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Sol-gel glass encapsulation of fish antifreeze proteins

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SOL-GEL GLASS ENCAPSULATION
OF
FISH ANTIFREEZE PROTEINS

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Genet H. Zemedu

May 2007

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ABSTRACT

SOL-GEL GLASS ENCAPSULATION OF FISH ANTIFREEZE PROTEINS

by Genet Zemedu

The cytoplasm of a living cell is a concentrated mixture of macromolecules. The structures and functions of biomolecules are sensitive to their environmental conditions, yet most biochemical studies of macromolecular properties are carried out in dilute solutions in which crowding effects do not occur. The sol-gel technique of encapsulating proteins in a silica matrix provides a method to simulate cellular crowding *in vitro*. Using sol-gel matrix encapsulation and characterizing the encapsulated protein by circular dichroism (CD) and fluorescence spectroscopy, this thesis project investigates the structure and conformational changes of fish antifreeze proteins (AFPs). Our results show that encapsulated type I and type III fish AFPs behave differently than the same proteins in dilute solution, which supports the idea that crowding influences protein structure. In the case of type I fish AFP, the protein had to be encapsulated in a xerogel to minimize leaching. In the case of type III fish AFP, the glass-entrapped protein showed enhanced secondary structure relative to that observed in dilute solution.

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LIST OF ABBREVIATIONS

AFP	antifreeze protein
AFGP	antifreeze glycoprotein
BSA	bovine serum albumin
CD	circular dichroism
EtOH	ethanol
GuHCl	guanidine hydrochloride
^1H NMR	proton nuclear magnetic resonance
IR	infrared
Kphos	potassium phosphate
MeOH	methanol
NMR	nuclear magnetic resonance
PEG	polyethylene glycol
TAMRA	carboxytetramethylrhodamine
TAMRA,SE	carboxytetramethylrhodamine succinimidyl ester
TEOS	tetraethoxysilane
TFE	trifluoroethanol
TMOS	tetramethoxysilane
Tris	tris (hydroxymethyl)aminomethane
UV	ultraviolet

CHAPTER 1:

GENERAL BACKGROUND

1.1 INTRODUCTION

Proteins are macromolecules that are essential to the structure and function of all living things [1]. Proteins are synthesized in cells in a linear fashion and fold into a native, active conformation. The driving forces behind this folding are provided by water and by the amino acid sequence (i.e., the distribution pattern of hydrophilic and hydrophobic residues). The hydrophobic side chains in a protein tend to be pushed together in the interior of the molecule, which enables them to avoid contact with the aqueous environment. By contrast, the polar side chains tend to arrange themselves near the outside of the protein molecule where they can interact with water and with other polar molecules. The folding process is temperature sensitive; a temperature either too low or too high causes inactivation [2]. Both temperature extremes compromise the structural integrity of active sites and binding sites and thus reduce activity.

Under physiological conditions, biochemical reactions occur in a crowded environment [3, 4]. Molecular crowding may have a big impact on protein folding and on macromolecular interactions, inducing large conformational changes within a macromolecule. The cell cytoplasm contains a high concentration of macromolecules, but experimental data are often obtained in dilute solutions [4]. Since molecular crowding is difficult to mimic outside of the cell, the role of crowding on the function of biomolecules is poorly understood. To understand protein behavior in the intracellular

environment, we have used the sol-gel glass encapsulation method to characterize antifreeze proteins.

1.2 MOLECULAR CROWDING

The cytoplasm is the gel-like fluid inside the cell that contains numerous types of biomolecules including proteins, carbohydrates, lipids, and nucleic acids [5]. The media is referred to as crowded rather than concentrated because no single macromolecule is at a high concentration. But, taken together, the total macromolecular concentration is 300-400 mg/mL [4-8], implying that between 30-40% of the total volume is physically occupied by these molecules [4-8]. Figure 1.1 provides a schematic illustration of crowding and confinement in the eukaryotic cytoplasm.

Figure 1.1: The crowded state of the cytoplasm. The drawing is approximately to scale and emphasizes the crowding in *E. coli* cells. RNAs are shown in blue, ribosomes in green, and proteins in red. Figure with permission from [17].

Molecular crowding is believed to have important consequences on the thermodynamics of the cell [9, 10] and to strongly affect diffusional processes [11]. The thermodynamics of the intracellular environment are influenced by the excluded volume effect which refers to the mutual impenetrability of solute molecules and strongly affects substances with large molecular weights [12]. Molecular crowding enhances protein association [13] and self-association of monomers [14]. It also increases the rate of folding and refolding [15]. The stability of proteins in crowded conditions is due to a decrease in the entropy of denaturation of proteins with no associated change in enthalpy [16].

