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Analysis of protein–surfactant interactions—a titration calorimetric and fluorescence spectroscopic investigation of interactions between *Humicola insolens* cutinase and an anionic surfactant

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Abstract

We have studied interactions of cutinase (HiC) from *Humicula insolens* and sodium dodecyl sulphate (SDS) by parallel calorimetric and fluorescence investigations of systems in which the concentration of both components was changed systematically. Results from the two methods exhibit a number of synchronous characteristics, when plotted against the total SDS concentration, $[SDS]_{tot}$. The molecular origin of several of these anomalies was assigned, and five intervals of $[SDS]_{tot}$ in which different modes of interactions dominated were identified. Going from low to high $[SDS]_{tot}$, these modes were: binding of (a few) SDS to native HiC, formation of oligomeric protein aggregates, denaturation of HiC and adsorption of SDS on denatured protein. For $[SDS]_{tot} > 3-6$ mM (depending on the protein concentration), the adsorption saturated, and no further protein–detergent interaction could be detected.

Two particularly conspicuous anomalies in the calorimetric data were ascribed to respectively denaturation and saturation. It was found that $[SDS]_{tot}$ at these points depended linearly on the (total) protein concentration, [HiC]. We suggest that this reflects the balance between bound and free SDS $[SDS]_{tot} = [SDS]_{aq} + [HiC]\ N_b$ where $[SDS]_{aq}$ and N_b are, respectively, the aqueous ("free") concentration of SDS and the average number of SDS bound per protein. Interpretation of the results along these lines showed that at 22 °C and pH 7.0, HiC denatures with ~14 bound surfactant molecules at $[SDS]_{aq} = 1.0$ mM. Saturation is characterized by $N_b \sim 39$ and $[SDS]_{aq} = 2.2$ mM. The latter value is equal to CMC in the (protein free) buffer. These results are discussed with respect to the SDS-binding capacity of HiC and the origin and location of the saturation point.

Keywords: SDS; Thermodynamics; Denaturation; Saturation; Apparent CMC

1. Introduction

Interactions of proteins and surfactants have been studied extensively for decades due to their importance in many biological, pharmaceutical and industrial systems [1–6]. One example is the SDS polyacrylamide gel electrophoresis (SDS-PAGE) [7,8] which is routinely used to estimate purity and molecular weight of proteins. Another example is the combined use of proteins and surfactants in food products to control the colloidal stability [9]. Enzymes are routinely used in diverse detergent [10] systems, where they must work in the presence of significant amounts of

Abbreviations: HiC, *Humicola insolens* cutinase; FsC, *Fusarium solani pisi* cutinase; ITC, isothermal titration calorimetry; MR, molar ratio; CMC, critical micelle concentration; SDS, sodium dodecyl sulfate; 2,6-TNS, 6-(p-Toluidino)-2-naphthalenesulfonic acid; 1,8-ANS, 8-Anilino-1-naphthalene-sulfonic acid; AOT, Bis(2-etylhexyl) sodium sulfosuccinate; BSA, bovine serum albumin

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surfactants. In these systems, protein–surfactant interactions will alter the functional properties of the proteins. In some cases, the interactions may be advantageous whereas in others they may have a damaging effect on protein stability or enzyme activity. An understanding of the mechanisms involved in protein–surfactant interactions provide a basis for rational strategies to optimize these applications.

Several methods have been used to characterize protein–surfactant interactions including equilibrium dialysis [11,12], spectroscopy [13,14], light scattering [15,16], surface tension [17,18] and surfactant sensitive electrodes [19,20]. Recently, a few papers have emerged, in which high sensitivity isothermal titration calorimetry (ITC) has been used to obtain the complete "interaction isotherms". This implies titration to high surfactant concentrations of the protein, and so far bovine serum albumin (BSA) [21], lysozyme [22,23] gelatine [23] and cellulase [24] have been investigated. The proteins showed complex interactions isotherms, or enthalpograms, which appeared to be rich in information, but was challenging to interpret in terms of molecular events. Each process involved will generate a characteristic thermodynamic "fingerprint" and may include contributions from one or more equilibria, like binding, conformational changes in the protein and micellization. In this context, it is usually necessary to combine the enthalpograms with structural information obtained by, e.g., spectroscopic techniques to resolve the underlying processes for each fingerprint. Thus, the combination of calorimetric and structural data provides a unique possibility to make both molecular and quantitative interpretation of the processes involved in protein–surfactant interactions.

