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Proteins in Saccharides Matrices and the Trehalose Peculiarity: Biochemical and Biophysical Properties

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Abstract: Immobilization of proteins and other biomolecules in saccharide matrices leads to a series of peculiar properties that are relevant from the point of view of both biochemistry and biophysics, and have important implications on related fields such as food industry, pharmaceutics, and medicine. In the last years, the properties of biomolecules embedded into glassy matrices and/or highly concentrated solutions of saccharides have been thoroughly investigated, at the molecular level, through *in vivo*, *in vitro*, and *in silico* studies. These systems show an outstanding ability to protect biostructures against stress conditions; various mechanisms appear to be at the basis of such bioprotection, that in the case of some sugars (in particular trehalose) is peculiarly effective.

Here we review recent results obtained in our and other laboratories on ternary protein- sugar-water systems that have been typically studied in wide ranges of water content and temperature. Data from a large set of complementary experimental techniques provide a consistent description of structural, dynamical and functional properties of these systems, from atomistic to thermodynamic level.

In the emerging picture, the stabilizing effect induced on the encapsulated systems might be attributed to a strong biomolecule-matrix coupling, mediated by extended hydrogen-bond networks, whose specific properties are determined by the saccharide composition and structure, and depend on water content.

Keywords: Biopreservation, glasses, myoglobin, protein dynamics, trehalose.

1. INTRODUCTION

1.1. Encapsulation of Proteins in Saccharide Matrices

In vivo, biological processes occur under highly non-ideal conditions, such as macromolecular crowding, that constitute a key factor in determining structure, dynamics, function, and stability of biomolecules [1, 2]. Indeed, the cell volume is occupied by biological macromolecules to a considerable extent, a large part being thus physically unavailable to other molecules. In addition, cells contain many surfaces, like membranes and cytoskeletal fibers, providing sites for specific and aspecific interactions. In such systems, the non-ideal thermodynamic behavior affects molecular interactions of both solutes and solvent, which, at variance with dilute solutions, is organized within few solvation layers.

Studies at molecular level on *in vivo* systems are difficult, in view of their often overwhelming complexity. For this reason, the behavior of biomolecules has been studied in matrices mimicking the conditions of thermodynamic non-ideality, *i.e.* in confinement. Roughly speaking, one can distinguish *soft confinement*, in which conditions of non-ideality are realized by increasing the biomolecule concentration and/or by the addition of co-solutes, from *hard confinement*, in which the biomolecule is entrapped within the pores of a solid, often glassy, matrix; hydrated powders constitute an intermediate case ranging from soft (at high hydration levels) to hard confinement (at hydration levels lower than ~60% w/w).

Incorporation of proteins in perturbed liquid matrices, as in solutions of monohydric alcohols [3-5], showed long time ago how these co-solutes affect both the static dielectric constant of the solvent and the hydrogen bond (HB) networks which regulates the hydrophobic interaction among suspended molecules. On the other hand, solid matrices, such as saccharide amorphous matrices or silica hydrogels, induce a series of structural, dynamical, and functional effects, whose study can contribute substantially to unravel general problems concerning protein-solvent, protein-cosolute, and also protein-protein interactions. Glassy matrices, and highly concentrated solutions of saccharides in particular, exhibit an unusual ability to shield biomolecules from harsh environmental conditions, e.g. freezing, heating, or dehydration. Saccharides are commonly employed by many specialized organisms which have evolved to survive extreme drought or temperatures. After being exposed to these stress conditions, in their cells a massive synthesis of specific carbohydrates is triggered, which provides the correct environment to sustain the successive state of suspended animation, called *anhydrobiosis* [6,7]. Increasing attention, with studies *in vivo*, *in vitro*, and *in silico*, has been devoted to molecular level studies of biomolecules in hydrated saccharide matrices, with the aim of understanding their preserving properties. This interest is also prompted by the possible applications in food, pharmaceutics, and medicine fields.

Ranging from monosaccharides to high molecular-weight polysaccharides, different properties and different mechanisms of interactions (specific and/or aspecific) between the biostructure and the matrix have been proposed [8]. However, neither the molecular mechanisms through which a specific sugar prevents structural degradation nor the basis of the superior effectiveness of certain

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saccharides are completely understood. Furthermore, many studies reported that mono/disaccharides and small oligosaccharides behave differently from large polysaccharides (see Section 1.3).

As regards the basic research, the study of protein-sugar systems at low hydration allows the investigation of their structure, dynamics and function in media in which the *solvent* rigidity can be regulated by changing the hydration level. As a result, the study of thermal effects on the dynamics and structure of biomolecules, as a function of the matrix water content, allows to disentangle matrix rigidity from temperature effects.

Among small sugars, large attention has been devoted to trehalose (Fig. **1**); this review will be mainly focused on the peculiar interactions of this molecule with biological systems.

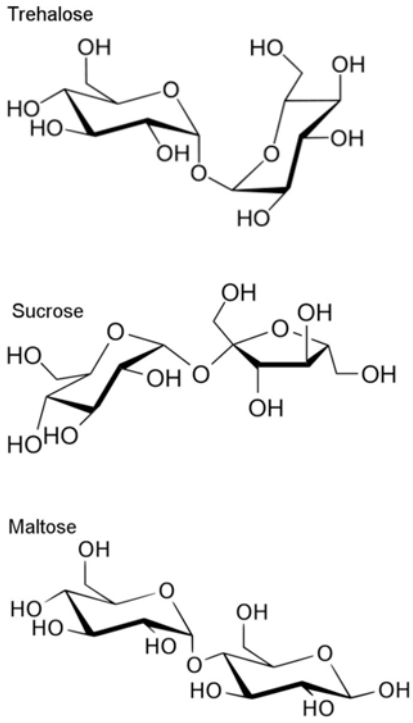

Fig. (1). Molecular structure of trehalose, sucrose and maltose.

1.2. The Trehalose Peculiarity

Trehalose (α,α-D,D-glucopyranosyl-glucopyranoside, Fig. **1**) is one of the most effective protectants [9-14]. It is widely known for its positive effects in preservation of biological molecules and structures; for this reason it is employed in food, pharmaceutics, and biotechnology industries for optimal long-term storage of biological samples (see Section 1.4) [15-17]. Trehalose *peculiarity* has been associated to its high glass transition temperature (T_g), that in conditions of about one water molecule per glucose ring is higher than in other disaccharides [18]; to specific interactions between sugar and biological systems in the dry state (*Water Replacement hypothesis* [19]) or among sugar, protein and residual water at the interface, preserving the native solvation (*Water entrapment* or *sugar preferential exclusion*) [20, 21]. Another hypothesis refers to the high viscosity of sugar matrices, which would cause a decrease of the internal protein dynamics of higher hierarchy, leading in turn to structure damages, inactivation of protein's function, and eventually denaturation [22]; in fact, the rigid carbon skeleton of disaccharides provides sterical restraints that affect the dynamic properties, promote the increase in viscosity and facilitate the formation of glassy structures. Trehalose, in particular, has a very rigid structure due to the inter-subunit α,α-1,1 bond, which connects the anomeric carbons in an axial-axial conformation, leading to a clam shell shape with higher stability than more open conformations. This reduces the molecule flexibility enhancing its glass-forming properties [23, 24].

The above hypotheses are not mutually exclusive [16]. It has been reported that drying with trehalose kept stable a restriction enzyme, even if heating was carried on well above T_g [25]; this confirmed the requirement of an amorphous state, although vitrification was not in itself necessary.

The occurrence of a glassy state in a saccharide matrix does not imply *per se* the presence of saccharide-biomolecule hydrogen bonds, as pointed out by data on polysaccharides, like dextran [26]. The trehalose efficiency could be then related to its ability to form glassy matrices also at relatively high hydration levels, maintaining a large capability of hydrogen bonding to the biomolecule. Actually, trehalose was found more effective than other disaccharides at high temperatures, when direct interactions between the sugar and the biomolecule should play a stronger role. However, this model cannot explain results on raffinose [27]: this sugar is less-effective, despite having a T_g similar to trehalose, and an even larger hydrogen bonding capability [28]. It has also been shown that glass formation alone does not provide the best suppression of protein motions: either liquid or glassy glycerol provides stronger effects than glassy trehalose at low temperature, while trehalose appears most effective at high temperatures [29].

Structural studies of trehalose-water binary systems propose that trehalose effectiveness stems from its polymorphism, both in crystal and amorphous solid [30]. Crystals of dihydrate trehalose slowly form by water evaporation, in conditions of at high temperature and low moisture; this allows water molecules to remain engaged in the same HB network of the sugar solution. This process would facilitate the capture of residual water molecules without disrupting the structures embedded in the matrix. The retention of water molecules in the stable crystalline form of trehalose allows good preserving performances as it does not cause depression of T_g value, even though this does not prevent crystallization altogether [31]. Further slow dehydration would produce anhydrous trehalose. The biostructure would therefore be protected by the inhibition of translational motions, preserving its active conformations. This process has bioprotective effects because of the existence of reversible paths among the different states, avoiding at the same time water crystallization [32].

Trehalose matrices were also studied by using positron annihilation lifetime spectroscopy. Results showed that, while in the amorphous phase the average intermolecular hole size increases with water content, in the dihydrate crystals water behaves as a one-dimensional fluid, able to diffuse in and out of the crystallites, in channels of fixed diameters. At low hydration, this mechanism can have a buffering effect, which would preserve the embedded structures [33].

Actually, in amorphous matrices water distribution is not homogeneous at the molecular level (e.g., water clustering effects). Lack of long range correlations in an amorphous state makes sugar matrices to exhibit a variety of hydration conditions. Indeed, the accessible conformational freedom of the sugar hydroxyl groups makes their interaction with the other matrix component, in particular with water molecules, to vary and causes a subtle dependence on the physical state of the matrix [34, 35].

The strength of trehalose-water interactions has been proposed as a basis for trehalose effectiveness. Raman and neutron scattering experiments on binary systems [36, 37] showed that the addition of trehalose disrupts the tetrahedral water network; as a consequence, water molecules reorder around the sugar, impairing ice growth and improving preservation. It has been shown that long-lived water bridges are formed and water dynamics is reduced, as evidenced by both the lower translational diffusion coefficients and the lower intermolecular electrostatic energy of the water molecules around the sugar. Other findings by the same authors revealed that the solute-solvent interaction strength has the highest values in trehalose, along with the hydration number [38].

The number and extent of HB among trehalose and water has been the subject of different speculations. The number of HB formed by disaccharides is roughly similar, irrespective of the specific sugar; they can induce sizable long-range collective structures in the solvent. In this respect, trehalose has a considerable effect on the surrounding water structure and induces a hydration layer whose thickness is consistently larger than its homologues, as shown by size-exclusion chromatography [39]. This solvent structuring is much more extensive than in liquid water or monosaccharide solutions; moreover, it appears to depend on the chemical structure of the solute. In the case of trehalose, it has been found that a "band of solvent density" runs around the region centered on the glycosidic bond, formed by water molecules coordinated to both rings [40].

The general hydration behavior observed is not dramatically different from that of maltose or sucrose (Fig. **1**). However, despite being a phenomenon common to many saccharides, above a definite concentration trehalose appears to reduce the water diffusion [38] and increase the water residence time to a larger extent than its homologues [41, 42]. Furthermore, water around trehalose has the longest residence times and is more structured than in the proximity of maltose and sucrose [43].

At odds with the above observations, Pagnotta *et al*. reported that trehalose-water H-bonding in solution is surprisingly limited. The authors speculated that trehalose protective effects could be based on mechanisms not involving water mediation. In their report, most of water molecules in the first shell of trehalose are not strongly coordinated; this leaves trehalose hydroxyls capable to direct binding to other solutes [44]. However, the concentration dependence of sugar effects should be also taken into consideration. Indeed, at low concentration sugar molecules insert into existing water networks: this breaks water-water HBs and promote sugar-water HBs (*structure breaking effect*), the effect depending on the number and spatial orientation of hydroxyls groups in the sugar molecule. On the contrary, above 25-30% concentration, the presence of sugar molecules increases water-water interactions, causing the formation of more stable and extensively hydrogen bonded water clusters. Therefore, above a certain concentration, sugars can act as "*structure-makers*" [45].

Incoherent neutron scattering experiments showed that the amplitude of the glass local, high frequency dynamics correlates with the ability to impart protein stability. Local relaxations are suppressed and collective vibrations, which occur in nanosecond and faster timescales, are slower, in correspondence with the coupling of dynamics of the protein to glass dynamics [46]. This suggested that a time–dependent friction approach could elucidate the effect of solvent dynamics on protein motions. To be effective in preservation, the medium should effectively reduce the local, fast motions which are probably precursors for protein denaturation or other deactivation processes [46]. In this respect, the reduction of water dynamics, as observed in binary systems [47], could be the mechanism for the invoked suppression of short scale motions.