Despite the physiological uniqueness of the intracellular environment, experimental data are often obtained in solutions with a total macromolecular concentration of 1-10 mg/mL or less, an environment where crowding effects do not occur [17]. To understand biochemical processes in the living cell, the crowding condition should be simulated in experiments *in vitro*. To mimic molecular crowding *in vitro*, crowding agents need to be added to the solution. The ideal crowding agent should have a molecular weight in the range 50 to 200 kD, be highly water soluble and not prone to self-aggregation, have the shape of globules to avoid making the solution too viscous, and not be prone to specific interactions with the test molecule of interest [18]. The most commonly used crowding agents are bovine serum albumin (BSA) [19], Ficoll 70 [18-20], dextran [15, 21], and high-molecular-weight polyethylene glycol (PEG) [21]. Because macromolecules occupy 30-40% of the volume of cell interiors, this range

defines the relevant concentration of crowding agents to be used to simulate biological crowding conditions *in vitro*.

1.3 THE SOL-GEL GLASS SYSTEM

1.3.1 Background

In this research, we used the sol-gel glass system instead of a soluble crowding agent. Sol-gel technology has been used extensively in the past. Sol-gel reactions have been shown to produce a variety of inorganic networks from metal alkoxide solutions [22, 23]. The overall sol-gel process involves two stages: (1) hydrolyzed precursors initially form high molecular weight but soluble oligomeric intermediates called “sol,” and (2) the intermediates further link together via condensation reactions to form a cross-linked network called “gel.” The precursors to these colloids consist of a metal or metalloid element surrounded by various reactive ligands. Metal alkoxides are most popular because they react with water. The most widely used metal alkoxides are the alkoxysilanes, such as tetramethoxysilane (TMOS) and tetraethoxysilane (TEOS) [23].

Tetramethoxysilane is used as an example below to illustrate the sol-gel process, where R represents the methyl group (Figure 1.2).

Figure 1.2: General reaction schemes for sol-gel formation (adapted from [24]).

In the first step, equation (1), hydrolysis is initiated by the addition of water to the TMOS solution under acidic, or basic conditions. In acidic conditions, a positive charge develops on the alkoxy silane through the attack of a proton. The alkoxy silane then is hydrolyzed to form a silanol moiety. In the second step, equation (2a) or (2b), the silanol groups undergo condensation with either an alkoxide or another silanol group, forming a stable siloxane linkage and producing either alcohol (ROH) or water (HOH). As the number of Si-O-Si bridges increases, the siloxane particles begin to aggregate. Gel formation occurs when the sol particles undergo sufficient condensation such that a gel is formed, trapping the water and alcohol by-products. These by-products remain in the pores of the silica but may be removed by evaporation or by rinsing with a new solution [25].

Through sol-gel processing, homogeneous, high-purity inorganic oxide glasses can be made at ambient temperatures rather than at the very high temperatures required

for conventional formation of glasses. The ability to form silica glasses under aqueous, room temperature conditions, in which proteins are active, opens up the possibility to extend sol-gel processing to the encapsulation of biomolecules [26].

1.3.2 Encapsulation of biomolecules in sol-gel matrices

The encapsulation of biomolecules into inorganic host materials using sol-gel processing has attracted considerable attention for its potential applications [26, 27]. Biomolecules are added to the hydrolyzed $\text{Si}(\text{OH})_4$ solution in the presence of a buffer with a pH of ~ 7 to induce the condensation step. The silica network then grows around the biomolecules which remain entrapped within the porous gel (Figure 1.3). A novel class of bioactive materials is thus obtained, made up of biomolecules physically trapped within silica matrices.

Figure 1.3: Protein encapsulation in sol-gel glass. (Figure from D. K. Eggers, with permission.)

The highly porous character of the sol-gel glasses allows small analyte molecules to diffuse into the matrix, whereas the large protein macromolecules remain physically trapped in the pores under most solvent conditions. The ultraviolet and visible transparency of the matrix enables the use of optical spectroscopy methods to characterize macromolecular structure in the pores of the glass.

In this research, we used type I and type III fish antifreeze proteins as model proteins to investigate the structural and conformational effects of encapsulation in the silica matrix.

1.4 FISH ANTIFREEZE PROTEINS

1.4.1 Background

Approximately forty years ago, researchers realized that several marine fish were able to survive cold environments in which their circulating systems were expected to freeze. After testing various components of Antarctic fish, it was discovered that the fish contain a compound that could protect them from freezing. Based on these studies, the activity was assigned to a family of plasma proteins, subsequently named antifreeze proteins. These fish plasma proteins, collectively termed AFPs, are primarily synthesized in the liver and secreted into the blood stream to be distributed throughout the extracellular space [28]. Antifreeze proteins are a group of structurally diverse proteins with the distinctive capability of binding to the surface of ice crystals and inhibiting ice crystal growth [28-32]. The mechanism of high-affinity ice binding is not yet fully understood, but the proteins are believed to bind irreversibly to growing ice crystals in such a way as to change the curvature of the ice-water interface, leading to freezing point depression [31, 33].