Analysis of protein–surfactant interaction data is typically done on the basis of either the surfactant concentration or molar ratio (MR) scale. The choice of concentration scales implicitly reflects whether a given process is presumed to be governed by the concentration (or activity) of the surfactant in solution independent of the protein concentration or by the formation of a protein–surfactant complex of a certain average stoichiometry. Consequently, combined analysis of protein–surfactant data using both scales provides valuable information about the character of molecular processes in protein–surfactant solutions. A full interpretation may require that both the concentration and MR scale are used in combination. Thus, analysis at low surfactant concentrations where specific binding has been observed suggests an interpretation in terms of MR. At high surfactant concentration, where non-specific interactions can be expected to dominate, it may be more relevant to use the concentration scale.

In this study, we have investigated solutions of *Humicola insolens* cutinase (HiC) and SDS in relation to both enzyme and surfactant concentration. High sensitivity ITC has been used in combination with steady-state fluorescence spectroscopy to resolve the complete interaction isotherm of HiC and SDS. Fluorescence spectroscopy have previously been used successfully to study structural changes in cutinases from *Fusarium solani pisi* (FsC) [25] and HiC [26] induced by the anionic surfactant Bis(2-etylhexyl) sodium sulfosuccinate (AOT). An important scope of the current study is to exploit the potential of ITC in combination with a spectroscopic technique to acquire detailed knowledge about protein–surfactant interactions from complex protein–surfactant enthalpograms. In addition, the results can be used to obtain a rational understanding of the effects on an anionic surfactant on HiC, an enzyme with potential industrial applications.

2. Experimental

2.1. Enzyme and chemicals

Recombinant HiC was expressed in *Aspergillus oryzae* and purified to >95%, determined by SDS/PAGE, at Novozymes A/S, Bagsvaerd, Denmark. The protein was extensively dialyzed, at 5 °C, against 50 mM TRIS, 2 mM EDTA, pH 7.0. The following chemicals were used: TRIS, (>99%, Merck, Darmstadt, Germany), ethylenediaminetetraacetic acid, EDTA (>99%, Merck, Darmstadt, Germany), sodium dodecyl sulfate, SDS (>99%, Fluka, Buchs, Switzerland), 6-(p-Toluidino)-2-naphthalenesulfonic acid, 2,6-TNS (>97%, Fluka, Buchs, Switzerland) and 8-Anilino-1-naphthalenesulfonic acid, 1,8-ANS (>99%, Sigma, St. Louis, MO, USA).

2.2. Isothermal titration calorimetric (ITC) experiments

The calorimetric measurements were conducted on a MCS-ITC (MicroCal Inc., Northampton, MA, USA) isothermal titration calorimetry equipment [27]. The reference cell was filled with water. In a typical experiment, the sample cell was loaded with a solution of 20–98 μM HiC. The cell solution was titrated with 50–104 aliquots of 5 μL of 30 mM SDS in 50 mM TRIS, 2 mM EDTA, pH 7.0 buffer. All calorimetric experiments were done at 22 °C, where SDS micelle formation is practically athermal [28]. Hence, the enthalpic contribution from demicellization of SDS upon injection can be neglected in data analysis. In experiments exceeding 50 injections, it was necessary to make an initial titration trial of approximately 50 injections. Upon refilling the syringe another 50 injections was made into the same solution. The two data files were merged into a single file using the ITC-merge software provided by Dr. Bent W. Sigurskjold, University of Copenhagen. The obtained heat signals from the ITC were integrated using the Origin software supplied by MircoCal Inc.

2.3. Steady-state fluorescence measurements

Equilibrium fluorescence measurements were made on a Perkin Elmer Luminescence Spectrometer LS-50 (PE Biosystems, Foster City, CA, USA). An excitation wavelength