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Incorporation of α-chymotrypsin into the 3D channels of bicontinuous cubic lipid mesophases

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Abstract

The effects of protein entrapment on the structure and phase behavior of periodically curved lipid mesostructures have been examined by synchrotron small-angle X-ray diffraction and FT-IR spectroscopy. The study was directed towards a better understanding of the effect of confinement in a lipid environment on the stability and unfolding behavior of α-chymotrypsin, and, vice versa, the effect of the entrapped protein on the lipid's mesophase structure and temperature- and pressure-dependent phase behavior. We compare the interaction of protein molecules of two different sizes (cytochrome *c*, 12.4 kDa, and α-chymotrypsin, 25.8 kDa) with the cubic Ia3d phase of monoolein (MO), which forms spontaneously in water. The cubic structure changes significantly when cyt *c* is incorporated: above a protein concentration of 0.2 wt.%, the interaction between the positively charged protein and the lipid headgroups leads to an increase in interfacial curvature which promotes the formation of a new micellar cubic phase, presumably of crystallographic space group $P4_332$, which the lipid system does not form on its own. The larger α-chymotrypsin leads to a different scenario. On the basis of an examination of the calculated geometric parameters and water volume fractions, it is concluded that the α-chymotrypsin molecules cannot be located exclusively in the water channels of the cubic Ia3d or $P4_332$ phases, but rather form new, less ordered (presumably cubic Pn3m) structures. The new structure disappears above the unfolding temperature of chymotrypsin and exhibits a pressure stability, which – in contrast to cyt *c* in MO – decreases with increasing chymotrypsin concentration in the system. While the secondary structure of cyt *c* remains unaffected in the confining lipid environment, the structure of α-chymotrypsin gets destabilized slightly, and the protein tends to aggregate even at relatively low concentrations.

Keywords: High pressure; SAXD; FT-IR; Lipid; Cubic phase; Monoolein; α-chymotrypsin; Cytochrome *c*

1. Introduction

The conformational aspects of proteins while under confinement and at membrane surfaces are essential to understand various biological processes including their assembly and function. In general, interfacial structure and lateral organization of lipids and proteins in biomembranes are of great importance for protein–membrane interactions [1,2]. Furthermore, physical parameters, such as temperature, pressure and confining geometrical restrictions have strong influence on the structure of the membrane and its interaction with peptides and proteins [1–5]. Besides lamellar phases, which are most frequently observed in biological systems, the phases with cubic symmetry are the most complex and intriguing [1,2,6–9]. Locally, cubic lipid structures may be involved in different biological processes (e.g., membrane fusion, fat digestion), and can occur in cellular and intracellular membranes [6,10].

The structures of bicontinuous cubic phases (Q) have been described in terms of triply periodic minimal surfaces (TPMS), that is, three-dimensional arrays of connected saddle surfaces with zero mean curvature at every point of the surface [6–8]. In inverse cubic structures (Q_{II}), the lipid monolayer is arranged across either side of the minimal surface, touching it with their terminal methyl groups [6–13]. This results in 3D periodic

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bicontinuous structures, formed by distinct water and lipid volumes. The bicontinuous cubic lipid/water phases have specific and controllable water channel sizes and large surface areas. The crystallographic space groups of the cubic phases observed here, Ia3d and Pn3m, are based on the G (Gyroid) and D (Diamond) minimal surfaces, respectively. An interesting aspect of these systems is that the physical properties of these cubic phases can be finely tuned by different means such as changing water content, adding charged lipids and surfactants, and application of temperature and pressure [14–16]. Furthermore, the biological activity and stability of proteins can be modified by embedding in lipid mesophases as well as by pressurization.

New applications of bicontinuous nanostructured cubic materials in biochip and biosensor technologies are being actively sought. While lamellar bilayer-forming lipids are already used in biosensor systems, lipids forming non-lamellar structures, such as MO, are anticipated in novel protein biochip developments [17,18]. Furthermore, since cubic lipid phases are biocompatible and digestible, such bioadhesive matrices are being developed for controlled-release and delivery of proteins, vitamins and small drugs in pharmacological applications [19–21]. Another important application is that they offer a 3D lipid bilayer matrix for successful crystallization of membrane proteins [22–24].