A further structural hypothesis, based on the peculiar bonding present in trehalose, has been long proposed and retains its validity. The 1,1 non-reducing bond between the trehalose subunits is very resistant to hydrolysis and is essential in preservation, as it prevents the occurrence of Maillard reaction [48] in protein-trehalose systems. By examining in detail the molecular parameters of the trehalose-water and trehalose-small solutes interactions, it has been noticed that this particular bond is a dynamically stable "hydrophobic patch", nearly unsolvated and able to interact with some hydrophobic surfaces. This patch gives trehalose an amphiphilic character, which could be at the basis of its multifunctional character as a stress protectant [23, 38, 49]. Furthermore, cryoprotectant properties of trehalose seem unaffected by potential diastereomeric differences, *i.e.* D,D and L,L stereoisomers have been reported to have the same effect, while D,L-trehalose has roughly the same properties of sucrose. These results imply that trehalose binding at the chiral biological surfaces is not restricted to definite forms, but enable to recognize the importance of the intrinsic three-dimensional structure of the molecule [50].

All the observations reported point for the peculiar effectiveness of trehalose with respect to other saccharides. However, a lot of sugars do have bioprotective properties: different organisms can use different saccharides as bioprotectant (e.g. glucose in frogs). In the framework of various hypotheses for biopreservation, this is to be expected because saccharides have a rather homogeneous chemical structure, and all have large hydrogen bonding capability, hence the ability to strongly influence HB networks. As we will see below, this similarity in saccharide structure gives flattened effects both in solution and rubbery states, where viscosity and solvent dynamics play the leading role, and the embedded biomolecules face an "average solvent". When the entire system is in the glassy state, a very effective suppression of biomolecules dynamics is present, which in this case largely relies on specific solute-protein-water interaction. In the latter condition effects due to the different saccharide chemical structure are enhanced, trehalose showing better properties in biomolecules preservation under suboptimal conditions [51] or at lower concentrations than other disaccharides [52].

In the following we will pay relatively minor attention to monosaccharides as they have been shown to be almost unable to efficiently immobilize embedded biomolecules, at least at environment temperature, both due to their low glass transition temperature and their inability to generate highly rigid matrices [53, 54]. Monosaccharide importance in biopreservation is essentially related to their use as components in mixed formulations with other, higher molecular weight, components. The limited effectiveness of monosaccharides could also be considered as a proof that sugar effect is exerted aspecifically by modifying solvent properties and not by direct interaction with the protein. Indeed, in this last case, monosaccharides should be more effective, at equal mass concentration (weight/weight), as they have a larger number of hydroxyls and a larger degree of conformational disorder than disaccharides [55].

1.3. Disaccharides and Polysaccharides: Specific vs Aspecific Matrix-biomolecule Interactions

Even though the results reported above depict disaccharides, and trehalose in particular, as ideal biopreservers, from the point of

view of applications small sugars reveal some unfavorable characteristics, when added to food or chemicals. These sugars are generally tight water-binding, hygroscopic and likely to locally crystallize, causing eventual massive crystallization if exposed to atmospheric moisture. Furthermore, disaccharides are not always valuable for food industry, because of their rheological properties. At variance, long oligo- and polysaccharides are widely used as additives, in order to improve selectively the systems rheology. These long chain carbohydrates (e.g. dextrans) may also have bio-preserving effects because of their high T_g, despite their lower ability to interact directly with protein or membrane surfaces [26]. They are indeed largely excluded from the biomolecule surface, because of their large free volumes and steric hindrance [56, 57]. In this respect, it is to be observed that the basic working mechanism of polymeric sugars is more or less the same as that of all the high molecular components *in vivo*, *i.e.* the molecular crowding. Indeed, while *in vitro* studies point out that the stability of solvated biomolecular compact structures is largely determined by interfacial interactions, *in vivo* macromolecular crowding would favor states in which the smallest volume is excluded from the highly concentrated macromolecular solution. In this way crowding, through excluded volume effects, might reduce the configurational entropy of the unfolded state.

Crowding is the reason for a bias against extended conformations and toward compact and associated states. Kinetically it would accelerate processes not limited by the rate of encounter or reactions with transition states more compact than initial states [2], thus accelerating protein aggregation and protein folding, although excessive crowding could prevent both folding and aggregation [58]. Moreover, it has been shown that in a crowded environment the denaturation process is determined by the transition between a compact denatured state with a preserved native-like fold (or molten globule) and the unfolded state, because this transition is associated with the largest variation in volume. It has been postulated that molten globule states, along with other non-native states, can be present for a durable time in crowded environments, as living cells, and can be involved in various processes [59].

The crowding mechanism appears to be the same irrespective of the chemical nature of the crowding agent: proteins are the main components in the cell, but polysaccharides or even synthetic polymers induce roughly the same effects [60]. The protective effect of crowding against protein denaturation is used by the cell, but can also be exploited in pharmaceutical, medical fields, by using edible or biocompatible polysaccharides. Their effect would however be a “blind” aspecific immobilization process, which generally is not able to take advantage of direct interactions with the biomolecule, and therefore its efficiency is limited. In this respect it has been shown that malto-oligosaccharides lose their ability to interact with biostructures in the dry state, with increasing molecular weight [53, 61]. However, some polysaccharides are able to show some degree of stabilization by direct interactions with the biomolecule, e.g. Ficoll (a sucrose-based copolymer) or trehalosyldextrin (trehalose-headed dextrin molecules) [60, 62].

It could however be considered that high mass polymers form flexible networks in solution. In this case, the solvent appears like a porous medium where smaller biomolecules can diffuse, despite the high viscosity of the solution [63]. In this scenario, mono- or disaccharides could synergistically contribute to bioprotection, because they can settle in the oligosaccharide matrix, enhancing the matrix capability to interact with embedded structures [57, 64]. Actually, disaccharides combined with long chain maltopolymers (e.g., maltodextrin or starch) are widespread as glass formers in food industry. Minor effects are instead observed either in dextrin combined with polymers or with mixed chain dextrins, as short chain dextrins cannot diffuse in the matrix formed by long chain ones [65].

In a mixed amorphous matrix, stabilization during desiccation mostly arises from low molecular weight components, while the improvement of the matrix physical stability originates from high molecular weight components. Such mixed matrices have been shown to be particularly effective in stabilizing labile proteins [8]. Studies on liposomes embedded in glucose/hydroxyethylstarch (HES) showed that glucose depresses the melting temperature in the dry lipid, but barely prevents fusion if not at very high concentration. Conversely, the polymer can inhibit fusion but has little influence on phase transitions. It is likely that combinations of low/high molecular weight compounds would occur naturally in anhydrobiotes. It appears that in the desiccation tolerant alga *Nostoc* a glycan works in tandem with oligosaccharides. It has also been shown that certain proteins might modify the sugar phase state, being a support or even a requirement for stabilization [66]. Carbohydrate-protein matrices can host a larger quantity of unfrozen water before triggering system crystallization; hence the mixed systems are stable in a wider hydration range. This effect was reported to be roughly independent on the protein type and molecular weight [67].

Mixed sugar and polymers can be used to stabilize some types of enzymes. It has been found that mixtures of sugars (trehalose, lactose or mannitol) with small amounts of polyethyleneglycol preserve the activity of freeze-dried lactate dehydrogenase or phosphofructokinase better than the single components [68]. Compared to simple sugars, polymers exhibit superior glass transition effects, while small sugars exhibit better water replacement effects; mixing would counteract the component limitations. This works also if the polymer is a protein: it has been shown that bovine serum albumin (BSA) or gelatin can work together with sucrose in protecting some enzymes, with the latter effectively providing a water replacement effect, while the former strengthens the glassy matrix [68]. At variance, in mixtures of polysaccharides and proteins (both high molecular weight components, e.g. mixtures of guar gum and soy protein), the components interact via weak electrostatic forces and hydrogen bonds. Networks are formed, allowing for greater water retention and resulting in a larger increase in viscosity than that induced by the individual components in solution [24].

However, it must be noticed that preservation by low molecular component occurs by direct interaction, and that sugars with limited preservative efficacy do not show synergistic effects when mixed with polysaccharides, as shown by dextran/glucose or dextrin/glucose mixtures [62, 69]. In mixed sugar matrices, water activity is determined by both molecular weight and saccharide type, and increases in the order sucrose < maltose < trehalose [70]. Activity is also partially dependent on T_g which, interestingly, has the same order as above.

1.4. Sugar Based Applications: Food, Medicine, Pharmaceutical & Biotechnology

Beside the use as a stabilizer, trehalose can be used as a drug delivery vehicle, a bulking agent or a binder in solid dosage forms; in cosmetics it has been utilized as a moisturizer and to reduce body odour. The advantages of trehalose rely mainly on its stability against high temperature or hydrolysis.

Trehalose is used in thousands of products, mostly in confectionery. Trehalose was approved as a food ingredient in the UK (as cryoprotectant, 1991), Korea and Taiwan (general use, 1998), USA (General Recognized As Safe, GRAS, 2000) and European Union (2001) [16]. Trehalose has lower and longer-lasting sweetness with respect to sucrose, and besides confectionery products, can be found in processed vegetables, fruits, and seafood, frozen food, baked goods, beverages and also refrigerated products.

This sugar is also naturally present in many unprocessed food products e.g. mushrooms, bean products, honey, shellfishes, leaven products and some seeds. Trehalose percentage of total dry mass is e.g. 10-23% in mushrooms and 7-11% in baker's yeast.

Trehalose is mainly used in food industry because of its stability and preserving ability, however it has been reported that trehalose is also particularly effective in preventing discoloration and preserving the aroma in many preserved products, also protecting from the effect of heating or cooking (e.g. suppression of buttery aroma in baked chocolate products) [17]. Trehalose improves also the retention of aroma in dried fruit products, as strawberry puree, with respect to other sugars. The best retention of aroma components in dehydrated strawberry puree, both instrumentally (head space mass spectrometry) and sensorially (panel test), was obtained by trehalose addition, either alone or in combination with maltodextrins, while only maltodextrin addition alters substantially the aroma perception [71]. As trehalose induces a slower rise in blood sugar level, with respect to other sugars, it has been proposed as long-lasting energy supply supplement for sport activities and in nutraceutical field [17].

Trehalose solutions and glasses have been used to preserve many complex biological structures, from part of cells to whole organisms. Trehalose solutions at low concentration have been used to improve the viability of bacteria against drying (*Escherichia coli* and *Bacillus thuringiensis*), with respect to analogous sucrose solutions, which however have preserving characteristics with respect to drying without sugars. With either trehalose or sucrose no change in the conformation of the whole bacterial proteins is obtained, indicating that the sugars maintain the dry proteins in their hydrated conformations. Both sugars protect *E. coli* and *B. thuringiensis* upon rehydration immediately after drying, but only trehalose exhibits the ability of long term protection after drying [72].

Trehalose was shown to be more effective than sucrose, glucose, dextran and dextran/glucose mixture in preservation of active recombinant retroviruses [69]. Trehalose combined with catalase was used as a supplement in the conventional freezing medium of hematopoietic cells and gave a better protection of functionality, hence a better graft quality, in particular for growth factor receptors and adhesion molecules [73].

Under definite experimental conditions, trehalose-containing liposomes were shown to be able to fuse with red blood cells (RBCs), delivering micromolar amount of trehalose directly into the cell cytosol, without damage of the RBC membrane. Stability may be ascribed to biomechanical effects, e.g by a coating of the membrane surface. Indeed, liposomes either adsorbed to RBC membrane with different strength or partially fused or even free floating liposomes may cover the membrane, providing a barrier that physically protect RBCs during slow cooling, avoiding cryoinjury [74].

Methods for freeze-drying RBCs at the same time maintaining a high degree of viability has important consequences in blood transfusion and in the clinical medicine field. It has been shown that RBC plasma membranes can efficiently uptake trehalose molecules at 37 °C in a 7 h timespan, from the extracellular medium. This is due to a combination of osmotic imbalance and modulation of the phospholipid phases, giving an intracellular concentration up to 150 mM. Higher values can be obtained by using mixtures with trehalose and polysaccharides (HES) [75, 76].

Rehydrated platelets freeze-dried with trehalose reacted normally to multiple agonists, and they had a thermal behavior resembling fresh platelets. On the contrary, membranes in platelets freeze-dried in the absence of sugar underwent a remarkable phase separation, with a pattern dissimilar from fresh platelets [77]. Also the uptake of trehalose has been suggested as a preservation mechanism, as in the case of RBCs [78].

The limited availability and relatively short shelf-life of blood have been the driving forces for the development of blood substitutes as synthetic alternatives to donor blood. One of the strategies that have been developed is that of encapsulating hemoglobin (Hb) in liposomes [79]. In order to be able to lyophilize, rehydrate at a later stage, and maintain the characteristics necessary for a viable red cell substitute, liposome encapsulated Hb has been modified by adding carbohydrates in order to preserve the integrity of the liposome bilayer even at very low hydration, both from the structural and functional point of view [6, 80]. In particular, Rudolph and coworkers showed that liposome encapsulated Hb prepared with trehalose can be effectively lyophilized and then rehydrated as a viable oxygen-carrying fluid [81-83].

