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REVIEW

A review of multi-domain and flexible molecular chaperones studies by small-angle X-ray scattering

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Abstract Intrinsic flexibility is closely related to protein function, and a plethora of important regulatory proteins have been found to be flexible, multi-domain or even intrinsically disordered. On the one hand, understanding such systems depends on how these proteins behave in solution. On the other, small-angle X-ray scattering (SAXS) is a technique that fulfills the requirements to study protein structure and dynamics relatively quickly with few experimental limitations. Molecular chaperones from Hsp70 and Hsp90 families are multi-domain proteins containing flexible and/or disordered regions that play central roles in cellular proteostasis. Here, we review the structure and function of these proteins by SAXS. Our general approach includes the use of SAXS data to determine size and shape parameters, as well as protein shape reconstruction and their validation by using accessory biophysical tools. Some remarkable examples are presented that exemplify the potential of the SAXS technique. Protein structure can be determined in solution even at limiting protein concentrations (for example, human mortalin, a mitochondrial Hsp70 chaperone). The protein organization, flexibility and function (for example, the J-protein co-chaperones), oligomeric status, domain organization, and flexibility (for the Hsp90 chaperone and the Hip and Hep1 co-chaperones) may also be determined. Lastly, the shape, structural conservation, and protein dynamics (for the Hsp90 chaperone and both p23 and Aha1 co-chaperones) may be studied by SAXS. We believe this review will enhance the application of the SAXS technique to the study of the molecular chaperones.

Keywords SAXS · Multi-domain proteins · Protein dynamics · Molecular chaperones · Mortalin Hsp70

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Introduction

The comprehension of biological systems depends on an understanding of highly dynamic macromolecules and processes. Multi-domain, flexible or intrinsically disordered proteins cannot be neglected in the genome (Fukuchi et al. 2011). In fact, 75 % of signaling proteins have more than 30 amino acid-long segments that are predicted to be disordered and/or flexible (Dunker et al. 2008). Flexible regions can work as linkers connecting folded domains in a multi-domain protein, specifying some preferred conformation. They can also work in inter-domain signal transduction (Ma et al. 2011). They serve as binding sites for interacting partners, helping in molecular recognition, working as activation/inhibition modules, exhibiting post-translational modification sites (Berlow et al. 2015; Dunker et al. 2002; Tompa 2005, 2011; Tompa and Csermely 2004; Trudeau et al. 2013; Uversky et al. 2000; Wang et al. 2011), among others. Hence, protein dynamics is closely related to protein function.

A direct consequence of flexible regions is an increase in protein conformational dynamics in solution, resulting in conformational polydispersity (Kikhney and Svergun 2015). Therefore, more than one biophysical technique is usually required to study flexible proteins (Putnam et al. 2007). Indeed, structural studies of such proteins by protein crystallization are limited by several factors including high conformational dynamics, protein degradation, and a low propensity

to crystallize. Nuclear magnetic resonance (NMR) spectroscopy is the method of choice to study protein structure and dynamics (Berlow et al. 2015; Lemak et al. 2014), but it also has its own limitations like protein size and concentration, as well as its high cost (Putnam et al. 2007).

Usually, eukaryotic multi-domain proteins are difficult to purify due to their instability and propensity to aggregate and degrade (Berlow et al. 2015). Alternatively, folded domains can be isolated and their structures studied by crystallization. However, despite the invaluable contribution of this approach, information about full-length protein dynamics is needed to understand protein structure-function-dynamic relationships. Thus, full-length protein behavior must be checked by some coherent method once is unknown how disordered regions participate in protein function in vivo.

In this context, small-angle X-ray scattering (SAXS) studies can provide structural information of flexible/disordered proteins, and contribute to knowledge on hydrodynamic behavior and shape/topology of macromolecules (Kikhney and Svergun 2015; Tompa 2011; Rambo and Tainer 2011; Barbosa et al. 2013).

Molecular chaperones from the Hsp70 and Hsp90 families and many of their co-chaperones are multi-domain and/or flexible proteins involved in protein folding and degradation, translocation through membranes, avoidance of aggregation–disaggregation, and other processes (Batista et al. 2015a; Borges and Ramos 2005; da Silva and Borges 2011; Kampinga and Craig 2010; Nillegoda et al. 2015). Therefore, these proteins exert critical control over cellular protein quality (Batista et al. 2015a). They are formed by one or more folded domains connected by flexible linkers, and they can present intrinsically disordered regions.

Figure 1 depicts the primary structure organization of the Hsp70 and Hsp90 families and some of their co-chaperones, which are the focus of this review. The presence of autonomous domains/regions as well as flexible/disordered regions is indicated by zigzag lines.

Here, we review the application of SAXS for studying multi-domain molecular chaperones containing flexible linkers and/or disordered regions. Briefly, our approach after SAXS data collection involves: (1) application of ab initio modeling strategies, such as DAMMIN or DAMMIF; (2) validation of the generated model by correlating hydrodynamic properties predicted for the ab initio model with the experimental ones determined by SAXS, analytical ultracentrifugation (AUC) and analytical size exclusion chromatography (aSEC); and (3) use of available three-dimensional structures for the proteins or their domains to infer about protein organization and dynamics, programs such as GENFIT, CRYSOL and EOM, to name a few, were used in the latter approach. In a general way, this approach provides an understanding of the domain organization of the target protein and insights into their mechanisms of action. However, some systems studied by SAXS do not conform to this general strategy, so here we show additional examples of how to use this remarkable technique to study proteins in solution.

SAXS: principles and applications

SAXS is an important tool in the analysis of biologically relevant macromolecules like proteins, micelles and liposomes (from surfactants or lipids) and nucleic acids in solution (Barbosa et al. 2013; Blanchet and Svergun 2013). The technique is based on the scattering of X-rays as they interact with the electrons from the scattering particle. Figure 2 illustrates the experimental set-up. First, a collimated beam of X-rays can be produced in a conventional X-ray tube, or in a synchrotron ring or even in a free electron laser source (FELS) like the one under construction in Hamburg, Germany. The main difference between the X-ray sources is the beam brilliance.

After the X-ray beam exits a monochromator, it passes through two or three slits to collimate the beam. It then hits the sample holder and is scattered to the detector positioned tens of centimeters to a few meters from the sample.