The present study is directed towards a better understanding of the effect of lipid confinement on the stability and unfolding behavior of α-chymotrypsin, and, vice versa, the effect of the entrapped protein on the lipid mesophase structure and temperature- and pressure-dependent phase behavior. As a model system, we chose the cubic Ia3d phase formed by an uncharged lipid, monoolein, at limiting hydration of 20 wt.% water. In our previous work [25,26], we studied in detail the incorporation of a small water-soluble membrane protein, cytochrome *c* (12.4 kDa, isoelectric point (p*I*)=10.1), into mesophases of monoolein/water dispersions (20 wt.%). Cytochrome *c* consists of a single polypeptide chain of 104 amino acid residues and is covalently attached to a heme group. It consists of ~45% α-helices, ~18% turns and ~37% random coil segments [25]. The molecule is slightly ellipsoidal with dimensions of 30 Å × 34 Å × 34 Å. The cationic side chains of several lysine and arginine residues are clustered at the surface on one face of the protein and are thought to provide a binding site for anionic groups of cytochrome oxidase. Likewise, on the opposite face of the molecule, a cluster of anionic residues including glutamic acid and aspartic acid may provide a binding site for a reductase or other components of the electron transport systems. Cytochrome *c* has 19 positively charged lysine residues and two arginines which are also positively charged, but only 12 acidic residues (aspartic acid and, glutamic acid). Furthermore, cytochrome *c* is weakly associated with the inner membrane space. We found that the interaction of the positively charged cyt *c* with the neutral lipid headgroup of MO leads to an increase in interfacial curvature which promotes the formation of a new micellar cubic phase, presumably $P4_332$ (Q^{212}), which the lipid system is not able to form on its own [25,26].

To study the effect of confinement of larger proteins in MO we chose α-chymotrypsin. It is a water soluble enzyme (25.8 kDa, p*I*=9.1) with an ellipsoidal structure (51 Å × 40 Å × 40 Å) containing about 50% β-sheets, 10% α-helices and the rest corresponding to disordered and turn categories [27,28]. The substrate binding site is selective for amino acid side chains. It catalyzes the hydrolysis of peptide bonds on the carboxyl side of the aromatic side chains in tyrosine, tryptophan, and phenylalanine, as well as in a few large hydrophobic residues such as methionine.

The influence of protein incorporation on the conformational properties of the matrix monoolein was studied by means of FT-IR spectroscopy. Information on the conformation and molecular order of the acyl chain as well as structural changes at the interfacial region of the lipid system was obtained. The effect of the cubic lipid matrix on the structure and stability of the embedded protein was also studied using FT-IR spectroscopy by monitoring changes in secondary structural properties of the protein by analysis of the amide I′ band region. Synchrotron small-angle X-ray diffraction (SAXD) was used to investigate the thermotropic and barotropic ordered phases of the system MO/water/α-chymotrypsin at T=10 to 80 °C and a pressure range from ambient to 4 kbar.

2. Materials and methods

2.1. Sample preparation

Synthetic monoolein (1-monooleoyl-rac-glycerol, M=356.55 g/mol) (MO) was obtained from Sigma-Aldrich (Taufkirchen, Germany) with a purity of 98% and used without further purification. α-Chymotrypsin (chym) and cytochrome *c* (cyt *c*) were obtained from Sigma-Aldrich (Taufkirchen, Germany) with purity of 95%. Aqueous dispersions of the lipid were prepared by mixing appropriate amounts of lipid and buffer solution; the protein samples were prepared by mixing appropriate amounts of MO and protein solution in buffer. The protein was dissolved at appropriate concentrations in 30 mM sodium phosphate buffer (pD 7) for all temperature-dependent experiments. The pressure insensitive 30 mM TRIS buffer at pD 7 was used for the high-pressure experiments. The level of hydration was kept constant at 20 wt.% and the protein concentration in MO/D_2O was varied from 0 to 14 wt.%. The samples were subjected to five freeze–thaw cycles (at 30 °C and −196 °C, respectively) for homogenization of the lipid dispersion. Then, the samples were centrifuged for 30 min at 8000 g. The samples obtained are transparent and highly viscous. For the FT-IR experiments on protein solutions, the proteins were dissolved at 7 wt.% (2.72 mM) for α-chymotrypsin, and 5 wt.% (4.04 mM) for cyt *c*, in 10 mM sodium phosphate buffer (pD 7) in 99.9% D_2O for all temperature-dependent experiments, or in 10 mM Tris buffer at pD 7 for the high-pressure experiments. D_2O was used as a solvent instead of H_2O to stay away from the spectral overlap of the strong bending vibration mode of H_2O at 1645 cm^{-1} with the protein amide I mode at about 1650 cm^{-1}.

2.2. Synchrotron small-angle X-ray diffraction

The structural data were measured at the BioCAT beam line at APS (Argonne National Laboratory, USA). A camera length of 1.5 m and 13.8 keV X-rays (λ=0.898 Å) were used and the data were collected as a series of 1 s-exposures by using a high-sensitivity charge-coupled device (CCD) detector. Diffraction intensity as a function of reciprocal spacing s ($s=(2/\lambda)\sin\theta$; λ wavelength of radiation, 2θ scattering angle) were obtained by radial integration and averaging of the 2-D CCD images using the software FIT2D and Igor Pro. The incident beam intensity was recorded and used to normalize the individual exposures. From the positions of the low-angle Bragg reflections, the mesophase structure and the