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Translational and rotational motions of proteins in a protein crowded environment

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Abstract

Fluorescence correlation spectroscopy (FCS) was used to measure the translational diffusion of labeled apomyoglobin (tracer) in concentrated solutions of ribonuclease A and human serum albumin (crowders), as a quantitative model system of protein diffusive motions in crowded physiological environments. The ratio of the diffusion coefficient of the tracer protein in the protein crowded solutions and its diffusion coefficient in aqueous solution has been interpreted in terms of local apparent viscosities, a molecular parameter characteristic for each tracer–crowder system. In all protein solutions studied in this work, local translational viscosity values were larger than the solution bulk viscosity, and larger than rotational viscosities estimated for apomyoglobin in the same crowding solutions. Here we propose a method to estimate local apparent viscosities for the tracer translational and rotational diffusion directly from the bulk viscosity of the concentrated protein solutions. As a result of this study, the identification of protein species and the study of hydrodynamic changes and interactions in model crowded protein solutions by means of FCS and time-resolved fluorescence depolarization techniques may be expected to be greatly simplified.

Keywords: Translational diffusion; Rotational diffusion; FCS; Macromolecular crowding

1. Introduction

Macromolecules occupy a substantial fraction (between 10% and 30%) of the total volume in physiological systems [1–3]. Such crowded environments might hinder the diffusion of biomolecules involved in crucial biological processes: metabolisms, transport of solutes, protein processing and signaling events, and may have large effects on their reaction rates and apparent equilibrium constants [4–8]. It is therefore important to incorporate crowding effects in the design and interpretation of *in vitro* studies [9,10], in order appropriately to understand and quantify how kinetic properties of specific macromolecules in physiological media are modified by the presence of other, neutral (non-interacting) macromolecules in their neighborhood. Among other biophysical techniques, methods based on fluorescence spectroscopy [11–13] appear to be very convenient for interaction studies in crowded media due to the possibility of specifically labeling only the tracer protein with extrinsic fluorescent dyes, which in this way can be distinguished easily from the crowding macromolecules. These techniques allow characterization and quantification of the hydrodynamic properties of free and bound species, both in crowded model solutions [14,15] and in whole living cells [6,16–18], using their fluorescence. Time-resolved fluorescence depolarization (TRFD) measurements provide a detailed description of the rotationally depolarizing motions of the tracer molecules that take place on the nanosecond time scale. Fluorescence correlation spectroscopy (FCS) measures translational diffusion times by autocorrelation analysis of fluctuations in the fluorescence intensity collected from a small group of diffusing molecules in an open sample volume element [19,20]. In (infinitely) dilute solutions, the rotational (D_r^0) and translational (D_t^0) diffusion coefficients are related to the hydrodynamic radius (R_T) of the labeled biomolecule (tracer), and to the absolute temperature (T) and viscosity (η) of the solution, through the Stokes–Einstein–Debye (SED) and the Stokes–Einstein (SE) relationships respectively [21–23], which assume that, in terms of its resistance to Brownian rotations or translations, the solvent behaves as a viscous continuum. However, the complexity of cellular

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systems makes the proper interpretation of kinetic data challenging, since the SE and SED relationships are not directly applicable to tracer molecule diffusion in these media. As a first approximation, however, it may be useful to generalize the SE and SED relations with η replaced by the *local apparent viscosity* or *equivalent viscosity* [24], an effective molecular parameter which would include all the neighboring effects of the crowder molecules upon the rotational (η_r) and translational (η_t) motions of the tracer in crowded media:

