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A study of the interaction between fluorescein sodium salt and bovine serum albumin by steady-state fluorescence

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ABSTRACT

The binding of fluorescein sodium salt with three kinds of commercially available bovine serum albumin (BSA) of different grades of purity was investigated at 288, 298 and 313 K by fluorescence and absorption measurements at pH 7.50. The association and dissociation constants K_a and K_d were determined by the quenching of BSA fluorescence in the presence of fluorescein sodium salt. The best results were obtained by fitting raw data by non-linear regression and Lineweaver–Burk equations. The modified Stern–Volmer and Scatchard plots gave less reliable data since the fitting was much more difficult.
The agreement of the constants for the three sets of measurements coming from the different BSA was not as good as expected. BSA binding properties differ depending on the different BSA grades of purity. Actually, the binding constants found for the three BSAs used differed in the same set of interactions, even by keeping the experimental conditions constant. These results are a novelty in the field of BSA–ligand binding studies and should be taken into account for future binding studies using BSA. Actually, a large number of aspects should be considered including the grade of purity and the presence of BSA covalent and non-covalent dimers, trimers and oligomers in solution which can affect the goodness of the binding results.

1. Introduction

Serum albumins are the most extensively studied and applied proteins because of their availability, low cost, stability and unusual ligand binding properties. For this reason, a huge number of papers dealing with albumins have been reviewed so far [1,2]. Albumin is the most abundant protein in blood plasma and serves as a depot protein and transport protein for numerous endogenous and exogenous compounds. Albumin is also the principal factor in contributing to the colloid osmotic pressure of the blood and has been suggested as a possible source of amino acids for various tissues. Without question, albumin is the most multifunctional transport protein and plays an important role in the transport and deposition of a variety of endogenous and exogenous substances in blood [3] due to the existence of a limited number of binding regions of very different specificity [2,4].

Albumin is clearly an extraordinary molecule of manifold functions and applications. Perhaps, the most outstanding property of albumin is its capacity to bind reversibly a numerous variety of ligands [2,4]. The physiological importance of albumin, with its properties of transporting protein, and the relative ease with which it can be isolated and purified on a large scale have resulted in a great number of binding studies. Reviews have previously appeared dealing, in relatively general terms, with binding of small molecules to albumin and other proteins such as fatty acids, lysolecithin, bilirubin, warfarin, tryptophan, steroids, anaesthetics and several dyes [2–4].

Most ligands are bound reversibly and typical association constants (K_a) range from 10^4 to 10^6 M^{-1}. Because of the incredible diversity of ligands bound by albumin, early researchers saw ligand binding to serum albumin as non-specific in nature and did not recognize that there were discrete sites *per se*. Instead they envisaged the ligands as randomly attached to the surface, somewhat like a sponge. This view of albumin has changed over the past years, and now it is generally recognized that there are a small number of distinct binding locations [2].

Bovine serum albumin (BSA) is constituted by 582 amino acid residues and on the basis of the distribution of the disulfide bridges and of the amino acid sequence it seems possible to regard BSA as

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Nomenclature

K_d	dissociation constant
K_a	association constant
B_{max}	maximum amount of the complex that can form at saturating the ligand
n	number of binding sites
F_0	steady-state fluorescence intensities in the absence of quencher
F	steady-state fluorescence intensities in the presence of quencher
[Q]	quencher/ligand concentration
[Q_f]	free quencher/ligand concentration
[Q_b]	bound quencher/ligand concentration
[R_t]	total protein concentration

composed of three homologous domains linked together. The domains can all be subdivided into two subdomains. As proposed by Kragh-Hansen [4], there are at least six binding regions and another characteristic feature of albumin–ligand interactions seems to be the presence of one or two high affinity binding sites (primary sites) and a number of sites with lower affinity.

Quenching measurements of albumin fluorescence is an important method to study the interactions of compounds with proteins [5–7]. It can reveal accessibility of quenchers to albumin's fluorophores, help to understand albumin binding mechanisms to compounds and provide clues to the nature of the binding phenomenon.

Dyes are being increasingly used in clinical and medicinal applications [8–10]. The discovery that some dyes would stain certain tissues and not others led to the idea that dyes might be found that would selectively stain, combine with and destroy pathogenic organisms without causing appreciable harm to the host. Actually, some azo, thiazine, triphenylmethine and acridine dyes came into use as antiseptic trypanocides and for other medicinal purposes [11]. It is also known that certain dyes like fluorescein and rose bengal are preferentially adsorbed by cancerous cells [10].