Eaton and coworkers suggested [84] that trehalose glasses could be effectively used to preserve hemoglobin-based blood substitutes considering that: (i) the expected slowing down of deoxyhemoglobin (deoxy-Hb) oxidation to methemoglobin (met-Hb) in a trehalose glassy matrix; (ii) the high protein concentration that can be achieved by slowly drying aqueous solutions containing trehalose; (iii) the lack of toxicity of trehalose [83, 84]. More recently, Eike and Palmer showed that oxidized saccharides can be advantageously used as Hb cross-linking reagents for the production of blood substitutes consisting of high affinity and high molecular weight Hb dispersions [85]. Among the different tested saccharides, an oxidized methylglucopyranoside was shown to be very effective, producing very large cross-linked Hb species with high oxygen affinity and low met-Hb levels [85].

During cryopreservation of biological samples, trehalose was able to protect cells and even whole organs viability [86-88]. It has also been shown to significantly improve the survival of stem cells (ES) from human embryos, which are notoriously difficult to cryopreserve [89]. Furthermore, together with dimethylsulfoxide (DMSO), the sugar enhances the viability of fetal skin cryopreserved *in vitro*, providing *in vivo* better engraftment and wound closure. Engineered epidermis has been preserved by using blends of trehalose and DMSO as cryoprotectant agents. These mixtures are more effective than DMSO alone on keratinocytes and engineered epidermis, improving their survival and proliferative capacities. Furthermore, engineered epidermal grafts treated with trehalose and DMSO were indistinguishable from fresh control grafts, and superior to DMSO-cryopreserved grafts, upon transplantation.

The optimal concentration at which trehalose has the best effect may vary with cell type, ranging from 0.5 M for fetal skin [88] down to 0.2 M for human and ES cells [89], and even 0.06 M reported for human hematopoietic cells [90].

Solutions containing trehalose and a polysaccharide (HES, typically) have been used with positive results in the preservation of endothelial cell lines against long term cold storage [91]. The

same, or very similar, solutions have been used to preserve skin flaps from rabbit ear, used as models for traumatically amputated tissues, with the aim to prevent ischemic damage under long term cold storage. Effective preservation was observed up to 72 h after amputation [92, 93].

Intact yeast cells can be vitrified in the presence of trehalose giving a system with T_g close to that of trehalose alone; after the sugar is de-vitrified, the cells are viable, proving that they survived in the dry state [94]. Trehalose was shown to be particularly effective in protecting yeast cells when it is present both inside and outside the cell. Metabolical intracellular trehalose does not show a clear effect, except when its amount is artificially increased and it is used together with external trehalose [95].

The ability of trehalose matrices in preserving the three-dimensional integrity of biological cells has been investigated in ultra-high vacuum mass spectrometry. The method has been exploited to provide the imaging *in situ* of lipid localization of cellular membranes at room temperature, beside ice matrices. In this way, it has been possible to study the sites of phagocytosis in macrophages at a submicrometre resolution; fine biological processes, such as those occurring in the nervous system, can also be resolved [96].

Guo *et al*. reported that human primary fibroblast cells at low concentrations of trehalose remained viable for days in the absence of water. The authors showed that the sugar is not only able to preserve cell components or prokaryotes, but also the complex eukaryotic nucleated cell, merely by inducing intracellular trehalose synthesis. This was achieved by infecting the cell with a recombinant adenovirus carrying the trehalose-biosynthetic genes from E. coli. [97]. These results have however been challenged by other authors, who claim that a minimum internal concentration of trehalose (~0.2 M) is needed to preserve the components of the eukaryotic cell.

Chen *el al*. reported results for plasma membrane of fibroblasts. It was shown that that 0.2 M trehalose was needed both inside and outside the cell for a correct preservation, besides cooling to ~4 °C; concentrations below 0.1 M did not preserve the plasma membrane during drying. In this study trehalose was introduced inside the cell trough a pore-forming protein used to reversibly permeabilize the membrane. Results showed how the plasma membrane could remain intact during storage under conditions of 22 °C and 5% relative humidity, even after several weeks of storage [98].

Corneal epithelial cells were protected from death by desiccation, in formulations at increasing trehalose concentration. This effect was peculiar: maltose, for example, had no effect under the same drying conditions. Indeed, there was no pattern for the response to maltose concentration, while a clear linear trend was observed for the response to trehalose concentration in the range 0.02 – 0.2 M [99].

Even whole bovine embryos can be preserved in trehalose solutions, before storage in liquid nitrogen. After storage, viability, after thawing and culture growing, was tested and it resulted significantly increased in the presence of trehalose [16].

Finally, canine lungs, preserved for 12 h using trehalose solutions, were examined using light microscopy for over two hours after transplantation. It was shown that this treatment kept the lungs significantly more functional and with normal histology, with respect to lungs stored in other preserving solutions [16, 100].

1.5. Biomolecules in Saccharide Matrices: A Biophysical Point of View

The perspective of this review is to set up a connection between the pharmaceutical/applicative approach, which is traditionally "*stabilization-procedure centric*", and the biophysical approach, which is generally "*protein-centric*", in line with other recent review works [101]. To this aim, we will review studies of bioprotective systems, which consist of concentrated solutions or glassy host matrices containing sucrose, maltose, trehalose, polysaccharides at different water content and rigidity, both in the presence and in the absence of proteins, through experimental and simulative techniques. Kinetic and thermodynamic properties of the systems will be analyzed. The attention will be addressed to the modulation of dynamics, to the molecular origin of this phenomenon and, mostly important, to its hydration, temperature, and composition dependence.

The review is organized as follows. In the second Section, we report on experimental and simulative insights on the effects on protein structure and dynamics. The data come from a complementary set of techniques, which provides a description of the involved processes from the atomistic to the macroscopic level. Results from each technique are separately presented and compared with the aim of drawing a unifying picture. In the third Section, the effects on protein stability are discussed. Also in this case, the data come from a complementary set of techniques, which provides a description of protein thermal/chemical denaturation and protein (or peptide) aggregation processes, from the microscopic to the thermodynamic level.

The effects on protein function will be discussed in the fourth Section, with focus mainly on ligand binding and electron transfer. In particular, some relevant flash-photolysis experiments on heme proteins, as well as photoinduced electron transfer experiments on photosynthetic reaction centers will be reviewed.

2. EFFECTS ON PROTEIN STRUCTURE AND DYNAMICS

2.1. Atomistic Level

It has been reported that saccharide matrices have a noteworthy impact on the properties of embedded proteins, as pointed out by neutron scattering experiments on carbonmonoxy myoglobin (MbCO) in very dry trehalose glasses and in a wide temperature range [102]. The Mean Square Displacements (MSDs) of the hydrogen atoms and the density of states resulted those of a harmonic solid, while in D_2O-hydrated myoglobin (Mb) a dynamical transition to a non-harmonic behavior was present at ~180 K [103-106], dependent on the hydration level. Therefore, it was suggested that, in the whole temperature range investigated, the trehalose matrix entraps myoglobin in a harmonic well. Similar results were obtained for C-phycocyanin embedded in a trehalose dry matrix. In neutron scattering experiments, the protein's atoms MSDs were found similar to those of the amorphous matrix, pointing out that their dynamics is strongly coupled [107, 108].

Elastic neutron scattering experiments were also performed to measure the MSDs of the hydrogen atoms of lysozyme (LSZ) embedded in glucose-water glassy matrices with different water content, as a function of temperature [109]. In the dry sample anharmonicity at ~100 K is still observed and attributed to the activation of methyl group reorientation [110, 111]. This suggests that very local protein dynamics is decoupled from the external environment on the investigated temperature range. In the more hydrated samples, larger anharmonic contributions appear at

higher temperature, just in correspondence to the dynamical activation of the surrounding glassy matrix. Raman scattering complemented neutron scattering experiments in the 100-350 K range, showing that both low and fast frequency protein motions in trehalose glasses parallel the ones of the matrix, pointing out how LSZ dynamics and activity are controlled by trehalose [112]. In particular, it has been found that trehalose is most effective in inhibiting protein dynamics at high temperatures. Protein dynamics is barely suppressed in glycerol, compared to aqueous solutions at ambient conditions; on the contrary, glycerol appears to better perform under cryogenic conditions [29, 113]. By adding low quantities of glycerol to trehalose, it is possible to reduce the amplitude of local fluctuations even at high temperature [114].

Temperature dependent optical absorption spectroscopy showed that, in MbCO-trehalose amorphous matrices, non-harmonic motions of Fe with respect to the porphyrin plane have a lower amplitude than in hydrated proteins systems, at room temperature. Results from Mössbauer spectroscopy showed that large scale quasi-diffusive Fe motions take place at ~180 K [115] at high hydration, while their onset is shifted to ~230 K in dry trehalose matrices. Moreover, these motions are largely suppressed even at room temperature [116].

Protein-matrix coupling has been investigated by Mössbauer spectroscopy also in MbCO embedded in 80% w/w sucrose-water system. The main heme displacements were controlled by the solvent viscoelastic properties. Furthermore, CO escape out of the heme pocket couples to the same type of solvent motions, while recombination involves heme deformation modes solvent-decoupled. With respect to glycerol, sucrose viscosity effects on heme displacements and on ligand binding kinetics are related to preferential hydration of the protein [117].

Results on spectral diffusion dynamics of cytochrome (cyt-c) suggests that large-scale motions are more impaired in a trehalose than in a glycerol glass [118]. Optical heme absorption and IR CO bands were also investigated in similar sugar-coated cytochrome systems. The width of the iron-bound CO IR band was found dramatically influenced by solvent conditions. In particular the band remained broad even at low temperature (<200 K), suggesting that the conformations of the protein arising from large-scale motions are 'frozen in' [119].

To understand at a molecular level how sugars, and in particular trehalose, protect biomolecules, Molecular Dynamics (MD) simulations have been carried out on binary hydrated saccharides and ternary, protein containing, systems. Simulations of binary systems were performed under various conditions, namely with different force fields and computational protocols, at many different temperatures and sugar concentrations; results revealed how the dynamics and the water HB network are sizably altered by trehalose. [40, 41, 120-128]. However, the slowdown of water dynamics around trehalose molecule can be observed only in the first solvation shell wherein it depends mostly on the local molecular topology [129].

It has also been shown that trehalose has a higher hydration number than sucrose. This could play a role in preservation processes; indeed, the trehalose glass would hinder molecular motions by binding water molecules more tightly than other sugars. This possibly leads to its superior cryo- and lyoprotective properties [130]. MD simulations have been also performed in ternary LSZ-sugar-water systems at various sugar concentration [131-134]. More recently, a simulation of a solid state system composed by several LSZ proteins embedded in a low water glassy trehalose matrix has shown the presence of a more tight molecular packing of the proteins, which impairs the formation of large voids present in the dry LSZ glass without sugar [46].

MD simulations have been performed on MbCO-saccharide-water systems at very low water content, roughly comparable to what can be experimentally obtained [135-138]. The Mean Square Fluctuations (MSFs) on the hundreds of picoseconds time scale, computed in the 100–300 K range, were compared in trehalose and water-solvated MbCO and with the above mentioned Mössbauer [116] and neutron scattering [102] data on trehalose-coated MbCO. Results showed that the amplitude of nonharmonic motions is lower than in aqueous solutions (0.04 $Å^2$ for the backbone heavy atoms, to be compared to ~0.2 $Å^2$ in water, at 300 K), and their onset is shifted toward higher temperature. This holds true for almost all the atomic classes, and in particular, it is shown that confined diffusive heme motions are present, leaving the underlying harmonic, small amplitude, vibrations unaltered. In other words, harmonic vibrations in the heme are not affected by trehalose, while large scale anharmonic protein motions are reduced.

Analogous MD simulations of a very concentrated liquid MbCO-trehalose-water solution [136] showed instead that the internal protein dynamics is not affected by trehalose (MSFs averaged on all the protein atoms 0.24 $Å^2$, in agreement with the values in water solution). Similar results were obtained from simulations of lysozyme in a trehalose aqueous solution: the calculated fluctuations for the Cα atoms showed no significant difference with or without trehalose, the corresponding averages over the protein residues being 1.02 $Å^2$ and 0.98 $Å^2$, respectively [131]. From these studies it can be concluded that *hard confinement* is needed to suppress the high temperature large scale protein motions, while *soft confinement* is almost ineffective.

Long time ago, it was performed a MD simulation of MbCO in aqueous solution, in which water and protein atoms were coupled to two different thermostats at two different temperatures, 180 and 300 K respectively [139]. Low temperature water in this way acted as a high viscosity solvent uniformly enclosing the protein. Protein atomic fluctuations were significantly reduced and, most importantly, their profile was uniform along the protein residues. A similar result has been obtained in the MbCO-trehalose-water systems above described [138]. In protein-water systems, it has been shown that water translational motions are necessary for the onset of protein large-scale motions and structural relaxations [140, 141]. These studies pointed out that reducing the dynamics of the solvent-matrix as a whole has a strong effect on the protein dynamics.