To date, five different types of fish AFPs have been identified (Table 1.1) [34-36]. The first AFP isolated from Antarctic nototheniids was found to contain carbohydrate moieties and, therefore, is called an antifreeze glycoprotein (AFGP) [34]. The second protein, isolated from winter flounder, is the most studied AFP and is named type I AFP [34, 35]. Type II AFP was isolated from sea raven [34], type III AFP was isolated from ocean pout [36], and type IV AFP was isolated most recently from longhorn sculpin [34]. Despite the marked differences in amino acid composition and protein structure, all

antifreeze proteins have a similar function; AFPs recognize ice crystals, bind to them, and prevent them from growing within the subzero temperature range in which the fish lives.

Table 1.1: Characteristics of several AFPs (adapted from 34-36)

	AFGP	Type I AFP	Type II AFP	Type III AFP	Type IV AFP
MW (kDa)	2.6 - 33	3.3 - 4.5	11 - 24	6.8	12.3
Sequence	Ala-Ala-Thr repeat	Ala-rich; 11 residue repeats	Cys-rich, disulphide linked	Relatively hydrophobic	17% glutamine, no disulphide bridges, general
Secondary structure	Polyproline Helix	All α -helix	Mix of α -, β -coil	Irregular, β -strands	All α -helix predicted
Tertiary structure	Extended	Single α -helix	Globular	Globular	Helix bundle
Interesting features	Di-saccharides are attached to Thr residues	Repeats thought to match ice lattice	Some isoforms require Ca^{2+}	Flat ice-binding surface	--
Species	Antarctic nototheniids; northern cods	Winter flounder; right eye flounders	Sea raven; smelt; herring	Ocean pouts; Eel Pouts	Longhorn sculpin

1.4.2 Ice and AFPs

It is believed that antifreeze proteins function at the ice-water interface and that the binding of antifreeze proteins to ice is essential for their function. A schematic picture of an ice crystal is shown in Figure 1.4. The two basal planes are located on the top and bottom of the hexagonal disk. The six equivalent prism planes are located around the sides of the hexagon. The water along these surfaces is oriented in such a way that

more water is added preferentially to the prism planes rather than to the basal planes [37]. The ice then grows as a disk. In the presence of an AFP, ice growth on the prism faces is inhibited, resulting in growth along the c -axis in a step-wise manner until a hexagonal bipyramid is formed.

Figure 1.4: Schematic representation of an ice crystal (adapted from [39]).

The adsorption of the AFPs to the ice surface has been examined by a variety of techniques. An ice-etching experiment shows that not all AFPs bind to steps on the ice [38]. The experiment is done by growing a single crystal of ice into the shape of a hemisphere that is then placed in a solution of a dilute AFP. The protein binds to the ice but does not inhibit its growth because the solution is too dilute in AFP. The hemisphere is then removed from the solution and allowed to sublime so that the bound, non-volatile protein remains behind. The bound protein is etched on the remaining ice hemisphere. This work has also been done for AFGP, type I AFP, and type III AFP. For AFGP and

type I AFP, the protein binds to pyramidal planes [38]. Type III AFP binds to a primary prism plane. From these results, it is clear that a hexagonal bipyramidal crystal should form. Even though the basal plane is not directly inhibited by bound protein, the crystal grows toward an apex, preventing further *c*-axis growth [39].

1.5 ANALYTICAL METHODS

Two analytical methods, circular dichroism and fluorescence spectroscopy, were used in this project to characterize and monitor the structure of type I and type III AFPs.

1.5.1 Circular dichroism

CD is a form of light absorption spectroscopy that measures the difference in absorbance of right- and left-circularly polarized light as a function of wavelength [40]. A CD signal is detected when a molecule is structurally asymmetric and when the bonds that define the asymmetry exhibit absorbance. Asymmetry can result from chiral molecules that have been linked together, such as the peptide backbone of proteins, from a non-chiral molecule covalently attached to a chiral molecule (e.g., a chromophore bound to protein) or from a non-chiral molecule in an asymmetric environment (e.g., aromatic amino acid side chains). In practice, the light generated from the lamp is divided into two circularly polarized components by a modulator and is passed through the sample. If the chromophore is chiral, the absorptions of the two polarized components are different. Thus, the resultant (i.e., the combined component) is now elliptically polarized. The CD instrument does not recombine the components but detects

the two components separately. It will then display the dichroism at a given wavelength of light expressed as either the difference in absorbance of the two components ($\Delta A = A_L - A_R$) or as the ellipticity in degrees [41].