Many drugs and other bioactive small molecules bind reversibly to albumin and other serum components, which then function as carriers. Serum albumin often increases the apparent solubility of hydrophobic drugs in plasma and modulates their delivery to cell in vivo and in vitro. Consequently, it is important to understand the mechanism of interaction of a bioactive compound with protein.

Many drugs, including anti-coagulants, tranquillizers, and general anaesthetics, are transported in the blood while bound to albumin [12]. Drug–protein interactions are important since most of the drugs and other bioactive small molecules are extensively and reversibly bound to serum albumin and they are transported mainly as a complex with protein. The nature and magnitude of drug–protein interaction influences the biological activity (efficacy and rate of delivery) of the drug [13]. It is then important to study the binding parameters in order to know and try to control the pharmacological response of drugs and design of dosage forms. This kind of studies may provide salient information on the structural features that determine the therapeutic effectiveness of drugs/dyes, and hence become an important research field in chemistry, life science and clinical medicine [13–15]. Serum albumin is considered as a model for studying drug–protein interaction in vitro since it is the major binding protein for drugs and other physiological substances.

The use of dyes for protein determination is well established [16,17]. However, other parameters such as mode of interaction, association constant and number of binding sites are important, when dyes are used as drugs. Several spectrophotometric methods such as fluorescence, UV–vis, circular dichroism, light scattering, FT-IR, nuclear magnetic resonance have been used to study the interaction of small molecules and proteins and clarify the conformational change of protein [7,18,19]. Some techniques such as electrochemical technique [20] and capillary electrophoresis [21] have also been utilized for the evaluation of binding mode and binding constants. Among them, fluorescence spectroscopy has been widely used due to its exceptional sensitivity, selectivity, convenience and abundant theoretical foundation. Critical literature survey reveals that attempts have not been made so far to investigate the mechanism of interaction of fluorescein sodium salt (Fig. 1) with BSA.

The present paper deals with the mechanism of binding of fluorescein sodium salt as a ligand with different BSAs by fluorescence steady-state measurements. This study highlights for the first time how binding properties can change for different BSAs. The three commercially available BSAs taken into account differ for the grade of purity and for the purification method (Table 1).

2. Results and discussion

2.1. Fluorescence intensity

Fluorescence-quenching measurements have been widely used to study the interactions of organic compounds with proteins [5–7]. This method can reveal accessibility of quenchers to protein fluorophores, help to understand protein binding mechanisms to compounds and provide clues to the nature of the binding phenomenon.

In principle both native protein fluorescence or ligand (if any) fluorescence can be exploited to monitor the complex formation. However, most of the studies rely on the quenching of protein fluorescence. Only few studies were performed by titrating the dye with the protein but they were mainly related to the use of absorbance instead of fluorescence measurements [22,23].

In this case, in order to investigate the binding of fluorescein sodium salt to BSA, protein concentration is held constant and increasing concentrations of ligand are added. Fluorescence spectra were recorded in the range of 300–500 nm upon excitation at 295 nm. This excitation wavelength avoids excitation of tyrosines and selectively excites tryptophans. Monitoring quenching of tryptophan fluorescence yields much better signal to noise ratio than monitoring increases in ligand fluorescence. As shown in Fig. 2, by increasing the fluorescein sodium salt concentration, there is a decrease of the BSA fluorescence intensity but the emission maximum does not move to shorter or longer wavelength. These results indicated that interaction between fluorescein sodium salt and BSA occurs and the fluorophore quenches the intrinsic fluorescence emission of BSA.

As a preliminary study, some BSA/fluorescein sodium salt concentration ratios were investigated and a binding curve was constructed in order to check which range of concentration has to

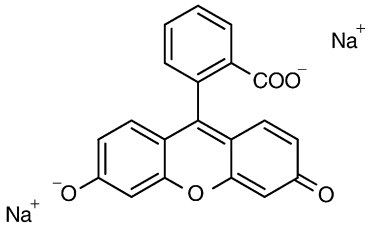

Fig. 1. Structure of fluorescein sodium salt.