For a binary system composed of sugar and water, it has been possible to disentangle the effects of the two components by shutting down the dynamics of one of them. To this aim nanosecond time scale simulations of MbCO have been performed in a dry trehalose-water system, in the range 50-400 K, with constraints imposed on the water molecules, either by blocking both the translational and the rotational motions of the molecules, or by blocking only translations, while allowing the water molecule to rotate [142]. Results showed that the dynamics of MbCO is severely impaired in the systems with fixed water molecules, and that, in particular, protein atomic fluctuations throughout the protein are strongly reduced when water translational degrees of freedom are suppressed. MSFs were similar to the ones in the same system at 200 K in which all water degrees of freedom are preserved. The average of the MSFs along the protein chain is ~0.06 $Å^2$, pointing out that

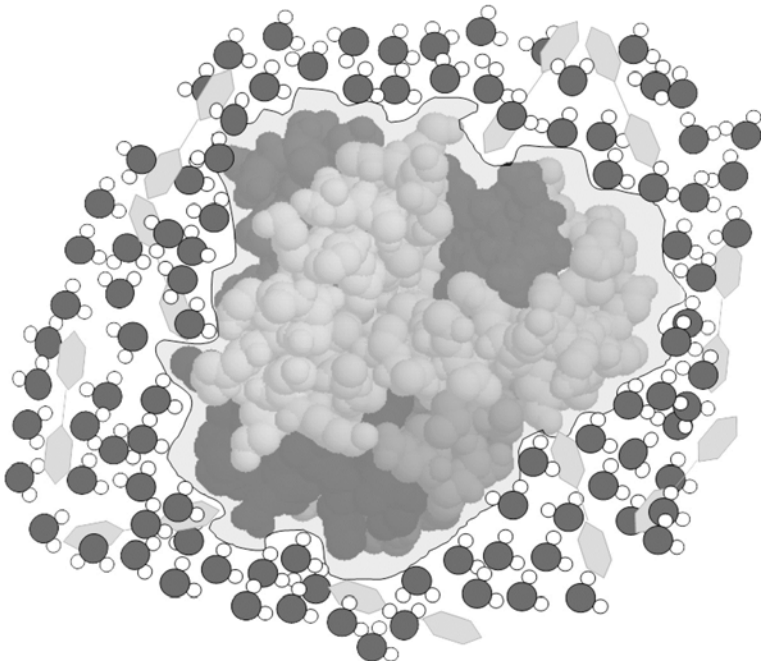

Fig. (2). Pictorial sketch of myoglobin (in vdW representation) surrounded by a layer of water molecules (dark grey); few trehalose molecules are also shown in light grey.

inhibiting water translational displacement at 300 K reduces the protein dynamics on the nanosecond time scale to that measured on lower (100 ps) time scale (see above). Furthermore, no protein dynamic transition is observed in the whole temperature range, even at the temperature of the matrix glass transition [142].

An analysis of the solvent partitioning at the protein-solvent interface revealed that a higher water is present than in the bulk solvent; few trehalose molecules are also present, forming single HBs with the protein (see Fig. **2** for a pictorial sketch). Results agree with previous experimental data [143] suggesting that trehalose or sucrose interact mostly with protein residues, where water is usually bound via single HBs. In other words, the sugar does not displace the primary bound water molecules. Furthermore, in agreement with IR data [26], it was found that sugar molecules are mainly bound to the carboxylic O atoms of GLU and ASP, and to the peptide O atoms. Upon dehydration, the number of water molecules bound both to protein and trehalose increases, as well as the average number of HBs a water molecule engages in.

According to the above observations, the water replacement and water entrapment hypotheses do not exclude each other. This was also pointed out by simulations on LSZ-trehalose solutions, where trehalose clusters were found at the LSZ surface, reducing the flexibility of the protein backbone [144].

As stated by Green and Angell "there is no clear structural explanation for the relative efficiency of trehalose over other sugars that also act as desiccation protectants except that it is not related to the number or the position of hydroxyl groups available for hydrogen bonding" [18]. As reported by these authors, at room temperature the trehalose matrix is still a glass, whereas sucrose and maltose matrices are already above their glass transition. Although different physical states may imply different effects on the protein's structure and dynamics, it must be considered that results for binary cannot be extrapolated to ternary systems. To better understand the trehalose peculiarity, MD simulations have been extended to the homologue disaccharides sucrose and maltose (Fig. **1**) [137, 138].

Moreover, the protein MSFs were compared with those in aqueous solutions at 300 and 400 K. The high temperature simulation in water was carried out with the following motivations: i) to find which regions of the main chain are involved in the onset of protein thermal denaturation and to which extent these regions are preserved by sugar; ii) to compare the effects of various disaccharides with respect to bioprotection.

Overall, the main chain MSFs were found similar in the three homologue disaccharides. However, the trehalose matrix imposes stronger and more uniform constraints on the protein, while the other disaccharides allow part of the chain to be more flexible; as pointed out in the water simulation, these regions are among the ones more involved in protein thermal denaturation.

Sucrose and maltose are largely excluded from the protein surface. However, results show that the number of water molecules bound to both the other components is lower than in trehalose, despite the larger subset of water still bound to protein. Under the hypothesis that the fraction of bridging water molecules mediates the protein-matrix coupling, results suggest that this is tighter in trehalose than in other homologues disaccharides.

To summarize, MD simulations provided hints on the effects of disaccharides on the protein internal dynamics, pointing out a key role for the residual water present in the systems on the local flexibility at residue level. This result was also strengthened by the HB analysis, which, once more, pointed out the key role of water molecules in mediating the protein-matrix interaction. Results suggest that, at very low hydration, the efficacy in bioprotection could be attributed to the sugar potential to anchor a thin water layer at the protein surface, hindering large-scale protein motions coupled to solvent. Small scale local motions, which do not imply displacement of the protein surface, would still be allowed. This hypothesis has been supported by vibrational echo experiments on proteins in trehalose matrices or silica gels (see below). Furthermore, water strongly bound to the protein surface would increase the surface tension, because of the increased free energy cost to displace water, brought about by the co-solutes.

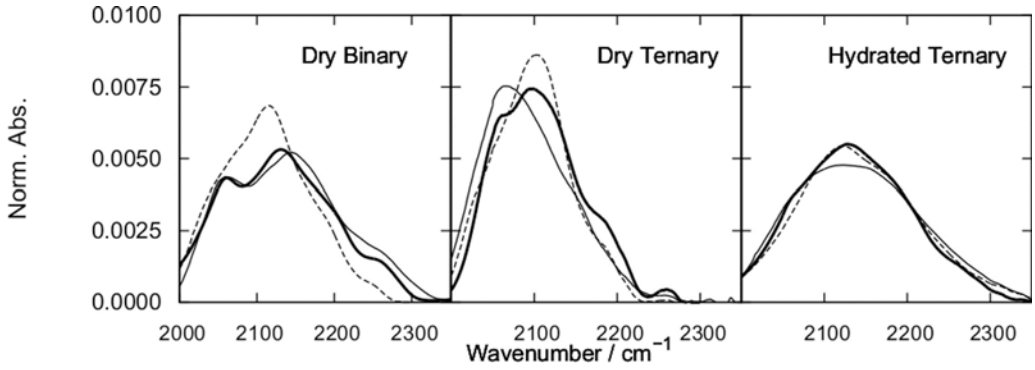

Fig. (3). Normalized spectra of the Water Association Band (WAB) at 300 K. Thick lines: trehalose [145]; dashed lines: sucrose [146,168]; thin lines: maltose [54]. Reproduced from Eur.Phys.J.E, 36, 2013, 79, *Proteins in amorphous saccharide matrices: structural and dynamical insights on bioprotection*, Giuffrida, S.; Cottone, G.; Bellavia, G.; Cordone, L. [148] with kind permission of The European Physical Journal (EPJ).

The protein-matrix coupling, as a function of hydration, has been also studied by Fourier Transform Infrared Spectroscopy (FTIR) in trehalose systems containing MbCO at different water content [54, 145, 146]. It has been analyzed the stretching band of the iron-bound CO molecule (COB), whose shape is dependent on the structure of the heme pocket and on the system water content [147], and whose thermal evolution reflects the internal dynamics of the protein. The matrix properties in the same systems have been studied through the Water Association band (WAB). This band arises from a combination of intramolecular bending with intermolecular modes of water, which may involve also non-water HB-forming groups [145]. Saccharides are very hydrophilic molecules, able to bind several water molecules in their hydration spheres; this makes the properties of the saccharide matrices strongly dependent on water content, the evaluation and control of the hydration playing an important role in the study of these matrices. The study of the properties of the infrared bands of water, such as the WAB, conveys deeper information on the properties of water in these systems. Furthermore, thanks to the intermolecular contributions, the WAB thermal evolution provides hints on the matrix thermal modifications.

FTIR measurements were performed also in the homologue disaccharides sucrose and maltose, in raffinose, and in glucose matrices, at various hydration levels in the range 20–300 K; results were compared with those obtained in the trehalose matrices [54, 148]. With the aim to rationalize the trehalose peculiarity with a model based on the characteristics of the HB network present in the system, ternary (protein-saccharide-water) system were also compared with corresponding binary systems (saccharide-water). This gave information on the molecular basis of the complex relation among sugar, water and embedded proteins. In what follows, we focus once again on the comparison among the three homologue disaccharides trehalose, sucrose, maltose (Fig. **1**) [148].

When comparing high and low hydration ternary samples a distinctive trend is present, irrespective of the saccharide: in the former the WAB loses almost all its saccharide-specific features and approaches the shape typical of pure water [149] (Fig. **3**, right panel). This was suggested to arise both from the higher contribution of water-water interactions on the band and from a higher mobility of the water molecules, which is expected for an amorphous but not glassy system, resulting in an averaged absorption.

WAB profiles in dry conditions are instead strongly dependent on the type of saccharide, as they arise from different pattern of population of HB local structures (Fig. **3**, left and central panels). In particular, in the three disaccharides here reported the band is divided in at least four components, whose frequency is roughly the same in all the samples, but whose population differs. The comparison of the band profile in binary and ternary systems shows that the studied sugars fall in two categories: in trehalose and maltose the band shape undergoes a huge change, indicating that the introduction of protein alters strongly the distribution of HB structures; in sucrose the band shape shows smaller changes. In particular, although the main component always corresponds to bulk-like water absorption, in both maltose and trehalose one can notice an increase in the low-frequency components, this effect originating from the presence of weaker hydrogen bonding groups, as expected in case of protein-water or protein-sugar interaction. This is less evident in sucrose, indicating that the distribution of water molecules is not strongly altered. Hence the presence of the protein has only a weak influence in sucrose matrices; conversely the matrix does not change its HB networks to better host the protein. This can be at the basis of the reduced effectiveness of sucrose as bioprotectant. At variance, in the maltose ternary system there is a larger population of the low-frequency component than in the trehalose one, indicating an average weakening of the HB network. Given that water-sugar HBs are, in average, stronger than protein-water, this would indicate either that more water is present in the protein domain, or that protein is involved in a larger number of HB. This enables to put the three sugars in an order of HB network strength (sucrose > trehalose > maltose), defined as the resistance to alteration upon protein addition. This order parallels the order of influence of these sugars on the homogeneity of the saccharide matrix on a larger scale as identified by Small Angle X-ray Scattering (SAXS; see below).

The concurrent study of the COB confirmed this behavior, probing directly the protein alterations. We reported that a shoulder appears in the low-frequency side of the CO spectrum in sucrose matrices [146]. The occurrence of this component could be ascribed to the presence of sucrose-like units which induce heme pocket structural alterations, as observed in MD simulations [137]. In addition, in this case the strong and scarcely alterable HB network in sucrose could be at the basis of the deformation of the protein, which is forced to adapt to this HB network. This is in agreement with the suggestion that sugar-sugar interactions could be so strong that sugar molecules would ultimately interact with themselves rather than with the protein [53]; in this case the sugar coating might not be adequate to preserve proteins.