The photons that cross the sample, but do not scatter, are called the direct beam, and the scattering angle is defined as the angle between a certain photon and the direct beam (Fig. 2). The scattering vector is related to the scattering angle, and is used to express the scattered intensity as follows:

$$q = \frac{4\pi}{\lambda}\sin(\theta) \quad (1)$$

where λ is the X-ray wavelength and 2θ the scattering angle (Fig. 2).

SAXS can be applied to a large number of systems and in this review we show the main features of the technique and its application to the study of modular proteins in solution, particularly molecular chaperones.

Guinier's law

One of the first interesting physical quantities that can be easily extracted from a SAXS curve is the protein Radius of Gyration (Rg). It is related to the way that the protein mass is distributed with respect to its center of mass. Andre Guinier (1911–2000) discovered the relationship between the scattered intensity in the small angle region ($q \to 0$) and the scattering particle Rg (Guinier 1939). Guinier showed that the scattered intensity in this region can be expressed as (Guinier 1939; Guinier and Fournet 1955):

$$I(q \to 0) \approx I(0)\, e^{\frac{-(qRg)^2}{3}} \quad (2)$$

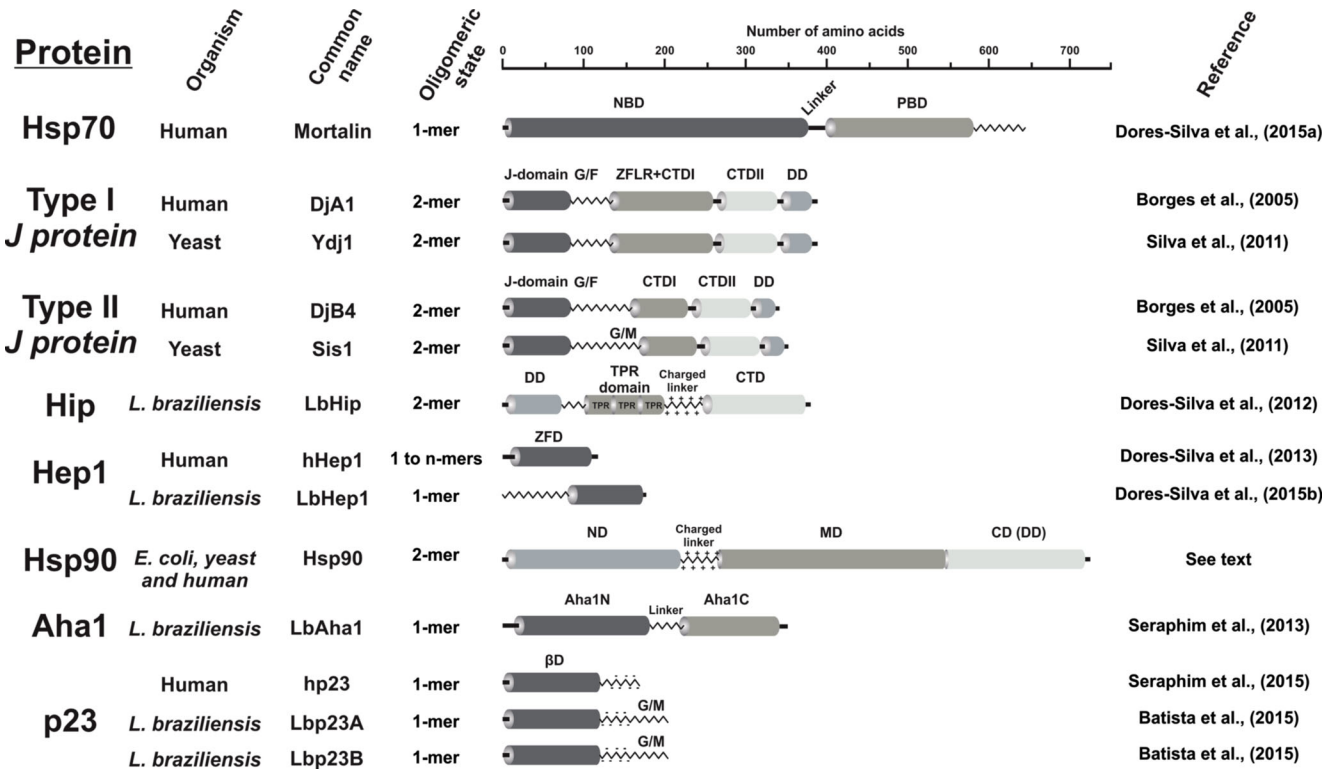

Fig. 1 Domain architecture of Hsp70 and Hsp90 molecular chaperones families and some of their co-chaperones that form the focus in this review. The abbreviated names of the domains are placed *above the domain* (see text for identification). The size of the domains and regions corresponds to their peptide lengths. Protein domains are described in the text. *Zigzag lines* represent flexible regions or intrinsic unfolded sequences

Thus, a plot of $Ln\ (I(q))$ versus q^2 should be a straight line and the modulus of the angular coefficient equal to $Rg^2/3$, whereas the linear coefficient is the forward scattered intensity, I(0), which is related to the protein molecular weight. Such an approximation is an interesting tool for studying proteins in solution, particularly when only the main structural features are of interest.

Kratky plot

The Kratky plot is another interesting tool that can be used to characterize globular or (partially) unfolded proteins (Kratky and Porod 1949; Glatter and Kratky 1982). It was first proposed by Kratky to study polymers in solution (Kratky and Porod 1949), but is now also widely used in the analysis of protein SAXS data. This plot is sensitive to the degree of compactness of a protein, thus it is particularly interesting for the study of folding/unfolding processes and of natively unfolded, intrinsically disordered proteins. The Kratky plot is obtained by simply representing the experimental data as $I(q)\ q^2$ versus q where a globular protein is represented as a bell-like shaped Kratky plot. For globular proteins, intensity is proportional to q^{-4} for large q values. For unfolded proteins, however, the Kratky plot should present a plateau, since scattering intensity is proportional to q^{-2} (Kratky and Porod 1949; Barbosa et al. 2013; Glatter and Kratky 1982).

The Kratky plot is indeed a useful tool in the study of proteins in solution, in particular because it does not require any pre-treatment of the experimental data except for the proper subtraction of the buffer from the protein scattering curve.