$$D_r = kT/(8\pi\eta_r R_T^3); \qquad D_t = kT/(6\pi\eta_t R_T) \tag{1}$$

Studies on the diffusion of several labeled dextrans, Ficolls and proteins of different size and charge in the cytoplasm of different cells [25–30] have shown their translational mobility to be hindered in a size-dependent manner with respect to their diffusion in aqueous buffer. Comparative translational diffusion studies of a series of globular proteins and dextrans in the same cultured cells [29,31] indicated that labeled dextrans diffused faster than proteins in the cytoplasm, *i.e.*, the viscosity experienced by diffusing molecules in cytoplasm, η_t (cytoplasm), appears to be a function of tracer size and tracer characteristics. Since the cell cytoplasm is a very complex environment, containing fluid medium, proteins, networks of cytoskeletal filaments, etc., *in vitro* studies conducted in well-defined solutions, in which the crowded cellular environment is mimicked by a high concentration of a macrosolute (water-soluble polymers such as dextrans and Ficolls or non-related proteins) [2], would constitute a first approach to the evaluation of macromolecular crowding effects on tracer diffusion. The diffusion of labeled Ficolls and dextrans have been carefully characterized in globular protein solutions of concentration comparable to the range reported for cytoplasm, by fluorescence recovery after photobleaching (FRAP) techniques [25,26]. These studies showed that the diffusion of these water-soluble polymers in concentrated solutions of globular proteins presented weaker tracer size dependence, in the opposite direction than observed in cytoplasm (dextrans diffusion), or not significantly size dependence (Ficoll diffusion), suggesting that the high protein content is not alone responsible for the observed diffusion behavior in cytoplasm. Muramatsu and Minton [32] studied the diffusion of tracer proteins in solutions crowded with proteins *via* measurements of boundary spreading, a macroscopic technique based on absorbance measurements [33]. They showed that the fractional reduction of the diffusion of the tracer increased with increasing size of tracer species, and with decreasing size of background species. On the other hand, in a recent FCS tracer diffusion study in solutions crowded with Ficoll-70 (0–60 wt.%), Dauty and Verkman [34] have found comparable percentage reductions in the translational diffusion of small solutes, proteins, DNAs, dextrans and nanospheres for each Ficoll concentration, suggesting that the relative reduction of tracer diffusion in Ficoll-70 solutions is not size-dependent. In addition, absorption anisotropy and FCS studies of different tracer globular proteins in solutions crowded with dextrans [35,36] showed that the local rotational and translational viscosities were a complex function of the size of both the tracer and the crowder molecules. Overall, these data indicate that the mechanism of steric hindrance to the diffusion of tracer globular proteins and soluble polymers of comparable size varies with the nature of the crowder molecule, and may be related to the different conformations they exhibit in solution. In general, globular proteins behave in water like hard spheres [37], whereas dextrans have a random coil structure with a high hydration and flexibility and Ficoll-70 molecules behave as relatively open structures rather than as hard-packed spheres in solutions at concentrations larger than 5 wt.% [38]. Recent studies have proposed that the observed size dependence on tracer mobility is an indirect indication that simple diffusion models may not apply and that diffusion dextrans diffusion in cytoplasm and protein diffusion in highly concentrated random-coil polymers and globular proteins solutions are anomalous [30,39]. It is important to note that this subdiffusive behavior was considerably weaker in protein crowder solutions (up to 350 mg ml^{-1}) than that was observed in the case of large dextrans [39].

The motivation for the present study was to provide an experimental basis for estimating the magnitude for the *local apparent viscosities* (η_t and η_r) of tracer proteins in solutions crowded with non-related proteins, to better understand *in vitro* protein interaction studies in protein crowded media. We have studied the translational diffusion of apomyoglobin (apoMb) in concentrated ribonuclease A (RNase A) and human serum albumin (HSA) solutions, using FCS. One of the advantages of this microscopic technique is the very low concentration of labeled protein required for the experiments (typically nM). HSA was selected as a crowder protein because it is the major macromolecular component in blood plasma. On the other hand, RNase A was chosen because of its heterogeneous nature [40], since heterogeneity is a notable property of *in vivo* biological systems which is frequently disregarded in the *in vitro* studies performed in model crowded solutions. We have measured the translational diffusion coefficients at different crowder concentrations relative to the corresponding values in buffer (buffer viscosity, η_0), and this ratio has been interpreted in terms of relative local translational apparent viscosities (η_t/η_0). In order to obtain a global view on the rotational and translational motions of tracer protein under the same crowded conditions, we have compared the relative translational viscosities as determined by FCS (this work) with the relative local rotational viscosities (η_r/η_0) taken from a TRFD study conducted in the same crowded solutions [15,41]. For each tracer, we have tried to correlate the local viscosities with the experimentally determined macroscopic (bulk) viscosity of the crowded solutions. In all the protein solutions studied in this work, local translational viscosity values were larger than the solution bulk viscosities, and also larger than the rotational viscosities for the same tracer protein under the same crowding conditions. Our FCS diffusion results agree well with the data of Muramatsu and Minton [32], and allowed us to propose preliminary empirical relationships for estimation of local translational and rotational viscosities in Eq. (1) directly from the determined bulk viscosity. As result, the determination of hydrodynamic changes and interactions in crowded protein solutions by means of FCS and TRFD techniques may be considerably simplified. However, further work