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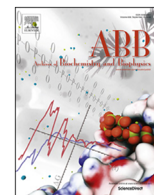

Effects of macromolecular crowding on a small lipid binding protein probed at the single-amino acid level

Silvia Pérez Santero, Filippo Favretto, Serena Zanzoni, Roberto Chignola, Michael Assfalg, Mariapina D'Onofrio*

Department of Biotechnology, Strada Le Grazie 15, University of Verona, 37134, Verona, Italy

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ABSTRACT

Macromolecular crowding is a distinctive feature of the cellular interior, influencing the behaviour of biomacromolecules. Despite significant advancements in the description of the effects of crowding on global protein properties, the influence of cellular components on local protein attributes has received limited attention. Here, we describe a residue-level systematic interrogation of the structural, dynamic, and binding properties of the liver fatty acid binding protein (LFABP) in crowded solutions.

Two-dimensional NMR spectral fingerprints and relaxation data were collected on LFABP in the presence of polymeric and biomolecular crowders. Non-interacting crowders produced minimal site-specific spectral perturbations on ligand-free and lipid-bound LFABP. Conformational adaptations upon ligand binding reproduced those observed in dilute solution, but a perturbation of the free oleate state resulted in less favorable uptake. When LFABP engaged in direct interactions with background molecules, changes in local chemical environments were detected for residues of the internal binding pocket and of the external surface.

Enhanced complexity was introduced by investigating LFABP in cell lysates, and in membrane-bounded compartments. LFABP was able to capture ligands from prokaryotic and eukaryotic cell lysates, and from artificial cells (water-in-oil emulsion droplets).

The data suggest that promiscuous interactions are a major factor influencing protein function in the cell.

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1. Introduction

In the cell, proteins are subjected to the influence of a heterogeneous, crowded environment which modulates their stability, diffusion, conformational dynamics, and binding propensity [1–3]. The recognition that molecular crowding could affect protein function dates back to more than four decades ago and has determined an amazing spread of investigations on proteins in crowded solutions. After the first theoretical models, several computational and experimental studies have contributed to significantly advance our understanding of protein behaviour in native-like environments [1,4–8]. For example, in spite of the observation that the overall impact of a cellular environment on protein chemistry can be moderate [4,9], distinct forces were recognized to operate simultaneously, often producing opposite effects [10–12]. Continued efforts have recently led to the concept of structured crowding [13] and to the description of crowding-induced perturbations to solvent properties [14]. However we note that most experimental studies on globular proteins have been focused on macroscopic properties such as stability, rotational/translational diffusion, and binding affinity, while local effects of crowding have been largely disregarded. Since polypeptide functions are intimately linked to the structural and dynamic features of active sites, surface recognition patches, ligand binding pockets, or hot spot regions, it is important to assess how internal motions and local structure are perturbed in a cell-like environment.

Apart from computational approaches, few techniques are available to detect the perturbations produced by a crowded environment on proteins at the single amino acid level. Among these, NMR spectroscopy plays a prominent role [15]. An arsenal of solution NMR methods allows the study of structure, dynamics, and binding of biomolecules in a broad range of time scales [16]. While suffering from unfavorable consequences of high solution viscosity

* Corresponding author.
E-mail address: mariapina.donofrio@univr.it (M. D'Onofrio).

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in a crowded solution [17], a strength of the technique is that, after isotope-labeling, the signals of a tracer molecule can be monitored without interference from a complex background such as the cytoplasm [18,19]. ^{15}N-enriched protein molecules can be detected through two-dimensional heteronuclear correlation (HSQC) spectra which display distinct signals from the majority of amino acid residues. Individual resonances contain information on local structure and dynamics, as well as on binding-induced perturbations. Accordingly, peak position changes were reported to reveal protein-crowder interactions [20], signal line widths were exploited to determine the viscosity of the cell interior [21], ^{15}N- and ^{13}C-spin relaxation rate measurements were found to be able to detect weak unspecific protein binding under crowding conditions [22,23], and NMR-detected amide proton exchange experiments enabled the evaluation of the effects of macromolecular crowding on protein stability [24]. With notable exceptions, previous work was prevalently concerned with the influence of crowding on global molecular properties such as thermodynamic stability and diffusive dynamics. However, the extent to which this phenomenon affects local structure, dynamics and stability of biopolymers as well as its impact on binding site organization, remains elusive.

Herein, we report an NMR-based study aimed at a systematic evaluation of the effects of cell-mimicking chemistry on individual amino acids along the polypeptide backbone of the liver fatty acid binding protein (LFABP). LFABP belongs to a group of abundantly expressed cytosolic proteins that have been implicated in intracellular lipid trafficking, lipid signal regulation, and systemic metabolic homeostasis [25–27]. These proteins, called intracellular lipid binding proteins (iLBPs), consist of a ten-stranded antiparallel β-barrel and a short helix-turn-helix motif surrounding an internal ligand-binding cavity [28]. The hollow structure and the measurable localized internal dynamics of the ligand-free form of iLBPs in dilute solution [28–30] suggest that they may be more susceptible to crowding-induced perturbations than a compact globular protein. Upon fatty acid binding, LFABP largely retains its tertiary structure and occupies a similar volume to that occupied by the free protein (21.33 nm^3 and 21.07 nm^3, respectively). However, the density of non-solvent atoms in the protein-ligand complex is enhanced compared to the unbound form, and a bound ligand-dependent dynamics has been reported [31,32]. Therefore, LFABP represents an intriguing model to test if a small dynamic protein is affected by macromolecular cosolutes as well as to investigate crowding-induced perturbations to the mechanism of small molecule binding.