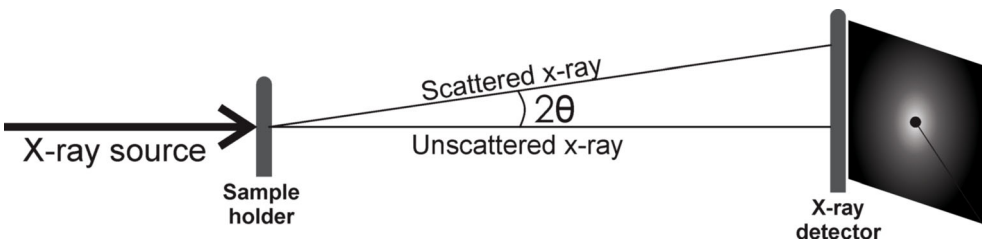

Fig. 2 Small-angle X-ray scattering experimental setup

In fact, if there are problems such as an uncorrected buffer subtraction, the Kratky plot should be avoided, since it can lead to a misinterpretation of the SAXS data.

The pair distance distribution function

This is probably the most used tool for the SAXS data analysis of proteins in solution. The pair distance distribution function (PDDF, p(r)), can be obtained from the experimental scattering curve without any geometrical model assumption.

For diluted systems, e.g., where the interference function does not take place over the SAXS curves or where the scattering system is monodisperse, an indirect Fourier transform connects the scattering intensity, I(q), to the PDDF, p(r). The PDDF function is related to the scattering particle inner structure such that it gives information on the protein size (maximum dimension, D_{max}) and shape (Glatter and Kratky 1982; Guinier and Fournet 1955; Svergun et al. 1987). The PDDF is of particular interest in the study of molecular chaperones since knowledge of their envelope is often relevant. Furthermore, SAXS allows the application of several different analyses. It is commonly used for the reconstruction of protein shape (ab initio methods), and can be combined with different methods such as molecular dynamics simulation (Avila et al. 2014). We usually use GNOM software to calculate the p(r) curves (Svergun 1992).

Ab initio low resolution shape reconstruction

The protein envelope is a key challenge in the study of molecular chaperones. They undergo conformational changes to perform their cellular activities, and thus the knowledge of protein shape under a variety of environments is of great interest. A few software algorithms can reconstruct the protein envelope using the PDDF. The most common are the DAMMIN (Svergun 1999) and DAMMIF (Franke and Svergun 2009), both developed by the research group of Prof. D. I. Svergun at the EMBL, Hamburg, Germany (Svergun 2015). Briefly, both search for an ensemble of a few thousand spheres ("dummy atoms") in certain positions in such a way that the theoretical SAXS curve calculated with this configuration is similar to the experimental one.

This procedure has several advantages, but it also has some disadvantages such as the number of possible mathematical solutions that can be achieved without physical meaning. In order to overcome this problem, the software applies penalties to ensure the protein envelope compactness and inter-connectivity (Svergun 1999). Moreover, to ensure that the final envelope is the most probable one, analyses should be repeated several (few dozen) times and averaged using DAMAVER software (Volkov and Svergun 2003) to get the final protein envelope. One should bear in mind that these methods require the monodispersity of the system, since the software looks for a mean structure that describes the scattering profile and the presence of more than one scattering particle in the system will disturb the final result.

High-resolution structures

There are some interesting methodologies in the SAXS data analysis that can be applied to study molecular chaperones in solution if their three-dimensional structures are known. Software is available to calculate theoretical scattering patterns, like CRYSOL (Svergun et al. 1995), SASMOL (Ortore et al. 2009) and GENFIT (Spinozzi et al. 2014). These programs rely on knowledge of the protein structure to calculate the theoretical scattering pattern. One should bear in mind that in order to apply these methods the protein structures in the crystals and in solution must be alike. This must be checked before any further analysis. Moreover, it is also important to check if the available protein three-dimensional structure (the PDB file) is complete (i.e., with all the residues). This is important because the absence of some specific regions in the crystallographic structure (like flexible regions that are generally missing in the PDB file) can affect the theoretical calculation of the SAXS profile.

Ensemble optimization

EOM is generally applied in the study of multi-domains proteins, with partially known three-dimensional structures (Bernado et al. 2007; Tria et al. 2015). This is of particular interest for proteins with compact domains linked by flexible regions, such that they could adopt several possible conformations in solution. EOM creates a pool of random conformations (around 10,000 structures), or conformers using the protein domain structures and the primary structure to account for flexible regions. An ensemble of possible conformations (around 50 structures) is then chosen based on the adjustment of the average theoretical scattering pattern of the ensemble on the experimental curve. Hence, the software chooses the best ensemble to produce the theoretical profile.

Several different methods can be applied to the study of proteins in solution. This review does not aim to cover all the SAXS methods and a more detailed description of these models can be found elsewhere (Barbosa et al. 2013; Hura et al. 2009; Jacques and Trewhella 2010; Koch et al. 2003; Mertens and Svergun 2010; Putnam et al. 2007; Rambo and Tainer 2010, 2011). In the next section, we show some examples based on our experience using SAXS to understand molecular chaperones.

Understanding molecular chaperones by SAXS

Mortalin: human mitochondrial Hsp70

Molecular chaperones belonging to the Hsp70 family perform a central role in proteostasis, defined as the concept of competing and integrated biological pathways within cells that control the biogenesis, folding, trafficking, and degradation of proteins, among others. It plays a pivotal role together with other molecular chaperone families (Batista et al. 2015a; Borges and Ramos 2005; da Silva and Borges 2011). Several activities have been ascribed to Hsp70, which can be executed by multiple induced or non-induced isoforms. Several co-chaperones control its mechanical cycle, different regulatory mechanisms, and organelle-specific isoforms (Arndt et al. 2007; Bukau et al. 2006; Daugaard et al. 2007; Hartl and Hayer-Hartl 2002; Mayer and Bukau 2005; Meimaridou et al. 2009).