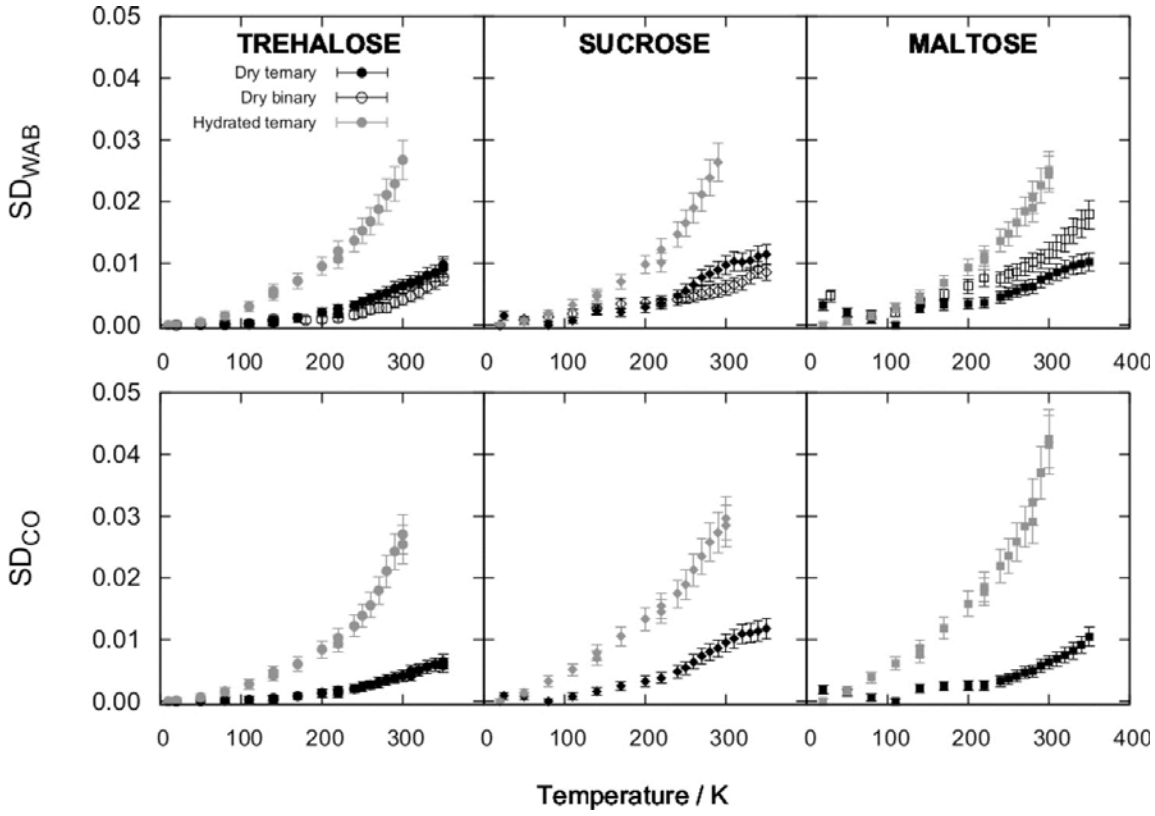

Fig. (4). Spectra distance for the WAB (SDWAB, upper panels) and the COB (SDCO, lower panels). Reproduced from Eur.Phys.J.E, 36, 2013, 79, *Proteins in amorphous saccharide matrices: structural and dynamical insights on bioprotection*, Giuffrida, S.; Cottone, G.; Bellavia, G.; Cordone, L. [148] with kind permission of The European Physical Journal (EPJ).

Both protein and matrix thermal evolution have been investigated by calculating the distance function of the spectra measured at various temperatures with respect to a reference spectrum measured at low temperature, which we called Spectra Distance (SD); for the quantitative definition of SD see in Refs. [54, 145, 149]. The quantity SD was proposed to reflect the thermally induced modifications of either the matrix (SDWAB) or the protein, at least as evidenced by the bound ligand (SDCO). In this respect, given that structure alterations originate from protein dynamics, SDCO could also hint on internal protein motions.

In dry ternary samples both SD have very low values up to ~ 200 K for all the sugars, while a very small increase is evident above 200 K (Fig. **4**). This indicates small structure modifications in both protein and matrix at all temperatures, suggesting that only harmonic atomic motions are present in both, at least within our sensitivity.

The SDWAB for binary samples has values comparable with dry ternary samples (Fig. **4**, upper panels). In particular in trehalose the thermal evolution of SDWAB is almost identical with or without protein, in the whole temperature range investigated. Taking into account that the protein clearly affects the population of the WAB components, and that both SDCO and SDWAB have similar behavior, it has been inferred the presence of a tight protein-matrix dynamical coupling. In the light of MD results, it was suggested that this coupling originates from the presence of a HB network connecting proteins side chains, sugar molecules and water. This network would anchor the protein surface to the surrounding medium, and its rigidity would depend on hydration (also referred to "*anchorage hypothesis*") [150-152].

Hydrated ternary samples show higher values for both SDs, which have a linear temperature dependence below to 200 K; above this temperature, a slope change is observed in all samples. The SDCO trend at low temperature reflects the hindrance of large scale dynamics of the protein, still allowing low tier conformational interconversions (Fig. **4**, lower panels). The same in SDWAB points to an analogous hindrance of water motions at the protein-matrix interface. Above 200 K, *i.e.* above the transition from a glassy to a rubbery state (see Section 3 below), the HB network encompassing the system loosens; protein-bound water starts to diffuse, enabling protein large-scale motions.

In this respect, the thermal behavior of COB has been compared with the thermal behavior of MSDs of protein hydrogen atoms as measured by Neutron Scattering experiments on dry and hydrated LSZ and tRNA [153], and on MbCO embedded in trehalose, also in view of MD simulation results [154]. It is worth noting that the onset of large scale interconversions (see arrow in Fig. **5**), detected by FTIR, almost coincides with the protein dynamical transition detected by Neutron Scattering.

In hydrated systems, the SDWAB plots relative to different sugars are roughly superimposable in the whole temperature range [54] (Fig. **4**, upper panels), confirming again the substantial similarity of the hydrated sugar-water matrices; on the other hand, the SDCO plots overlap only at low temperatures but start to differ at higher temperatures (Fig. **4**, lower panels). This suggests differences in the heme pocket thermal dynamics with respect to the matrix dynamics, hence a weakening of dynamical coupling. The different behavior of trehalose and sucrose samples indicates that sucrose mixtures are a better plasticizer of protein motions responsible for the protein dynamical transition, which is consistent

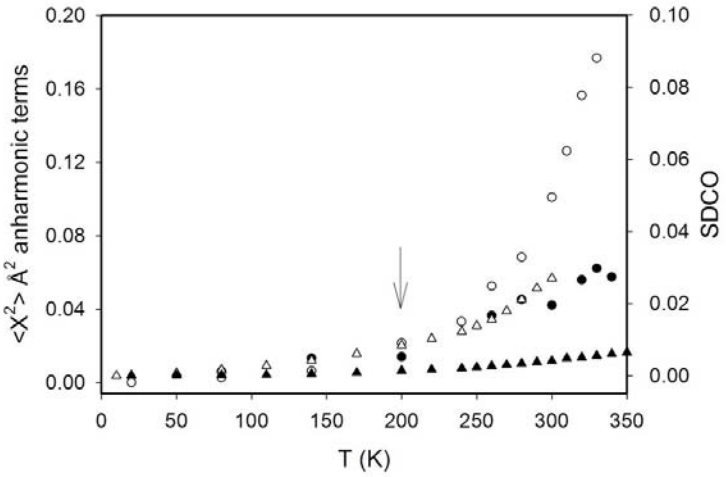

Fig. (5). SDCO (on the right axis) and non-harmonic contributions to the hydrogen MSDs measured by elastic neutron scattering for trehalose-coated MbCO [154] (on the left axis). Open circles: MSDs for humid sample; full circles: for dry sample. Open triangles: SDCO for humid sample; full triangles: for dry sample. The arrow marks the temperature of anharmonic activation. Partially modified from Fig. **5** in Ref. [156].

with sucrose having a lower T_g value than trehalose [101, 151, 155].

To summarize, FTIR spectroscopy has been exploited as a valid technique to explore, at one and the same time, properties of matrix and protein, by making use of suitable bands. This allowed examining in detail the HB network alterations. The thermal behavior of these bands has also been fruitfully exploited to obtain information on protein and matrix thermal evolution, complementing information from neutron scattering measurements [156]. With this technique it has been possible to point out *i)* the peculiarities of different disaccharides toward different aspects of the matrix-protein coupling; and *ii)* that in a mixed protein-cosolvent system, the global protein dynamics is neither solvent slaved, nor it exhibit inherent features independent of its solvent environment [151, 157].

Rigidity in dry matrices of oligo- or disaccharides might arise from the formation of domains encompassing the whole system, where an extensive water-sugar cross-connection is present, and matrix stiffness is modulated by water content. On the contrary, FTIR results [54] have shown that in the case of monosaccharides sugar molecules do not form extended structures.

Relevant results are provided by Terahertz Spectroscopy measurements in aqueous solutions of trehalose, lactose and glucose [158]. It was found that sugars affects fast collective motions of solvent, even far away the first hydration shell, and that the absorption depends on both solute concentration and number of hydrogen bonds between sugar and water molecules. Solvent collective motions could set up, depending on the solute; if solute molecules are not involved in hydrogen bonding, such long-range coherence reduces or even fails. In this respect, light scattering measurements on trehalose-water solutions in a wide frequency range (GHz to THz) [159] reveal two separate solvent relaxation processes: a slow one, related to the solute dynamics, ascribed to hydration water, and a fast one ascribed to bulk solvent. These results were confirmed by MD simulations [160] and in presence of protein [161].

Overall, FTIR and MD results suggested that HB networks, mediating the protein-matrix coupling, stiffen on drying. Therefore, saccharides protect proteins not just preserving their native solvation; likely they lock the protein surface either with direct protein-sugar interactions and/or via bridging water molecules. The presence of extensive HB networks in amorphous sugar matrices then increases the energy costs of the solvent rearrangements necessary to the onset of large-scale protein motions involving surface displacements [162]. This hypothesis was also supported by vibrational echo experiments [163]. The vibrational dephasing of the CO stretching mode was measured for native and mutated heme-proteins in trehalose matrices and aqueous solution [163]. The vibrational dephasing of the heme-bound ligand was sizably reduced in trehalose with respect to aqueous solutions. Spectral diffusion in proteins was slower when the protein surface was locked by the glass. As stated by Massari et al. *"...some dynamics are coupled to the hydration shell of water, supporting the idea that the bioprotection offered by trehalose is due to its ability to immobilize the protein surface through a thin, constrained layer of water"* [163], in full agreement with results obtained by MD simulations of MbCO (Fig. **2**).

Although internal protein dynamics is globally hindered in trehalose matrices, local, small amplitude changes of some protein regions may still occur. Indeed, MD simulations [135] showed that even though room temperature MSFs of the heme heavy atoms were reduced, the heme hydrogen atoms were more mobile than in water solutions. It was also found that one trehalose molecule was persistently bound to the heme propionate atoms [136]. These results point out the need for a more thorough study of the local scale structural and dynamic effects of the matrix. To this aim, X-ray absorption fine structure (XAFS) spectroscopy is particularly suited to probe the local environment of metal ions in proteins and the associated thermal fluctuations and static disorder [164, 165]. XAFS measures relative MSDs between the absorbing and back-scattering atoms; therefore results provide information different but integrating those given by Mössbauer and X-ray diffraction [166]. A XAFS Fe K-edge study of cyt-c was performed in solution, in a soft confinement system (hydrated polymer) and in trehalose glasses at various hydration levels (hard confinement). Results showed that the polymer interacts weakly with the embedded protein, giving results similar to protein in solution. Incorporation in trehalose leads to severe structural changes. In particular, in a very dry matrix, the MSDs of the Fe atom relative to the first coordination shell were reduced, and porphyrin ring was distorted.

It was then suggested that the matrix induces protein conformational changes, leading to local deformations of the heme structure.

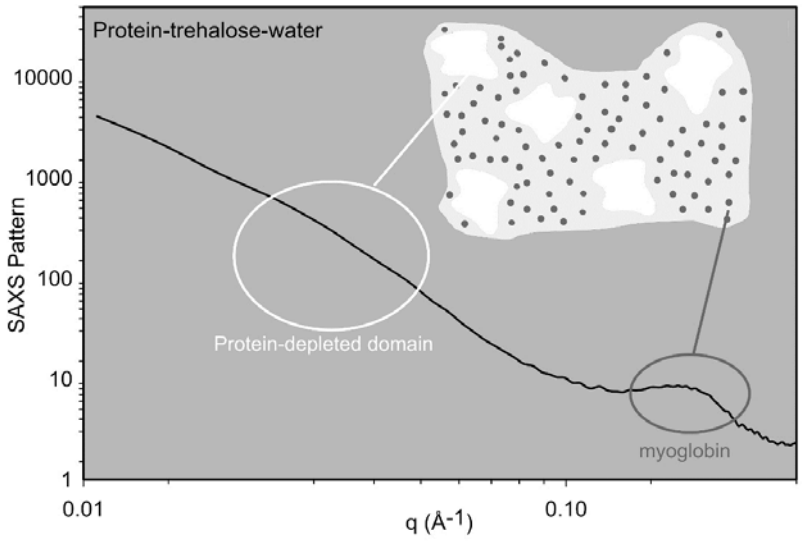

Fig. (6). A typical SAXS pattern for a trehalose-myoglobin-water dry sample. The protein and the protein-poor domains signals are highlighted. Sample features are pictorially sketched in the upper right region inside the plot: the protein poor domains are in white; proteins in black. Reproduced from Ref. [168] with permission from the PCCP Owner Societies.

