UV spectrophotometric data for determining the heat denaturation degree of bovine serum albumin (BSA) was assessed. Five different parameters of difference-UV spectra were obtained by subtracting spectra of unheated and denatured protein solutions at different temperature-time combinations. BSA was found to exhibit a maximum degree of heat denaturation of about 17% compared to the complete unfolding caused by 6 M guanidine hydrochloride. This low degree of heat denaturation is probably caused by the aggregation of the initially unfolded protein molecules. The kinetic analysis exhibited discontinuities in the Arrhenius plots, distinguishing the unfolding and aggregation phases of the denaturation process, whereas such a discrimination could not be obtained by differential scanning calorimetry analyses. The proposed method is accurate, fast, simple and sensitive enough to detect changes in the protein heat denaturation even at short temperature-time intervals.

1. Introduction

Difference-UV spectroscopy has been applied by a number of studies investigating protein conformational changes induced either by heat or other denaturing agents, such as urea and guanidine hydrochloride (GdHCl) (Apenten, Khokhar, & Galani, 2002; Bonincontro, Cinelli, Comaschi, & Onori, 2004; Busti, Gatti, & Delorenzi, 2005, 2006; Doyle & Bello, 1968; Glazer, McKenzie, & Wake, 1963; Glazer & Smith, 1961; Kella & Kinsella, 1988; Krzyzanowska, Lisowski, & Kochman, 1998; Poklar, Vesnaver, & Lapanje, 1993, 1994). In applying this method, the conformational changes of the protein due to a heating process is monitored either by a) obtaining the sample UV-spectrum against the solvent and by comparing (or subtracting) the spectra obtained after different heat treatments, b) obtaining the difference spectrum by means of a scan across a range of wavelengths, with the heat-treated sample in the reference cuvette and the native protein in the sample cuvette, or c) performing the heating step directly by means of a temperature scan at a specific heating rate, while obtaining the spectroscopic data at a certain wavelength. The difference-UV spectroscopic method, where the difference spectra were obtained against the solvent in the reference cuvette was applied by Glazer and Smith (1961). Doyle and Bello (1968) recorded conformational changes of collagen, with the heat-treated collagen solution in the reference cuvette and a sample of the untreated collagen solution in the sample cuvette. Apenten et al., 2002 monitored the UV-difference absorbance at a specific wavelength of 293 nm and the heating of the sample was performed using thermostated cuvettes, connected to a water bath.

In the case of difference-UV spectra, where absorbance is measured over a certain wavelength range, the estimation of the difference in absorbance between samples with a different degree of denaturation, is based on the value of a peak, which is attributed to the perturbation of specific aromatic groups during the unfolding of the polypeptide chain, i.e. tyrosine, tryptophan, phenylalanine (Schmid, 2001). However, these peak values are small and their use to distinguish small changes in the degree of denaturation, e.g. changes caused by small differences in the heating time and/or temperature of the protein samples, may become rather challenging to extract useful and reliable information. This situation may be further exacerbated due to drifting of spectra baselines, which is commonly observed in UV-spectroscopy. However, there are studies in the literature, where the plot peak values (peak values without taking into account the peak height below zero) are used to describe a denaturation process without discussing the relevance of the baseline drifting (Kella & Kinsella, 1988). To the best of our knowledge, there is no work reporting the measurement of the degree of denaturation or of other conformational changes of proteins, performed by integration of

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these peaks or by measuring their total height instead of reporting the reading at a particular wavelength, which may carry any errors caused by baseline drifts. In this study a new approach in utilizing difference-UV spectroscopy data was applied, where the protein denaturation degree was determined by measuring the area or the height of a characteristic peak of difference-UV spectra obtained after heat denaturation of aqueous solutions of bovine serum albumin.

2. Materials and methods

2.1. Materials

Bovine Serum Albumin (BSA), Fraction V, was purchased from Merck (Darmstadt, Germany). The protein powder was used without any further treatment for the preparation of the different aqueous solutions. Guanidine-HCl (GdHCl), 98% and pure urea were purchased from Alfa Aesar (Lancashire, UK) and ChemLab (Zedelgem, Belgium), respectively. Double distilled water was used in the preparation of all solutions. By mixing appropriate amounts of an 1% (w/w) aqueous stock solution of BSA, of an 8 M GdHCl stock solution, and double distilled water, solutions of 0.2% (w/w) BSA in 1.6, 2.4, 3.2, 4.8 and 6 M GdHCl were prepared. An aqueous solution of 0.2% (w/w) of BSA in 8 M urea was prepared in a similar way.