Human mortalin is the mitochondrial Hsp70 that acts mainly in importing and folding cytosolic proteins to the mitochondrial matrix (Bohnert et al. 2007; Dolezal et al. 2006; Fan and Young 2011; Mokranjac and Neupert 2009). It is also present in other subcellular locations related to its action in p53 "kidnapping" and cancer (Kaul et al. 2007; Ran et al. 2000; Wadhwa et al. 1993). As observed for all Hsp70 isoforms, mortalin is a modular protein formed by two conserved domains (Fig. 1). The N-terminal nucleotide-binding domain (NBD) has ATPase activity critical for their cycle mechanism, while the C-terminal peptide-binding domain (PBD) is involved in the interaction with client proteins (Batista et al. 2015a; da Silva and Borges 2011). A highly conserved hydrophobic linker works as a bidirectional allosteric signaling component connecting both domains (da Silva and Borges 2011; Kampinga and Craig 2010). Nevertheless, the study of the recombinant human mortalin, as with other mitochondrial Hsp70 proteins, is limited by its tendency to aggregate at higher concentrations (Dores-Silva et al. 2013; Sichting et al. 2005; Zhai et al. 2008). Thus, to investigate the structure and function of this protein, a special co-chaperone and dilute concentrations, which are usually appropriate for some spectroscopic techniques such as circular dichroism and fluorescence, are necessary (see below).

Recently, we reported on structural analyses of the recombinant human mortalin by SAXS and other biophysical tools (Dores-Silva et al. 2015a). The mortalin SAXS curves were obtained at dilute and aggregation-free concentrations (~0.6 $mg.mL^{-1}$) allowing the determination of structural parameters like MM (Molecular Mass), Rg and D_{max} as well as the construction of an ab initio model. MM and Rg were ~70 kDa and 36 Å, respectively, indicating protein was both homogeneous and monomeric. The p(r) curves generated by GNOM software (Svergun 1992) showed mortalin has a D_{max} of around 130 Å. Thus it was possible to create a reliable ab initio model for this protein using DAMMIN and DAMAVER (Svergun 1999; Volkov and Svergun 2003). The first program generated 20 independent models that were merged using the latter. The final mortalin ab initio model had an elongated shape that presented hydrodynamic properties predicted by the HydroPro program (de la Torre et al. 2000), and similar to those experimentally observed by AUC and aSEC. Both NBD and PBD crystallographic structures were well accommodated into the mortalin ab initio model required by the above assumptions. Moreover, GENFIT (Spinozzi et al. 2014) and the crystallographic structures of DnaK in open (PDB ID: 4B9Q) and closed (PDB ID: 2KHO) conformations were used to show that apomortalin behaves as an equilibrium of these conformations that shifts to the closed state in the test conditions (Dores-Silva et al. 2015a). Therefore, the SAXS technique is a powerful tool for unveiling the structure of limited amounts of recombinant human mortalin at spectroscopic concentrations (Dores-Silva et al. 2015a).

J-proteins: elongated and flexible proteins

J-proteins form a large group of proteins considering the domain organization and cellular localization (Kampinga and Craig 2010; Mayer and Bukau 2005). They are involved in protein folding, since they interact with client proteins through hydrophobic surfaces and avoid protein aggregation (Borges and Ramos 2005; Cyr and Ramos 2015; Fan et al. 2003; Kampinga and Craig 2010; Mayer and Bukau 2005; Summers et al. 2009). J-proteins are Hsp70 co-chaperones that stimulate ATPase and act as client protein scanning factors (Kampinga and Craig 2010; Karzai and McMacken 1996; Laufen et al. 1999; Rudiger et al. 2001; Summers et al. 2009).

From the structural point of view, J-proteins are characterized by the presence of an α-helical domain (the J-domain), which binds to Hsp70 and stimulates its ATPase activity (Greene et al. 1998; Kampinga and Craig 2010; Szabo et al. 1994). Based on domain organization, the J-protein family can be divided into four types of which the best studied are types I and II (Cyr and Ramos 2015; Kampinga and Craig 2010). They have the J-domain at their N-terminal regions (Fig. 1) followed by a flexible and disordered G/F-rich region (Szyperski et al. 1994), which works as a spacer between the J-domain and the rest of the protein (Borges et al. 2012; Kampinga and Craig 2010; Silva et al. 2011; Summers et al. 2009).

The central and C-terminal regions of type I and II J-proteins are formed by the C-terminal domain (CTD), which can be shared into two similar β-sheet folded subdomains (CTDI and CTDII) (Li et al. 2003; Sha et al. 2000). The CTDI is the main client protein binding site (Li et al. 2003; Sha et al. 2000) and delivers client protein to Hsp70 (Borges et al. 2012; Kampinga and Craig 2010; Rudiger et al. 2001; Silva et al. 2011; Summers et al. 2009). In type I J-proteins, there is a Cys-rich region forming a zinc-finger-like region (ZFLR;

Fig. 1) between the G/F-rich region and CTDI. Some type II J-proteins have a Gly/Met (G/M)-rich region instead of a ZFLR. Type III J-proteins only have the conserved J-domain in a part of the protein, not necessarily in the N-terminal region (Mayer and Bukau 2005), and select client proteins for Hsp70 (Kampinga and Craig 2010). Type IV J-proteins have the J-domain with a degenerated HPD motif responsible for interaction with Hsp70 and activation of its ATPase activity (Botha et al. 2007).

Types I and II J-proteins are structurally the most similar and form homodimers through the DD located in the C-terminus. Except for some type II J-proteins, the homodimers are critical for their intrinsic chaperone activity (Cyr and Ramos 2015; Kampinga and Craig 2010; Li et al. 2003; Sha et al. 2000). The main differences lie in the central portion of these proteins (Fig. 1).

Recently, the importance of type I and II J-proteins in metazoa was emphasized by their synergic effect in the disaggregation action of Hsp70 plus Hsp110, a Hsp70 nucleotide exchange factor (Nillegoda et al. 2015). Indeed, these proteins interact with each other through a mechanism that depends on J-domain coupling in the CTD (Nillegoda et al. 2015).

Interestingly, human J-proteins type I (DjA1) and II (DjB4) were studied by SAXS and hydrodynamic approaches, and the results indicate they are modular/multi-domain, highly elongated proteins with different structures (Borges et al. 2005). AUC and SAXS data indicate that DjA1 and DjB4 are both elongated homodimers in solution and long enough (>150 Å) to simultaneously interact with both Hsp70 domains (Borges et al. 2005). In spite of the higher MM, DjA1 had a smaller D_{max} than DjB4. Analyzing the ab initio models developed for these proteins and considering the dimerization site lies at the C-terminal region, the J-domains of DjB4 were positioned at opposite ends of the ab initio model, while they were side-by-side in the DjA1 (Borges et al. 2005). Therefore, the central portion of these proteins might be responsible for their divergent structures. Based on additional interaction sites between type I and II J-proteins with Hsp70, the SAXS results suggested different interaction mechanisms for these proteins (Borges et al. 2005).