Indeed, a persistent direct interaction of the heme with the matrix was already pointed out by MD simulations (see above). The distortion of the C heme atoms located in the fourth shell from iron, as probed by XAFS measurements, could be then attributed to protein-matrix interactions extending from the surface down to the protein interior, to a significant length scale [167].

2.2. Meso- and Macroscopic Level

Results discussed so far enabled to study the relation between protein and matrix structure and dynamics at molecular level; however they have been obtained with techniques not suitable to obtain information on larger structural alterations. In particular, because of their spatial scale, they are not able to mark the presence of micro- or mesoscopic inhomogeneities. To this aim, SAXS measurements on trehalose, maltose or sucrose systems, with and without embedded myoglobin, were performed to detect the reciprocal protein-matrix effects on a larger spatial scale [168, 169]. SAXS was chosen as a very convenient technique to study structures at the micro-nano scale, if a contrast of electronic densities subsists.

SAXS results indicated that saccharide-water systems, either with or without protein, are rather homogeneous on a micrometric scale, irrespective of the specific saccharide (see Fig. **6** for a pictorial sketch). The only difference is found in sucrose samples, where a number of smaller inhomogeneities can be detected, probably stemming from the formation of nanocrystals. This effect could be expected by considering that sucrose samples have lower residual water content, with respect to the other sugars; this promotes crystallization since sucrose crystallizes in anhydrous form [170]. Similar results were found in a recent study of matrices of sucrose or trehalose at various water contents, performed with high-field W-band EPR and FTIR spectroscopy [171]. Results pointed out a substantial difference between trehalose and sucrose in both structure and dynamics of the dry matrices, that holds during rehydration. At low hydration, trehalose appears to form a homogenous matrix, in both distribution of water and spin-probe molecules. The sucrose matrix appears instead to be heterogeneous, and composed by clusters of crystalline sucrose and domains of bulk-like water.

In the case of protein-containing samples the protein signal is clearly present, with a radius compatible with the native globin fold (Fig. **6**). Because of the high protein concentration, interference among Mb molecules is present, giving a peak corresponding to an average protein-protein distance of ~ 31 Å, irrespective of the saccharide. This homogeneous behavior indicated that average distribution of myoglobin molecules is not significantly altered, hence no definite phase separation or aggregation occurs [169]. However, the volume fraction of interacting proteins was found different in the three sugars. This difference stands for the amount of denatured protein in the system, as only proteins with similar shape and dimension can interfere. It has been possible to establish an order of efficiency in preserving the native protein structure: trehalose > sucrose > maltose [169]. The low efficiency of maltose was attributed to its reducing properties, which alter the protein structure and trigger intensive molecular cross-bridging.

In the presence of proteins, large, broadly distributed structures were also detected, whose size depends on the specific saccharide [169] (see Fig. **6**). The difference between these domains and their background was attributed to a difference in the local protein to sugar ratio. They would be constituted by sugar-water cross-connected networks, whose stability relies on a partial exclusion of the saccharide, *i.e.* a strong HB-former, from the weak HB-former protein. These domains are sensitive to hydration, increasing in size with increasing water content. This could be justified by taking into account that the incoming water tends to concentrate in the sugar-rich domains, which are more hydrophilic.

At very high water content, the Mb signal broadens and shifts since increasing protein hydration makes the domains to merge with the background (a sort of “melting”). Indeed, the protein-protein distance reaches a value consistent with that of myoglobin surrounded by (at least) a single layer of water [168]. Upon further hydration, interference peak disappears, as all correlation among proteins is lost (true melting).

The nature of these domains is slightly different in the different saccharides. In particular, in the presence of maltose the inhomogeneities are the smallest; their dimension is twice the protein one, in agreement with other studies at molecular level, which pointed out

that maltose clustering is at the basis of this behavior [54, 126]. In MbCO-sucrose systems larger inhomogeneities are present; along with the similar protein-protein average distance and the small contrast, this indicates that the background and the inhomogeneities have little difference in protein concentration. Trehalose systems lay between these two extremes. The presence of a major perturbation of the system in trehalose or maltose ternary matrices well agrees with the FTIR results, which show that in trehalose and maltose, but not in sucrose, protein addition sizably alters the WAB shape [148].

The model above presented depicts, for ternary systems, the presence of domains lacking in protein within a background where protein is more concentrated. In particular, in the background the protein would be locked into its environment through a water-saccharides HB network; on the contrary, the domains would be primarily constituted by saccharide-water regions, wherein few (or even no) Mb molecules might be present. Unduly importance should not be credited to the specific nature of the saccharide. The main player is the HB networks it forms, which could also be modulated by other components of the systems as well as by different drying procedures. In this respect, it must be noticed that a similar inhomogeneous structure, obtained from Small Angle Neutron Scattering measurements, has been recently reported in frozen solutions of LSZ, both containing and not containing saccharides [172], suggesting that the segregation is a general feature of these high concentrated protein solutions. Also Raman imaging in freeze-dried samples reveals the existence of high sugar-concentration regions separating broader protein-rich regions, in which the protein was found slightly more denatured [173].

It has been reported [30, 174] that, in dry trehalose-water systems, trehalose dihydrate domains may develop by capturing water from amorphous regions, which therefore are water source and sink based on need. In this respect, it was proposed that the protein-poor regions act, in nature, as a water buffer contrasting the fluctuations of atmospheric moisture during the day. This water buffering effect might be the basic mechanism to restart the vegetative cycle in anhydrobiotic organisms. The random absorption of water molecules likely causes a random onset of the various functions of the organism, in its different compartments, leading ultimately to damages or death. These domains would hence contrast the effects of moisture variations, by preferentially absorbing water molecules, and then slowly "melting" in a homogeneous phase above suitable hydration levels. This gentle transition would prevent a random onset of function and dynamics in different regions of the sample.

^{13}C and ^{1}H solid-state NMR were exploited to study sugar effects on protein in lyophilized powders stored at different relative. Spin-lattice relaxation times, which probe motions in the kHz-MHz range, were shown to increase with increasing sugar concentration, pointing out the strong interaction between sugar and proteins and reduced molecular mobility for the protein [175, 176]. An analysis of the spin-lattice relaxation time in terms of two components was needed in the presence of the protein, indicating local *phase separation* at a different extent for different sugars. Since the proton distribution in the samples did not correlate with the corresponding distribution of relaxation times, it was suggested that sugar molecules partition between sugar-only and protein-sugar phases, in agreement with above reported SAXS and Raman results.

NMR experiments were performed also on a cold shock protein embedded in a glassy trehalose matrix, at various levels of hydration [177]. The protein structure was found more native in trehalose than in dehydrated lyophilized powders; nanosecond and microsecond time scales motions of N-H groups become slower, and on rehydration water molecules build up around proteins forming a layer at the protein-matrix interface.

On a larger space scale, low resolution NMR relaxation times showed the contribution of saccharides in inhibiting the molecular mobility in preserved bacteria (*Lactobacillus paracasei*), thus allowing the reduction of detrimental effects on the organism. In this case, dextran was shown to be particularly effective in blocking the low-frequency, large-scale, motions [178].

To probe the effects of sugars at a macroscopic level scanning electron microscopy was used, and the effects of sucrose and trehalose on LSZ and Mb in the so-called supercritical fluid drying was analysed. Protein stability and other physical properties of the dry powders were investigated, and the results compared with the same after usual freeze-drying. Sucrose-containing samples showed agglomerated crystalline particles, while trehalose-containing systems consisted of amorphous spherical particles. A large fraction of LSZ could be readily reconstituted; protein secondary and tertiary structure was well preserved. A significant fraction of Mb did not dissolve, especially in the absence of sugars. Surprisingly, during the supercritical fluid drying process trehalose appeared to be less effective than sucrose in the case of Mb [179].

3. EFFECTS ON PROTEIN DENATURATION AND AGGREGATION

3.1. Protein Stability and Denaturation

According to Green and Angell [18] the efficiency in bioprotection can be related to the glass transition temperature of sugar-water systems. Results described so far have clearly demonstrated that the introduction of protein molecules in a sugar-water environment brings about significant effects on the system structure and dynamics. With the aim to understand how the presence of protein might alter the glassy properties of the matrix, and how protein stability is related to its T_g, Differential Scanning Calorimetry (DSC) measurements were performed on sugar-water and MbCO-sugar-water samples, for maltose (reducing), trehalose and sucrose (non-reducing) disaccharides [180] (see Fig. **7** for a case).

For all these systems two behaviors can be identified in different hydration ranges, as expected for solids with an equilibrium between glass and crystalline phases. In the low hydration range a transition between a homogeneous glassy state and a liquid (rubbery) state is observed by increasing the temperature, and the T_g dependence upon hydration can be described with the Gordon-Taylor equation.

In the high hydration range the observed transition is between a heterogeneous solid, composed by a mixture of ice and glass and a homogeneous liquid, and T_g is constant. For all the disaccharides investigated, it has been possible to obtain homogeneous glasses independent of the cooling rate below a definite critical water/sugar mole ratio. This value has been found to be 20, 18, and 15 mol/mol for trehalose, maltose, and sucrose, respectively [180]. This order parallels the order reported for the perturbation of the HB network by sugars [36, 37, 181]. It is noteworthy that trehalose binary matrices exist as a homogeneous glass in a wider hydration range than sucrose ones, wherein phase separation and crystallization might occur even at relatively low water content, in agreement with results obtained by other techniques [168, 169]. Analysis of DSC data in the low water range suggested that, in protein containing samples, the T_g extrapolated at zero water

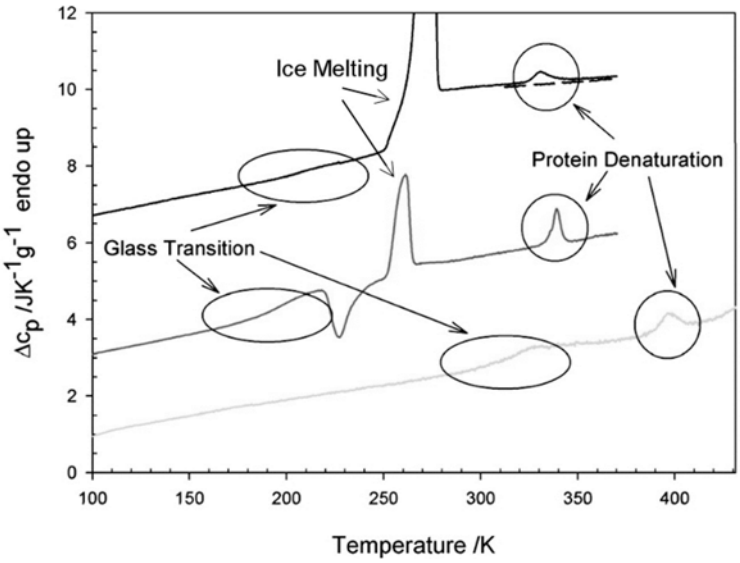

Fig. (7). DSC upscans in typical trehalose-water samples, normalized to sample mass (data refer to BSA in trehalose matrices). The curves are shifted along the ordinate scale for the sake of clarity. Bottom to top: 4, 41 and 277 water-trehalose mole ratio. The glass transition, the water melting and the protein denaturation are highlighted. Partially modified from Fig. **2** In Ref. [182].

content is lower than in samples without protein, hinting for a reduction of the underlying HB network strength. This could be attributed to water-sugar bonds weakening owed to the competition between protein and sugar for the few water molecules, in agreement with FTIR [145] and MD [136]. A monotonic increase of the Mb denaturation temperature (T_{den}) upon dehydration has been observed for nonreducing sugars, while for reducing sugars regularity is lost at lower hydrations, where Maillard reaction occurs [48].

In order to investigate the protein size or charge effects, DSC measurements were also performed in trehalose matrices containing LSZ, Hb and BSA, at constant sugar-protein mole ratio [182]. At high-to-intermediate hydration ($\geq$ 5 water-sugar mole ratio), a protein size effect on T_g was detected: T_g increases with protein size at constant water-trehalose mole ratio. This was attributed to a stiffening of the HB network, probably due to the solvent confinement induced by the protein. At very low hydration (< 5 water-sugar mole ratio), a T_g decrease is observed independent on the protein size: this could be interpreted as the onset of sugar-protein competition for water, as above mentioned.

While for T_g the effects of different sugars or proteins are of the same order of magnitude, T_{den} is much more sensitive to the kind of protein, as it is expected given that T_{den} is a protein property. The effective protein stabilization was found to depend also on charge effects. The T_{den} increases monotonically for all proteins, following the order: Mb<<LSZ < BSA ~ HB. For BSA, the stabilizing effect has been attributed to the strengthening of the matrix HB network, due to electrostatic interactions. Interestingly, in all the systems studied T_{den} and T_g were found to be linearly correlated. This finding has been proposed as a marker of the protein-matrix coupling [54, 138, 150, 156]: collective properties regulating the glass transition are directly related to small scale properties controlling protein structure alterations, the stabilization degree depending on the peculiar interaction with the matrix. Trehalose stabilizes better than sucrose at equal T_g, while maltose is similar to sucrose, beside the occurrence of Maillard reaction.