2. Results

2.1. Selection and characterization of macromolecular crowders

The protein of interest, LFABP, was presented with macromolecular crowding agents displaying different physicochemical properties, chosen among those that have been used most frequently in crowding research. We selected two synthetic polymers, polyethylene glycol (PEG 20k) and poly(sucrose-co-epichlorhydrin) (Ficoll 70k), and two proteins, bovine serum albumin (BSA) and hen egg-white lysozyme (LSZ).

Both PEG and Ficoll are highly water-soluble nonionic polymers. Ficoll is densely branched and displays an elevated number of hydroxyl groups, it behaves as a nearly spherical crowder and does not interact significantly with most proteins. Using Dynamic Light Scattering (DLS) we measured a hydrodynamic radius, $r_H = 3.24 \pm 0.07$ nm on a 300 g/L sample (Fig. 1A), in agreement with a reported range of 3–5 nm (concentration dependent) [33,34]. PEG contains both hydrophobic (-CH_2-) and hydrophilic (-O-) parts in its backbone, it is more flexible than Ficoll, it has greater hydrophobic character and less hydrogen-bonding capacities, thereby increasing its potential for unspecific attractive interactions with proteins [20,35]. The hydrodynamic radius of PEG 20k (300 g/L in phosphate buffer) was found to be 4.00 ± 0.16 nm, as determined by DLS (Fig. 1B).

The two biomacromolecules give rise to highly monodisperse solutions and their shape is much better defined than that of the synthetic polymers. The conformation of BSA can be approximated by an equilateral triangular prism with two triangular facets and three smaller rectangular sides [36], resulting in an apparent hydrodynamic radius $r_H = 3.66 \pm 0.07$ nm (Fig. 1C). While BSA is an acidic protein (pI = 4.7), LSZ displays a net positive charge at neutral pH (pI = 11). LSZ is not a commonly used background solute, however it was selected as it has a similar size (14.3 kDa) to that of LFABP and has different surface properties from those of BSA. The size distribution plot obtained by DLS indicated a hydrodynamic radius $r_H = 1.90 \pm 0.02$ nm (Fig. 1D).

2.2. Macromolecular crowding effects on ligand-free LFABP

LFABP was prepared as ^{15}N-enriched protein and dissolved in concentrated solutions of the selected macromolecular crowders. HSQC spectra were recorded and analyzed to detect amino acid-specific perturbations in comparison to the spectrum of the protein in buffer. The crowders were present in concentrations of 50, 100, 200, and 300 g/L, whereby the latter condition in most cases prevented the obtainment of a good quality spectrum due to viscosity-induced line-broadening.

The HSQC spectrum of LFABP in phosphate buffer displays well-dispersed peaks, reflecting a unique chemical environment of distinct amide atoms. The spectrum is shown in Fig. 2, overlaid with the HSQC spectra of LFABP in 100 or 200 g/L crowder solutions. The spectrum collected in Ficoll is completely superimposable with that obtained in the absence of cosolutes (Fig. 2A), as confirmed by residue-by-residue analysis of backbone amide peak position changes (chemical shift perturbations, CSP) (Fig. 2B). The plot of relative peak intensities (Fig. 2C) indicates generally less intense peaks due to sample viscosity. It can be noted, however, that linker αII-βB and turns CD, FG, HI, and IJ display less attenuated peaks in Ficoll compared to the rest of the protein. Indeed, the signals of amides in these regions are very weak when the protein is dissolved in buffer, due to fast hydrogen exchange with solvent, but in Ficoll solution the dynamics becomes more favorable for HSQC detection. The mentioned loops all contain glycine residues (at positions 37, 55, 87, 106, 116), which potentiate local main-chain flexibility due to their lack of a Cβ.

Very weak unspecific interactions may escape CSP analysis, therefore we also evaluated ^{15}N-spin relaxation rates. The product of the longitudinal relaxation rate, R_1, and the transverse relaxation rate, R_2, was shown to report on macromolecular interactions in a viscous solution [22,37,38]. The R_1R_2 values measured in 200 g/L Ficoll were of the same magnitude as those measured in dilute solution (Fig. 3A,B), thereby excluding direct solute-protein interactions. With few exceptions, the inter-residue variability of R_1R_2 values closely matched that measured in dilute solution, indicating that motions accessible by this analysis were largely unperturbed by the presence of Ficoll.

At variance with the case of Ficoll, PEG produced significant CSP already at 50 g/L. The spectrum obtained with 100 g/L is shown in Fig. 2D, while the spectrum recorded with 200 g/L polymer displayed excessively broadened signals. Both the CSP and the intensity perturbations were not homogeneous along the protein backbone (Fig. 2E,F). The residues displaying the largest CSP, together with the residues for which the peaks moved to a distant unknown position, mapped to one half of the protein structure that

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