The yeast J-proteins belonging to types I and II, Ydj1 and Sis1, respectively, diverge in terms of selectivity to client proteins (Cyr 1995; Lu and Cyr 1998), which was related to their central portion, since Sis1 has a G/M-rich region while Ydj1 has a ZFLR. In 2004, Cyr and colleagues (Fan et al. 2004) exchanged the central part of Sis1 and Ydj1, and vice-versa, yielding two chimeras (called SYS and YSY). Briefly, the chimeras exhibited exchangeble specificity for client proteins (Fan et al. 2004). Through SAXS, it was showed that Ydj1 and Sis1 have a similar domain organization to the human ortologues, i.e. Sis1 is more elongated than Ydj1 (Ramos et al. 2008). Indeed, the chimeras had their shapes changed. YSY became similar to Sis1 and was more elongated than SYS, which resembled Ydj1. Therefore, the central portions of these proteins are responsible for their structure and function (Ramos et al. 2008; Summers et al. 2009).

The crystallographic structure of the Sis1 CTD indicated its dimeric structure resembled a bent horseshoe with a hydrophobic cleft capable of binding client proteins. It lies on the surface of the CTDI (Sha et al. 2000). The CTD of the Ydj1 protomer has an L-shape where the ZFLR is the small leg and they might be "looking to each other" in the dimeric structure. Like Sis1, the Ydj1 CTDI has a hydrophobic cleft that binds client proteins (Li et al. 2003). In both cases, the flexible G/F-regions guarantee independence to the J-domain from the CTD and are also close to the hydrophobic cleft and can influence its interaction with client proteins (Johnson and Craig 2001; Sondheimer et al. 2001; Yan and Craig 1999).

In order to study the central portion of Sis1 and Ydj1, deletion mutants of these regions were prepared and purified in the folded state. They revealed elongated homodimers, as evidenced by AUC experiments (Silva et al. 2011). SAXS experiments indicated that all proteins studied are elongated and flexible, probably due to the presence of disordered and flexible regions. Even the truncation of the G/M+CTDI regions from Sis1 did not alter the mutant flexibility as evaluated by the Kratky plot (Silva et al. 2011). NMR experiments confirmed that the G/M region of Sis1 is indeed disordered (Borges et al. 2012). Furthermore, the modular behavior of J-proteins is critical for client protein transfer to Hsp70 (Borges et al. 2012; Silva et al. 2011) allowing the Hsp70 ATPase activation to be executed by the independent J-domain (Summers et al. 2009) in an "anchoring and docking" mechanism (Cyr and Ramos 2015; Hu et al. 2008). The deletion of the central region of Ydj1 resulted in a protein with similar shape and D_{max} to that observed in the Sis1 equivalent deletion, confirming this is the region responsible for the Type I J-protein characteristic shape (Silva et al. 2011).

Using SAXS data obtained for yeast J-proteins, truncated mutants as well as crystallographic structures available for Sis1 and Ydj1 domains, different modeling routines and rigid body simulations were performed to understand their organization (Silva et al. 2011). Similar aproaches were used for human J-proteins (Borges et al. 2005), yeast J-proteins and chimeras (Ramos et al. 2008). All cases took into account the dimeric structure of the dimerization site located at the C-terminal region (Borges et al. 2005; Sha et al. 2000). Therefore, the CTD of Sis1 or Ydj1 were positioned at the center of the ab initio model and the J-domain of each protomer were placed at opposite ends. In this position, the J-domain is fully available to stimulate the ATPase activity of the yeast Hsp70 as experimentally observed, despite a reduction of the protein D_{max} (Silva et al. 2011).

These results agreed with the literature since the J-domain can stimulate Hsp70 ATPase activity (Laufen et al. 1999; Pierpaoli et al. 1998). Therefore, this activity depends on the

availability of the J-domain to interact with Hsp70. Nevertheless, the truncated mutants could not interact with model client proteins nor cooperate with Hsp70 to refold luciferase (Borges et al. 2012; Silva et al. 2011). These results indicated that the main client-protein binding site in CTDI was eliminated, and the reduction in the Sis1 and Ydj1 maximum dimensions, harmed the client protein transfer to Hsp70 (Borges et al. 2012; Silva et al. 2011).

HIP: a highly elongated Hsp70 co-chaperone

Hsc70-Interacting Protein (HIP) is a co-chaperone to enable cognate Hsp70 to stabilize two chaperone molecules bound to the same client protein (da Silva and Borges 2011; Hohfeld et al. 1995). As depicted in Fig. 1, HIP is a dimeric multidomain protein composed by three conserved regions consisting of an N-terminal DD, a tetratricopeptide repeat (TPR) domain, and a C-terminal region, the latter two being involved in Hsc70 interaction (Lamb et al. 1995).

Using SAXS and other biophysical tools, our research group reported the structure of HIP from the protozoan *Leishmania braziliensis* (LbHIP) (Dores-Silva et al. 2012). It behaves like a highly elongated dimer in solution with MM and Rg values of 83 kDa and 54 Å, calculated using the Guinier analysis. Moreover, the Kratky plot indicated that LbHIP is slightly flexible in solution. Such behavior is probably due to the presence of several linkers connecting the α-helical folded domains and some disordered regions. The p(r) curve showed that LbHIP is a prolate particle in solution with D_{max} of around 200 Å. The protein envelope was consistent with the average ab initio model that displayed hydrodynamic properties (predicted by HydroPro) in agreement with the experimental data determined by AUC and aSEC. Furthermore, the ab initio model allowed the prediction of the relative position of the LbHIP conserved domains.