Summing up, DSC has been utilized to complement the results obtained with the other techniques with a thermodynamics point of view. It has been possible to observe the different effects that disaccharides have on the denaturation properties of an embedded probe protein and, reciprocally, how different proteins shape the properties of the trehalose-protein glassy system. A linear dependence of T_{den} as a function of T_g was found in the whole range of hydration, where secondary processes (ice formation, Maillard reaction) do not take place; this relation enabled to show that trehalose better protects the protein, for a given T_g value. A strong effect of the protein size was found on T_g values, while both protein size and charge affect T_{den} for given T_g values.

A systematic study of the structure and enzymatic activity of two other protein models, α-chymotrypsin and catalase, has been performed in samples at high sugar concentration, by using fructose, sucrose, trehalose, maltotriose, and dextran. Results obtained by IR and CD measurements provide detailed information on the effects of freeze-drying and heat treatments on protein secondary structure, when embedded in different sugars. While the activity of α-chymotrypsin is almost recovered after lyophilization with or without carbohydrates, catalase lyophilized without carbohydrates loses almost half of its activity and significant structural changes are observed in the dry state. Both these alterations of catalase are significantly reduced in the presence of low molecular weight carbohydrates, and in particular of trehalose. Furthermore, in glassy systems of low water content, the Maillard reaction between carbohydrates and proteins, which is know to cause enzyme inactivation, is suppressed [183].

Static and dynamic light scattering measurements were performed on BSA at low (1 mg/ml) and moderate (30 mg/ml) concentrations, in solutions containing different water-trehalose or water-sucrose ratios, at temperatures near the onset of protein aggregation [184]. At 1 mg/ml protein concentration a parallel shift towards higher temperatures with increasing sugar-water ratio was observed for both the total scattered intensity and the protein hydrodynamic radius, this shift being larger for trehalose than for sucrose. The fractal dimension of aggregates was unaffected, indicating a similar growth mechanism in which an initial growth with fractal dimension d ≈ 1.5 (characteristic of partially reversible ag-

gregates of very low compactness, as observed in the literature [185]), is followed by a mainly Gaussian coil-like growth characterized by a fractal dimension $d \approx 2$ and consistent with the formation of more compact aggregates, as already observed in previous studies on BSA aggregation [185,186]. The data show also that in dilute solutions the effect of sugars on protein aggregation is a water mediated a-specific effect and does not depend upon direct protein-sugar interactions [184].

At 30 mg/ml protein concentration different effects of the two sugars were observed. Indeed, while trehalose caused temperature shifts almost identical to those observed in the 1 mg/ml sample, significant differences are observed in sucrose solutions. These differences could be related to the different sugar-water interactions and structure of their aqueous solution. More specifically, the different capability of the two saccharides to form intra-molecular HBs make sucrose, on average, less available than trehalose to fit the HB network at low water content. Therefore at high protein and sugar concentrations, *i.e.* at gelling conditions, the sugar and protein molecules start competing for hydrogen bonding to residual water; in the case of sucrose, the formation of internal HBs may reduce the strength of the water-sugar network in the protein domain, promoting aggregation [187] and causing the different temperature shifts observed. In short, the sugar-induced changes observed in the early stages of aggregation could originate from modifications of statistical thermodynamics of the systems, more specifically from changes of underlying microscopic structures and dynamics of the whole solvent.

Interestingly, the sugar induced temperature shifts observed in the aggregation process of BSA are linearly related to the corresponding shifts of the glass transition temperature, in close analogy with what observed for denaturation temperatures [182]. This relation, that links thermodynamic parameters pertaining to very different temperature intervals, suggests that the solvent perturbation caused by the addition of sugars is able to regulate the glass transition of the system, the protein denaturation and the initial stages of protein aggregation. It is worth mentioning that, in agreement with the above suggestion, the sugar-induced solvent perturbation is also able to cause a modulation of the free energy barriers that determine the rate of solvent exposition of the amino acid residue Cys34, a key structural determinant for the cross-linking, aggregation and denaturation of BSA [188].

Raman scattering experiments were performed on trehalose-, sucrose- and maltose-LSZ systems, both in the amide I band region and in the low-frequency range, providing a microscopic description of the process of thermal denaturation. In particular, amide I band appears very sensitive to secondary structure unfolding, whereas the low-frequency range hints for the protein-water dynamics coupling. In these studies, trehalose was found the best stabilizer of the protein secondary structure [132, 181, 189-192]. By comparing protein-trehalose solutions of different composition [181, 189, 191], it has been observed that the main effect of trehalose is a perturbation of the tetrahedral organization of water molecules and the stiffening of the intermolecular O-H interactions necessary to stabilize the tertiary structure [190]. In this way, the thermal stability of the water HB network makes the native structure stable. Moreover, trehalose hinders the denaturation first stage, when the tertiary structure evolves in a quite disordered state still keeping undamaged secondary structure. Trehalose is also proven to better stabilize the folded secondary structure, by shifting the unfolding process, *i.e.* the denaturation second stage, toward high-temperatures [181].

It was also reported that trehalose effectively prevents thermal inactivation and aggregation of LSZ [193]. In fact, following heat treatment, LSZ forms insoluble aggregates, which are almost completely absent in the samples incubated in the presence of the disaccharide. FTIR measurements suggest that in 1.0 M trehalose solutions the loss of α-helix structure is reduced and less intermolecular aggregates form. Electrospray ionization mass spectrometry (ESI-MS) was also utilized to investigate on protein structural transition, showing that trehalose is able to influence the solvent accessibility to the amide peptide backbone upon heat treatment, therefore decreasing local changes in the protein environment.

DSC and quasi-elastic light scattering measurements have been performed to study trehalose, sucrose and glucose effects on structure and colloidal stability of LSZ, in three different buffer solutions. Data suggest that structural and colloidal stabilization depends on many other factors, such as pH, salt concentration, beside obviously the type of sugar [194].

The stability of β-lactoglobulin against chemical denaturation has been investigated in the presence of trehalose, with time-resolved fluorescence of a polymeric probe covalently bound to a protein cysteine, both in native and denaturating conditions [195]. Fluorescence anisotropy measurements show that trehalose strongly affect the protein internal dynamics, making more rigid the probe environment. Results suggested that local and specific effects induced by the sugar cannot be ascribed totally to preferential exclusion and/or preferential binding if cosolvents are present, as proposed in the Timasheff model [21].

In this respect, the stabilizing effect of sugar glasses on protein or nucleic acids has been attributed to a 'replacement' effect by sugar molecules or to the kinetic effect of slow processes (α relaxation) associated with matrix vitrification. Each of these hypotheses have been experimentally supported, but they cannot adequately explain the observed stabilization in sugar-glasses. On the contrary, stability in sugar glasses seems directly related to β relaxations in the matrix, *i.e.* to high frequency processes. It has been shown that the decrease of the degradation rates of some proteins, namely LSZ, horseradish peroxidase and yeast alcohol dehydrogenase, parallels the reduction of fast motions in the protein-sugar glassy samples, indicating the importance of these short length scale, high-frequency, motions in protein stabilization by glassy matrices, allowing to maintain the enzymatic activity upon rehydration [196- 198].

IR and optical spectroscopy and tryptophan fluorescence and phosphorescence were also used to study different proteins (parvalbumin, melittin, staphylococcal nuclease) confined in sugar films and in glycerol-water. It was found, in particular, that in trehalose-sucrose films proteins retain their overall folding over the 20-300K temperature range [199].

Interestingly, trehalose appears to be a bioprotectant against damages induced by exposure to electromagnetic fields [200, 201]. Samples of BSA in H_2O and D_2O solutions were exposed to a static magnetic field at 200 mT and to a 50 Hz electromagnetic field at 1.8 mT. Effects on the protein secondary structure were studied by FTIR; results showed that the amide A and amide I band intensities decrease in the aqueous solutions, while in trehalose aqueous solutions the spectra were not significantly altered after the exposures. This confirmed the hypothesis of a possible bioprotective effectiveness of trehalose against electromagnetic fields. Similar experiments performed on Hb fully confirmed the BSA results.

3.2. Protein Oligomerization, Polymerization and Amyloid Aggregation

The effects of sugars on association and binding of concanavalin A have been investigated as well. This protein undergoes a reversible tetramer-to-dimer equilibrium as a function of pH. Relative to the dimer, sucrose and trehalose, among many other osmolytes, stabilized the tetramer to varying extents [202]. The ability to shift the equilibrium toward more compact shapes has been also shown in the case of BSA by circular dichroism [203].

The stabilization of tubulin freeze-dried with trehalose has been recently investigated [204]. Microtubules, polymers of tubulin, are indispensable for many cellular activities such as maintenance of cell shape, division, migration, and ordered vesicle transport within the cell. Tubulin is a thermolabile protein that rapidly becomes unable to associate and polymerize. To preserve its conformation and polymerization properties, tubuline is usually stored at -80 °C. Freeze-drying of tubulin in the presence of trehalose prevents the alteration of its biological activity, allowing its long-term storage at ambient temperatures.

Trehalose has been found effective also in inhibiting aggregation of beta-amyloid peptides, reducing their cytotoxicity. In particular, as observed with fluorescence staining and Atomic Force Microscopy, in the case of Aβ40 trehalose prevents the formation of both fibrils and oligomers. When trehalose is co-incubated with Aβ42, the sugar inhibits only the formation of fibrils, while the oligomers are still present [205]. It has been also found effective towards the insulin amyloid formation in vitro, as pointed out by many techniques, such as fluorescence induced by thioflavin T, circular dichroism, and atomic force microscopy. The trehalose effect seems to be totally non specific and due to the preferential hydration mechanism; furthermore, enhanced surface tension makes insulin monomers to assume a more packed configuration against the partial unfolding, a prerequisite for the amyloid formation [206]. The trehalose effects on W7FW14F apomyoglobin and insulin amyloid aggregation were also investigated by FTIR spectroscopy, far-UV circular dichroism and absorption, and thioflavin T fluorescence. Results showed that trehalose is more involved in the fibrillization process rather than directly preventing aggregation. The effects is dose-dependent and depends on the protein. Also the lag phase of insuline was increased in the presence of trehalose [207].

It has been reported that in the presence of trehalose, wild-type α-synuclein, the protein implicated in Parkinson's disease, *in vitro* forms huge amorphous aggregates and not the neurotoxic β-sheet-rich fibrillar aggregates. Furthermore, trehalose hindered the overexpression of wild-type α-synuclein in the transduced PC12 cells, protecting in this way the cells against neurotoxicity [208]. Results come from Quasi Elastic Neutron Scattering experiments performed to study both soluble α-synuclein and fibrillar aggregates. Measurements were performed in a large temperature and momentum transfer range [209], with and without trehalose. Results show that the soluble and fibrillar α-synuclein respond in a different way to thermal stresses. In particular, trehalose was found to influence protein fibrillation, by stabilizing wild-type α-synuclein into conformations with lower tendency to assemble.

Various disaccharides have been shown to reduce the polyglutamine aggregation *in vitro,* preventing cell death in polyglutamine-related diseases, as e.g. Huntington disease. Trehalose was found efficient in avoiding aggregation of truncated huntingtin, hence improving motor dysfunction in a transgenic mouse model. The formation of intranuclear aggregates was reduced consequently to the ingestion of 2% trehalose in the drinking water of mice. It must be pointed out that trehalose treatments did not induce cellular stress because neither ingestion nor over-expression of trehalose-6-phosphate synthase/phosphatase have effects on the levels of heat-shock proteins in the cell models [210].

Results on the aggregation mechanism of polyGln proteins suggest that trehalose acts either binding to polyGln domains or hampering protein aggregation during the first phase of the process jut increasing the protein stability [210]. It was also reported that trehalose may also inhibit aggregation of proteins containing expanded polyalanine repeats in cell models, decreasing toxicity of mutant proteins. Tests *in vivo* against Oculopharyngeal muscular dystrophy, a poliAla disease, showed that the sugar delays the disease onset and death of cells in mice, by reducing the formation of aggregates [211].

Trehalose protection has been exploited also against prion-based diseases. It has been shown that cells infected by prions are protected against oxidative stress, which triggers the production of insoluble protein aggregates. However the sugar does not alter the resistance to protease and the solubility of the aggregates prion isoforms [212].

To better understand the molecular basis of these inhibitory effects on the protein folding, MD simulations of a β-hairpin peptide have been also performed in trehalose-containing systems. At a proper sugar concentration, it has been found that the potential energy barriers along the folding pathway are decreased, facilitating the peptide folding. Furthermore, peptide-sugar HBs are present to stabilize the fold [213]. MD simulations of Aβ16−22 and Aβ40 peptides at different trehalose concentrations were also performed. Results showed that peptides aggregation is prevented by trehalose, depending on the sugar concentration; preferential exclusion has been supposed to be at the origin of the trehalose inhibitory effect.