2.2. BSA solutions

For the UV-spectroscopic analyses, the protein concentration of the solutions used was 0.2% (w/w). This concentration was not suitable for DSC (Differential Scanning Calorimetry) measurements, where the solution has to be of a significantly higher density in order to obtain clear endothermic peaks during conventional DSC testing. The concentration of the solution used for the DSC measurements was 10% (w/w). The pH of the untreated protein as well as of the different heat-treated solutions was monitored with a Hanna Instruments pH 211 microprocessor pH-meter (Hanna Instruments, Padova, Italy).

A 1% (w/w) BSA stock solution was prepared and diluted, accordingly. The stock solution was stirred using a magnetic stirrer for more than two (2) hours and then kept at refrigeration temperature overnight, in order to facilitate protein solubilization prior to performing the experiments. The maximum absorbance value at 278 nm of the 0.2% (w/w) solutions was recorded during all experiments as a verification of the accuracy of the dilutions.

2.3. Heat treatment of samples

Aliquots of approximately 10–15 ml of the 0.2% BSA aqueous solutions were transferred into Schott gl18 glass tubes with screw caps. During preliminary tests, the efficient sealing of the tubes was checked under the experimental conditions, i.e. heating temperatures in the range of 55–87 °C, for loss of water through evaporation, and no weight changes were recorded.

The heat treatments were performed by placing the tubes containing the BSA solution in a Memmert water bath (Germany), equipped with a temperature controller, which was set to the desired temperature. The temperature of the water bath was adjusted to the target temperature prior to immersing the tubes containing the protein solutions. At each time interval, a glass tube was immediately removed from the water bath and transferred to iced water for approximately 3–5 min in order to achieve abrupt interruption of the denaturation process. Subsequently, the solutions were left to equilibrate to room temperature prior to UV-spectroscopic analysis.

2.4. UV-spectroscopy

The UV spectroscopic analyses of the samples were performed using a PC-controlled Shimadzu UV1800 double beam spectrophotometer (Tokyo, Japan). Quartz cuvettes with a 10 mm path length were used throughout the UV-spectroscopic analyses. The sample was transferred to the sample cuvette and the reference cuvette was filled with double distilled water. The wavelength range scanned was 200–400 nm. Prior to analyzing the samples, the spectrum of the unheated solution was obtained, with double distilled water in the reference cuvette. The spectra of the heat-treated samples were subtracted from that of the unheated protein solution. For the difference spectra of protein solutions in GdHCl or urea, the spectrum of 0.2% (w/w) BSA solutions in GdHCl (or urea) against the solvent (GdHCl or urea solution of the same molarity) in the reference cuvette was subtracted from the spectrum of the protein solution against double distilled water in the reference cuvette (untreated protein solution). The analysis of the spectral data was performed with the OriginPro software.

2.5. Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was used to verify the difference-UV data. Since DSC is unsuitable for monitoring protein denaturation in dilute solutions, as those appropriate for UV spectrophotometric analysis, BSA solutions of a concentration of 10% (w/w) were used. The DSC equipment employed was a Perkin Elmer Precisely Diamond DSC (Waltham, Massachusetts, USA) with an Intracooler 2P cooling assembly. Small amounts (40–50 mg) of unheated 10% (w/w) BSA aqueous solutions were placed into stainless steel pans, covered with matching caps and pressure sealed. The sealed pans were weighed before and after each scan to ensure that the pressure sealing was effective and there was no leakage during the measurements. Different scan rates were used, i.e. 5, 10, 15, 20, 25 and 30 °C per minute. In order to obtain activation energy (E_a) values, the methods of Kissinger (1956) and Borchardt and Daniels (1957) were employed. According to the former method, the denaturation temperature (transition temperature peak) varies with the heating rate applied and the change in the maximum deflection temperature is controlled only by the activation energy, E_a. The second method is based on the relationship between the vertical deflection of the DSC endothermic peak and temperature.

A pan containing approximately 50 mg of double distilled water was used as reference for all the scans performed. The temperature range of each scan was set from 30 to 90 °C, since it is known that BSA has a denaturation temperature of around 64 °C (Michnik, Michalik, & Drzazga, 2005) and all endothermic events related to its denaturation fall within this temperature range. After the end of each initial scan, the DSC was programmed to repeat the scan in order to confirm the irreversibility of the denaturation through the absence of the previously recorded endothermic peaks.

3. Results and discussion

3.1. Calculation of the degree of denaturation of BSA using difference-UV spectrum analysis

For the calculation of the absorbance coefficient of the BSA solution, expressed as $A^{1\%}_{1cm}$, maximum absorbance values at 278 nm were obtained as a function of concentration in the range of 0.02 to 0.2% (w/w). The value of the absorbance coefficient obtained was 6.5, which was in good agreement with reported values of coefficients of BSA aqueous solutions by other studies (Glazer et al., 1963).