We concluded that both mammalian HIP and LbHIP have N-terminal DDs located at the center of the ab initio model. The C-terminal region of each protomer was found at opposite ends of this model (Dores-Silva et al. 2012). The TPR domains of each protomer were supposed to be located between the N- and C-terminal regions. In addition, chemical-induced unfolding followed by SAXS and AUC indicated that LbHIP dimer is quite stable and the unfolding transition observed at 2.6 M urea should be related to a part of the protein other than the DD, e.g., the TPR and the C-terminal portion (Dores-Silva et al. 2012). In summary, SAXS studies were critical for understanding the LbHIP structural features, validating it as a scaffold protein for stabilizing two Hsc70 molecules and interacting with the same client protein, since it has two independent binding sites and it is of sufficient size to cooperate with two Hsp70.

Hep1: polydisperse or partially unstructured?

Hsp70-escort protein 1 (Hep1) is an essential protein for mitochondrial biogenesis since it acts by solubilizing and keeping the mtHsp70 protein, like mortalin, in a functional state (Burri et al. 2004; Sichting et al. 2005; Szklarz et al. 2005; Yamamoto et al. 2005). This small protein has a zinc-finger motif formed by four cysteines in a conserved zinc-finger domain (ZFD) almost 100 amino acids long (Fig. 1) (Momose et al. 2007; Vu et al. 2012; Yamamoto et al. 2005).

We reported the structure–function relationship of the human Hep1 (hHep1) and, surprisingly, it was a mixture of at least four species in which the monomer was the main species as shown by AUC data (Dores-Silva et al. 2013). Therefore, information regarding hHep1 shape and envelope were not obtained by SAXS, since it was not possible to evaluate the p(r) function. One should bear in mind that this requires monodispersity of the proteins. It is well known that the Guinier law can be used for calculating apparent MM for the scattering particles (Glatter 1977; Guinier and Fournet 1955). The SAXS data obtained indicated a MM for hHep1 different from that expected for a monomer or any other monodisperse oligomer, confirming that hHep1 behaves as a polydisperse system. Thus, we analyzed the SAXS data by simple visual inspection using the $I(q\rightarrow0)/c_{prot}$ value (Eq. 2) to obtain information on the protein oligomerization process. The increment of hHep1 concentration led to a gradual increase in $I(q\rightarrow0)/c_{prot}$, suggesting that hHep1 oligomerizes in a concentration-dependent manner. In addition, the presence of EDTA modified the increment effect in the $I(q\rightarrow0)/c_{prot}$ value as a function of hHep1 concentration, suggesting that some hHep1 oligomers were destabilized by EDTA (Dores-Silva et al. 2013). Hence, the SAXS technique provides not only structural aspects of a protein but also information on the system itself, like eventual interparticle interactions.

LbHep1 is the Hep1 orthologue found in the genome of the *L. braziliensis* protozoan. It has a conserved ZFD in its C-terminal region, but it has a 70 amino acid N-terminal sequence of unknown function that shares similarities with no known protein. SAXS experiments showed that, unlike hHep1, LbHep1 behaves as a highly elongated monomer in solution but in a rather different way from hHep1, at least at the concentrations tested. SAXS data were collected at several LbHep1 concentrations and the Guinier approximation was used to determine the MM and Rg values. The data revealed that LbHep1 behaves as a monodisperse system with MM and Rg of around 20 kDa and 33 Å, respectively, which agrees with the MM value for the monomeric species (18 kDa) obtained from the primary structure. Furthermore, the p(r) curve suggested that LbHep1 has D_{max} of about 130 Å, and had a maximum value of p(r)

for r ~30 Å, suggesting a quite elongated shape consistent with hydrodynamic data for LbHep1.

These results were explained by the presence of an unstructured region in the N-terminus (Fig. 1) as observed from circular dichroism data (Dores-Silva et al. 2015b). Therefore, the LbHep1 model consists of a disordered N-terminal region of still unknown function connected to the ZFD domain located in the C-terminal region, which in turn retains the solubility action over *L. braziliensis* mtHsp70 (Dores-Silva et al. 2015b). LbHep1 has dimensions compatible with that observed for *L. braziliensis* mtHsp70 (unpublished data) allowing us to hypothesize that LbHep1 might interact with both domains of that protein.

Hsp90: a multi-domain, dimeric and elongated protein

Hsp90 is a 80- to 90-kDa highly-conserved chaperone that plays a central role in the cell physiology of eukaryotes. It participates in protein folding, degradation, protein maturation, signaling, membrane translocation, and others functions (Batista et al. 2015a; Eckl and Richter 2013; Li and Buchner 2013; Silva et al. 2013). A plethora of proteins are known "clients" of Hsp90 including kinases, glucocorticoid hormone receptors, transcription factors, and proteins involved in intracellular traffic, genome maintenance and RNA metabolism (Eckl and Richter 2013; Flynn et al. 2015; Li and Buchner 2013; Mayer and Le Breton 2015; Zhao and Houry 2005). Since the function of Hsp90 is closely related to critical signaling pathways, it emerged as a potential therapeutic target against cancer and other diseases such as those caused by protozoan parasites (Pallavi et al. 2010; Patki and Pawar 2013; Seraphim et al. 2014; Shonhai et al. 2011; Young et al. 2001).

Hsp90 is a homodimer in solution where each monomer has three domains (Fig. 1) (Krukenberg et al. 2011; Nemoto et al. 1995). At the N-terminus, the N-domain (ND) contains an ATP-binding site with a low ATPase activity. It dimerizes transiently with the ND of the adjacent Hsp90 protomer during the Hsp90 functional cycle (Chadli et al. 2000; Obermann et al. 1998; Panaretou et al. 1998; Pullen and Bolon 2011). In Fig. 1, the ND is a site for Hsp90 interaction with some co-chaperones (Ali et al. 2006; Olesen et al. 2015). The M-domain (MD) is involved in ATP hydrolysis and forms a platform for client proteins and co-chaperones interaction (Cunningham et al. 2012; Lotz et al. 2003; Meyer et al. 2003, 2004). The C-domain (CD) is the Hsp90 DD and interacts with client proteins and TPR-based co-chaperones (Nemoto et al. 1995; Onuoha et al. 2008; Worrall et al. 2008).