4. EFFECTS ON PROTEIN FUNCTION

4.1. Flash-photolysis Experiments on Heme Proteins

Encapsulation of proteins in glassy saccharide matrices based on trehalose is able to greatly slow down even at room temperature protein motions that require significant structural rearrangements, as reviewed in the previous Sections. This peculiar effect offers the possibility of exploring how protein reactivity is modified when protein dynamics is selectively altered. Hagen *et al*. were the first to investigate the effect of encapsulation in trehalose glasses on protein function [214, 215]. In particular, they studied recombination kinetics of CO rebinding to Mb after flash-photolysis in MbCO-trehalose samples. The kinetics at room temperature was found highly non-exponential in time. Moreover, the heme absorption spectrum shifts progressively with ligand rebinding in accordance with the so-called kinetic hole burning mechanism [216-219]. These two observations demonstrated that the encapsulation in a trehalose glass was able to inhibit significantly interconversion motions between Mb conformational substates [220]. The average rate of CO rebinding to Mb from within the heme pocket (geminate rebinding) was found to be faster in a trehalose glass than in a 75% glycerol-water mixture at room temperature and no bimolecular rebinding of CO to Mb was observed in the trehalose glass. Hagen *et al.* attributed their observations as due to the high solvent viscosity able to inhibit a series of conformational changes (at the level of both the polypeptide chain and the heme) that were suggested to increase the inner barriers for ligand rebinding in liquid solvents [214]. Sastry and Agmon, however,

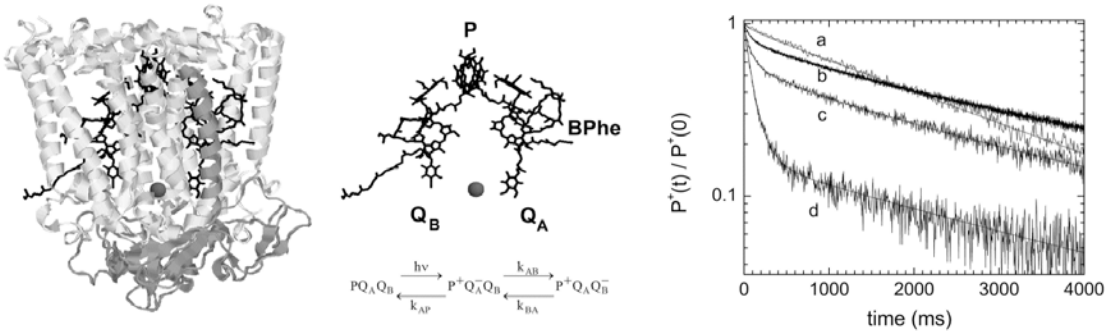

Fig. (8). Left panel: photosynthetic Reaction Centre from *Rhodobacter sphaeroides* in cartoon representation. Light grey: L and M protein chains; dark grey: the H subunit. Central panel: bacteriochlorophyll special pair (P), the two monomeric bacteriochlorophylls, the two bacteriopheophytins (BPhe), the ubiquinones QA and QB and the iron atom. A kinetic scheme describing the light-induced charge separation and recombination reactions involving P and the quinone acceptors QA and QB is also shown. Right panel: kinetics of charge recombination following laser flash excitation of QB-reconstituted RCs in trehalose–water matrices at water- trehalose molar ratios: 2.3 (trace a), 1.3 (trace b), 1.0 (trace c), and 0.8 (trace d). Continuous lines are best fit according to the sum of two power laws. Reprinted from Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1749, Lorenzo Cordone, Grazia Cottone, Sergio Giuffrida, Gerardo Palazzo, Giovanni Venturoli, Cristiano Viappiani, *Internal dynamics and protein–matrix coupling in trehalose-coated proteins*, 252-281, 2005, with permission from Elsevier [150].

Agmon, however, argued that the sugar matrix ability to prevent full protein dehydration is likely to retain a high protein internal flexibility. Accordingly, such flexibility would be responsible for the enhanced geminate protein reactivity observed by Hagen *et al*. [221]. Friedman and coworkers [222] studied trehalose effects on the reactivity of human Hb. As in the case of Mb, even at room temperature, CO geminate rebinding to the protein is highly non-exponential and no bimolecular ligand rebinding is observed. Moreover, time-resolved resonance Raman and fluorescence data indicated that, although the ultrafast iron out-of-the-heme plane motion is not affected, the opening of the heme pocket distal side is damped in a trehalose glass.

Kleinert *et al*. [223] encapsulated Mb in a sucrose glass and analyzed the CO rebinding kinetics from room temperature down to cryogenic temperatures. Bimolecular rebinding is suppressed below the solvent T_g of sucrose-water mixtures (T_g=227 K for 80% sucrose-water). Moreover, in the case of 92% sucrose-water glasses, a bimodal CO rebinding rate distribution at temperatures below 140 K is observed. This was interpreted as rebinding to Mb molecules with different protonation states of the heme pocket distal histidine (so-called A substates). By monitoring both kinetics of ligand rebinding after flash-photolysis and FTIR equilibrium spectra of MbCO embedded in trehalose glasses Librizzi *et al*. demonstrated that the interconversion between different protonation states of the distal histidine is almost fully hindered in conditions of high dehydration [224]. Upon slight increase of the sample hydration level, the interconversion between A substates is activated, nevertheless, a bimodal CO rebinding rate distribution is observed also in these conditions. This rules out the attribution of the two geminate rebinding phases to Mb in different protonation states that was instead attributed by the authors to CO rebinding from different ligand docking sites [225-228]. Independently, Friedman & coworkers [162, 229, 230], on the basis of flash-photolysis experiments on wild-type and mutant Mb embedded in trehalose glasses, showed that the room temperature ligand rebinding kinetics are indeed significantly affected by the ligand migration process [227, 228] and that the interplay between ligand rebinding and migration can be tuned by changing the sample water hydration level. Further evidence of the effect of ligand migration on CO binding in Mb embedded in dry trehalose glasses was obtained by pre-illuminating the sample before flashing-off the CO ligand [231]. In these conditions, a clear increase of the slow geminate phase component with respect to the fast one was observed indicating that the excess energy absorbed by the protein in the pre-illumination phase is able to facilitate the CO migration to the proximal side of the heme pocket.

4.2. Electron-Transfer in Bacterial Photosynthetic Reaction Centers (RCs)

Studies on the electron transfer kinetics in bacterial photosynthetic reaction centers (RCs, Fig. **8**, left and central panels) in different amorphous matrices pointed out the role of trehalose in altering the internal protein dynamics coupled to the electron transfer processes. Results show that protein motions are mostly unaffected in dry films without trehalose [232] or in polyvinylalcohol matrices [233], even in very dry samples (see also in [234]). Electron transfer processes involve different cofactors (electron transfer from quinone Q_A^- to quinone Q_B) and therefore distinct protein regions, each process with peculiar kinetics dependence upon matrix dehydration [235] (Fig. **8**, right panel). This suggests that different protein motions take place in the trehalose-water-protein system. In particular, in samples extremely dried, the after-flash kinetics of recombination of the primary, light-induced, charge separated state $P^+Q_A^-$ sharply accelerates, and the rate constants distribution broadens; actually, the behavior at room temperature appears the same as observed in a glycerol−water mixture at cryogenic temperatures [232]. Moreover, uninterrupted pre-illumination of RC embedded in trehalose brings about a gradual, but not complete, reversal of the above mentioned effects, suggesting a partial increase of the protein conformational freedom [236].

The same studies showed also that, in dry trehalose matrices, both structure and activity can be preserved at room temperature for long time.

As already suggested in the case of carbonmonoxy myoglobin, one could assume the presence of a HB network mediated by water locking RC surface to the matrix. The matrix effects would therefore propagate to the interior by residue-residue interactions. The internal protein rigidity would shape how the constraints imposed to the surface are transferred down to the protein interior, hence the extent of inhibition of protein dynamics [151]. To validate this hypothesis, kinetics of P^+Q^- recombination were investigated in carotenoid-containing RCs in dehydrated trehalose matrices [237]. This allowed to test how the absence or presence of

voids in the protein interior affects protein flexibility and the protein-matrix dynamical coupling, at least as hinted by $P^+Q_A^-$ recombination data.

The recombination kinetics of $P^+Q_A^-$, as well as RC thermal stability, have been studied also in sucrose matrices at different hydration [152]. Results show that protein dynamics is mostly similar to the one in solution, despite the very high viscosity and rigidity of the host medium. As for the protein integrity, it is mainly lost in sucrose after storage at 37 °C for few days, while it is maintained for long time in trehalose; moreover, the kinetics of loss of structural integrity is close to the one observed in protein dried without sugar. These results support the hypothesis that, contrary to trehalose, the RC complex is not coupled to the matrix, and depict a scenario in which the sucrose matrix and the protein are *dynamically* separated ("*nanophase separation*"). The two phenomena, either coupling or nanophase separation, are evident only at low hydration, when competition for the few water molecules starts. If less sugar-water-protein connections are present, as could stem from the lower propensity of sucrose to form HBs [130] with respect to trehalose [122], a separation of the mixture components might arise. In this case, the proteins could try to make HB with other partners in the system; this could led to protein clusters, in which the protein are kept together via inter-protein HBs connections, trapped in structures distorted and with a dangerous aggregation potential following rehydration [101]. This different behavior of sucrose and trehalose formulations at very low water content is also in agreement with the observations that in sucrose systems the WAB is scarcely altered by the insertion of the protein, while it is significantly red shifted in ternary trehalose-protein-water compared to binary trehalose-water systems [148].

As discussed above, redox active proteins are able to function even when they are embedded in polysaccharide glassy matrices, and they typically show increased stability over thermal denaturation [10]. J. Friedman & coworkers suggested that these properties could be exploited for developing new solid-state devices such as tunable batteries, switches, and solar cells. To this aim, a series of studies on the properties of electron transfer in sugar based glassy matrices have been performed. In particular, by doping trehalose based solid matrices with the reducing sugar glucose, it was possible to reduce metmyoglobin to deoxymyoglobin (or met-Hb to deoxy-Hb) by thermal inducing the electron transfer from the doping glucose molecules to the iron Fe^{3+} ions contained in the active site of the embedded proteins [238].

The same process could be obtained using two distinct trehalose sandwich layers, one containing met-Hb without any glucose, the other doped with glucose (or other reducing monosaccharides such as fructose or tagatose) but free of any protein; if the two glassy sandwiches are put in contact and heated at 75 °C for 45 min, full reduction of met-Hb to deoxy-Hb is obtained [239]. This long range electron transfer process can also be activated with light: if a protein free glassy sandwich is doped with deazaflavin rather than with glucose, put in contact with a met-Hb glassy sandwich, and the two are illuminated with low intensity light at 390 nm, again full reduction of met-Hb to deoxy-Hb is obtained [239]. Interestingly, a similar two-layer sandwich device can be used to induce the formation of ferrous nitric oxide hemoglobin (HbNO) if one of the layers contains initially met-Hb and the other layer contains nitrite and a thermal electron source (such as the monosaccharides glucose or tagatose): as the sample is heated at 50 °C, the hemoglobin optical spectrum gradually turns to the ferrous NO form indicating that: (i) NO can be easily generated in the second glassy layer by thermal reduction of nitrite; (ii) NO is able to diffuse across the interface separating the two layers [240]. By modulating the hydration level of the glassy layers it is also possible to trap intermediates in the reaction pathway between metHb and HbNO [240, 241]. In this respect, trehalose-water glass films have been proven useful to trap bovine rhodopsin intermediates in the light-activation process, at environment temperature. In particular, it was possible to trap conformations after photolysis whose UV-Vis spectra resemble those of the photointermediates metarhodopsin I and metarhodopsin II [242].

5. CONCLUSIONS

In this work we discussed the effects of saccharides on protein structure, dynamics and function, from the atomistic to the thermodynamic point of view. We reviewed results on saccharide systems in different physical states, from solutions to solid amorphous matrices, obtained with a large set of complementary experimental techniques, and from MD simulations. We focused on simple sugars, in particular disaccharides and among them trehalose, comparing them with mixed and polysaccharide systems where needed. The different mechanisms by which trehalose protects proteins were pointed out.

The emerging picture, at least in solid glasses, is that of an extended protein-water-saccharide HB network, encompassing the whole system, whose rigidity is modulated by water content. The behavior of the protein and the matrix are coupled, and both components concur in defining the whole system properties. The comparison among various sugars points out that, despite their structural similarity, the molecular basis of the interaction between saccharides and the other components in the system is peculiar to each saccharide. This effect is particularly evident in the glassy state at very low water content. Slight differences in molecular interaction lead to different mesoscopic structures of the amorphous matrices.

As far as the enhanced biopreserving properties of trehalose are concerned, they can be understood, at a molecular level, in terms of stronger interactions both with water and protein, that induce a stronger protein-matrix coupling. At a mesoscopic level, trehalose provides a host system characterized by protein poor domains, which might play as water buffers contrasting the variation of atmospheric moisture during the day. Such property is clearly beneficial in restoring to life the organisms in anhydrobiosis.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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