Hsp90 proteins are remarkably flexible macromolecules undergoing conformational changes related to ATP binding and hydrolysis during its conformational cycle (Krukenberg et al. 2011; Richter et al. 2008). Hsp90 exists in multiple conformational equilibria subtly influenced by ATP/ADP-binding (Mickler et al. 2009; Ratzke et al. 2012). At least three conformational states have been observed for Hsp90 proteins in which apo-Hsp90 fluctuates between open and closed conformations. ATP-binding Hsp90 tends to favor the closed state (Southworth and Agard 2008). The third discrete and compact state corresponds to Hsp90 ADP-bound (Mickler et al. 2009; Southworth and Agard 2008). Hsp90 conformational changes upon nucleotide binding are species-specific (Southworth and Agard 2008).

In the recent years, SAXS has been widely used to understand the structure and dynamics of full-length Hsp90 in solution (Bron et al. 2008; Cunningham et al. 2012; Krukenberg et al. 2008, 2009a, b; Partridge et al. 2014; Southworth and Agard 2008; Street et al. 2010, 2011). Using ab initio modeling strategies, as discussed above, Bron et al (2008) pointed out that Hsp90 has an elongated V-shaped solution structure, resembling the "flying seagull" conformation seen in the open state (Bron et al. 2008). The effects of nucleotides, osmolytes, temperature and pH on the Hsp90 conformation have already been monitored by SAXS (Street et al. 2010; Cunningham et al. 2012; Partridge et al. 2014; Krukenberg et al. 2009a). Due to the multi-domain and flexible nature of the Hsp90, it adopts several conformations in solution and changes in the Hsp90 structure are easily detected by inspecting the p(r) function. The Hsp90 presents a wide p(r) distribution when in its open state, whereas a closed state shows a sharper p(r) function (Cunningham et al. 2012). In spite of these large changes in the p(r), subtle shifts in the conformational equilibrium of the Hsp90 might also be detected and even different Hsp90 conformations might be estimated (Street et al. 2010; Partridge et al. 2014; Krukenberg et al. 2009a). Lastly, SAXS has been used to compare species-specific Hsp90 structures and conformational changes (Krukenberg et al. 2009b), highlighting the use of this technique to understand the features of orthologous Hsp90 proteins.

p23: structured conserved proteins

The p23 co-chaperone is a small protein formed by a folded N-terminal β-sheet domain (βD) and an acidic C-terminal disordered region (Weaver et al. 2000; Weikl et al. 1999). It is involved in a plethora of cellular processes like proteostasis, protein transport, ribosome biogenesis, transcriptional, and genome stability (Echtenkamp and Freeman 2014; Echtenkamp et al. 2011). The p23 interacts with the Hsp90 ND and MD, and the ND dimerization upon ATP binding stabilizes the interaction between the two proteins (Ali et al. 2006; Karagöz et al. 2011; Martinez-Yamout et al. 2006). This interaction allows p23 to regulate Hsp90 function by inhibiting its ATPase activity (McLaughlin et al. 2006); however, the exact role of this inhibition remains controversial. Some authors described p23 as a stabilizer of Hsp90–client-protein interaction (Dittmar et al. 1997; McLaughlin et al.

2006). On the other hand, p23 may be a release factor for Hsp90 (Young and Hartl 2000). p23 βD is involved in preventing aggregation by holding partially or fully unfolded client proteins (Forafonov et al. 2008; Seraphim et al. 2015; Weikl et al. 1999). The function of the C-terminal tail is unclear. Martinez-Yamout and colleagues reported that the C-terminal region of hp23 is disordered and proposed that it works like "fly casting" to increase hp23 solubility when bound to a client protein. Moreover, it could work as a flexible binding site for partners (Martinez-Yamout et al. 2006). A role in p23 regulation in apoptotic cells and telomerase complexes has been suggested for this region (Mollerup and Berchtold 2005; Woo et al. 2009).

Recently, we used SAXS to study the structural properties of p23 proteins from human (hp23) (Seraphim et al. 2015) and *L. braziliensis* (Lbp23A and Lbp23B) (Batista et al. 2015b). To understand how the C-terminal segment of hp23 affects its structure/function, four hp23 constructs were prepared: full-length hp23; $hp23_{1\text{-}142}$; $hp23_{1\text{-}131}$; and $hp23_{1\text{-}117}$. Circular dichroism spectra of hp23, $hp23_{1\text{-}142}$ and $hp23_{1\text{-}117}$ were typically of β-sheet-rich proteins, whereas $hp23_{1\text{-}131}$ presented some α-helix content. In addition, hp23 and $hp23_{1\text{-}142}$ had a higher content of disordered structure compared to $hp23_{1\text{-}131}$ and $hp23_{1\text{-}117}$ (Seraphim et al. 2015). These results pointed to the disordered C-terminal tail of hp23 and suggested that the region between the amino acids 118–131 could be prone to α-helical structure (Seraphim et al. 2015).

Thermal-induced unfolding experiments revealed that the thermal stability of hp23 decreased as the length of the deletion increases. Thus, we hypothesized that the hp23 C-terminus stabilizes the βD structure. SAXS experiments showed that all hp23 constructs were elongated monomers in solution with $hp23_{1\text{-}160}$ presenting higher Rg and D_{max} than $hp23_{1\text{-}142}$. $hp23_{1\text{-}131}$ and $hp23_{1\text{-}117}$ were smaller than the two other constructs and had similar dimensions despite their different sequence lengths. This was observed by AUC and aSEC experiments which showed that the C-terminal region is responsible for the elongated shape of the hp23 and suggests 118–131 α-helix region likely interacts with the βD (Seraphim et al. 2015). Thus, we proposed a mechanism where the C-terminal segment works as an auto-inhibitory module (Trudeau et al. 2013) regulating the structure and function of the hp23 (Seraphim et al. 2015).

In contrast to human, *L. braziliensis* protozoan possesses two p23 proteins named Lbp23A and Lbp23B that share 21 % of identity. Both structures were obtained folded with the same characteristic p23 signatures as their human and yeast counterparts (Batista et al. 2015b). Notwithstanding the Lbp23A and Lbp23B similar secondary structures, they were significantly different. Lbp23A was more stable than Lbp23B as shown by thermally and chemically induced unfolding (Batista et al. 2015b). In addition, Lbp23A had different substrate specificity compared to the Lbp23B in chaperone activity assays. SAXS profiles of Lbp23A and Lbp23B revealed both are elongated monomers in solution with a similar size and dimensions. The p(r) curves also revealed similar overall structures, confirmed by generating ab initio models using DAMMIN (Svergun 1999) and DAMAVER (Volkov and Svergun 2003). Despite the low conservation, slightly different secondary structures, stability and substrate specificity, Lbp23A and Lbp23B had a highly conserved solution structure (Batista et al. 2015b).

The same comparison was done between Lbp23A and Lbp23B comparing human p23 with the *L. braziliensis* proteins. All three had highly conserved solution structures, despite their differences in sequence and functional features. This strongly suggests that SAXS is valuable for studying the solution structure of evolutionarily distant proteins.

Aha1: a multi-domain flexible protein

Aha1 is a 38-kDa protein formed by two independent regions, the N-terminal (Aha1N) and the C-terminal domains (Aha1C) joined by a flexible disordered linker (Koulov et al. 2010; Meyer et al. 2004; Panaretou et al. 2002; Seraphim et al. 2013). Aha1 stimulates Hsp90 ATPase activity, participating in processes such as protein kinase (Lotz et al. 2003; Sun et al. 2012) and glucocorticoid receptor maturation (Harst et al. 2005). In addition, Aha1 acts on metabolic enzymes, mitochondrial proteins, membrane transporters, vesicle trafficking, and protein degradation-related proteins. However, it is not known if this occurs in an Hsp90-dependent or -independent manner (Sun et al. 2015).

Aha1 stimulates Hsp90 through a mechanism where its Aha1N domain interacts primarily with the Hsp90 MD, followed by an interaction between the Aha1C domain and the Hsp90 ND (Koulov et al. 2010; Lotz et al. 2003; Meyer et al. 2004; Retzlaff et al. 2010). The crystal structure of the yeast Aha1N domain complexed to Hsp90 MD (Meyer et al. 2004) and the NMR structure of the human Aha1C domain (PDB ID: 1X53) have been solved. In spite of this, the overall structure of Aha1 and its behavior in solution remained unknown. In order to fill this gap, we produced and characterized the recombinant *L. braziliensis* Aha1 (LbAha1) protein (Seraphim et al. 2013). LbAha1 is formed by two domains with different chemical stabilities that behave as a highly elongated monomer in solution, as attested by circular dichroism, fluorescence, aSEC and AUC techniques (Seraphim et al. 2013).

Using SAXS, we confirmed the protein behaves as an elongated monomer in solution with Rg of 36 ± 2 Å and D_{max} of 140 ± 10 Å (Seraphim et al. 2013). This dimension was compatible with that observed for the *L. braziliensis* Hsp90NM construct (unpublished data) allowing LbAha1 to interact with both *L. braziliensis* Hsp90, ND and MD. The LbAha1 ab initio model was reconstructed using the DAMMIN and

DAMAVER programs (Svergun 1999; Volkov and Svergun 2003). The final model emphasized the LbAha1 elongated feature and presented hydrodynamic properties in agreement with the experimental ones (Seraphim et al. 2013).

To go further into the LbAha1 dynamics, we wondered if LbAha1 could adopt a preferential organization in solution. To answer this question, we applied the EOM (Bernado et al. 2007; Tria et al. 2015). This strategy assumes that the scattering curve of a protein is consequence of a mixture of conformational species due to flexibility. Based on available Aha1N and Aha1C domain three-dimensional structures, the EOM generated 10,000 random conformers that could be adopted by LbAha1. From this pool, the EOM selected the best ensemble (composed of 20–50 structures) that had an averaged theoretical SAXS curve similar to the experimental one. It is important to mention that with this method all the structures in the ensemble contribute the same weight to the final theoretical SAXS curve (Seraphim et al. 2013).

Such structures were also analyzed by the Hydropro program and showed average hydrodynamic properties in agreement with the experimental ones. The Rg and D_{max}-values observed for the selected LbAha1 conformers displayed the same behavior as the 10,000 initial conformers. We concluded that LbAha1 has a high flexibility in solution with a relative independence between Aha1N and Aha1C domains (Seraphim et al. 2013). Lastly, we found that LbAha1 domains likely possess some distention-contraction behavior once the hydrated volume of LbAha1 was similar to that of a hydrated particle of the same MM, but it is slightly smaller than those estimated for the ab initio model and EOM selected conformers (Seraphim et al. 2013). In summary, our SAXS approach revealed that LbAha1 has dimension, flexibility and domain independence in agreement with the proposed general mechanism for Aha1–Hsp90 interaction.

Conclusions

Comprehension of biological systems relies on a knowledge of how macromolecules behave in aqueous environments. A network of dynamic interactions between dynamic macromolecules, such as proteins, drives processes that are essential for cell developmental and cell physiology. A plethora of proteins are formed by more than one domain connected through unstructured or flexible regions, or they present disordered regions, or they can be intrinsically unstructured. These regions are important sites for regulation and usually mediate protein–protein interactions. Thus, the study of these highly flexible, multi-domain and dynamic proteins is needed to comprehend how their structures are intrinsically linked to their cellular function.

Few techniques allow the study of the behavior of an overall protein in solution without regard for molecular size, dynamics or sample preparation. Here, we presented an overview of an old, but remarkably up-to-date, technique to understand the behavior of macromolecules in solution. This review is the result of our understanding of structures and functions of molecular chaperones of the Hsp70 and Hsp90 families by applying the SAXS technique and other biophysical tools. These proteins belong to a multi-domain class of proteins and they exemplify the potential of SAXS to study proteins in solution, well beyond the generation of ab initio models. SAXS can predict and interpret domain organization in a large and flexible protein. It can also determine macromolecular behavior such as oligomeric state, conformational changes, dynamics, and even structure conservation. We show that the combination of SAXS, NMR and X-ray crystallography provide an invaluable understanding of the structural features that drive protein function (Berlow et al. 2015; Lemak et al. 2014; Putnam et al. 2007). Moreover, a comparison of SAXS-generated models with hydrodynamic measurements (AUC, aSEC) can validate the SAXS theoretical analyses.

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Compliance with Ethical Standards

Conflict of interest Júlio C. Borges declares that he has no conflict of interest.

Thiago V. Seraphim declares that he has no conflict of interest.

Paulo R. Dores-Silva declares that he has no conflict of interest.

Leandro R. S. Barbosa declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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