Ellipticity is defined as the tangent of the ratio of the minor to major elliptical axis [41]. Modern CD instruments measure the difference in absorption of right and left circularly polarized light as a function of wavelength. In accordance with the Beer – Lambert law ($\Delta A = \Delta \epsilon c l$), the difference in absorbance is equal to the difference in molar extinction coefficients multiplied by the product of path length and concentration. Mean residue ellipticity, for which the molar ellipticity is divided by the total number of amino acid residues, is the most common unit for proteins ($\text{degree cm}^2 \text{ dmol}^{-1}$). Depending on the range of analysis, a CD spectrum can be divided into three parts:

Far-UV region (240-190 nm): A spectrum in this region can be used to analyze the overall secondary structure content of the protein (α -helix, β -sheet, and random coil). Each type of secondary structure has its own characteristic far-UV CD spectrum (Figure 1.5). The spectra of α -helices have two minima at 208 and 222 nm. The spectra of β -sheets have a single minimum at 216 nm. A random coil exhibits a minimum in CD signal at 200 nm [41, 42].

Figure 1.5: CD spectra for various secondary structures in aqueous solution; an α -helix (■), antiparallel β -sheet (●) and random coil (▲). Figure with permission from [43].

Near-UV region (320-260 nm): This region provides a fingerprint of the tertiary structure of proteins. Aromatic amino acid chains and disulfide bonds make the majority of contributions in this region of the spectrum. Each of the aromatic amino acids tends to have a characteristic wavelength profile: tryptophan, a peak close to 290 nm with fine structure between 290 nm and 305 nm; tyrosine, a peak between 275 and 282 nm; phenylalanine, sharp fine structure between 255 and 270 nm [42]. The rigidity of the protein structure, interactions between the aromatic residues and their neighboring residues, and the extent of solvent exposure also have effects on the spectrum [42].

Near-UV and visible region: CD in the near-UV, visible and near-IR region often gives information on the environment of co-factors (which play a role in the biological activity of protein, eg. pyridoxal-5'-phosphate, flavins and heme) and other protein-bound ligands. In many situations, the free co-factor or ligand is optically inactive, but, upon binding to the protein, becomes immobilized in an asymmetric environment, thus giving rise to CD signals in a characteristic absorption region [42, 40].

1.5.2 Fluorescence spectroscopy

Fluorescence spectroscopy is a method that is complementary to CD spectroscopy in studies of protein structure and that may be used to determine the dynamic properties of biomolecules.

Fluorescence is an optical phenomenon in which a molecule absorbs a high-energy photon and reemits it as a lower-energy photon with a longer wavelength. Upon absorbing the higher energy photon, the molecule is excited into a higher energy electronic and vibrational state. In the condensed phase, the excited molecules almost immediately lose excess vibrational energy and return to the lowest vibrational level of the excited state. The molecule is then able to relax back to the ground state with simultaneous emission of fluorescent light. The transition from the first singlet state (S_1) back to the ground state (S_0) is generally fast ($\sim 10^{-8}$ sec), and is likely to be coupled to emission of a photon with a slightly longer wavelength than the one originally absorbed [44] (Figure 1.6).

Figure 1.6: Fluorescence spectroscopy, transitions between molecular electronic energy levels (adapted from [44]).

A typical fluorometer contains a radiation source, a monochromator for selecting the excitation wavelength, a monochromator for selecting the emission wavelength, and a detector [44, 45]. Molecules in solution are usually excited by UV light, and the excitation source is usually a deuterium or xenon lamp. Broad-band excitation light from a lamp passes through the excitation monochromator that transmits only a selected wavelength. The fluorescence is then dispersed by the emission monochromator and detected by a photomultiplier tube. In order to determine the emission spectrum of a molecule, the wavelength of maximum absorption is determined, and the molecule is often excited at that wavelength. The excitation spectrum of a given molecule is determined by monitoring fluorescence emission at the wavelength of maximum intensity. The excitation and emission spectra show the relative intensity of fluorescence

and correlate to the electronic structure of the molecule. Typically, the intensity is plotted on the vertical axis versus wavelength on the horizontal axis.

1.5.3 Solvent effects on emission spectra

The emission of fluorescent light occurs at wavelengths that are longer than those of absorption. This loss of energy is due to the dissipation of vibrational energy, redistribution of electrons in the surrounding solvent molecules induced by the changed dipole moment of the excited fluorophore, and specific interactions between the fluorophore and solvent or solutes, including hydrogen bonding and formation of charge-transfer interactions [44-48].

Effects of the solvent on the fluorescence spectrum can be divided into general and specific categories [46, 47]. General solvent effects depend on the polarizability of the solvent, as measured by a parameter such as the refractive index (n) or dielectric constant (ϵ), reflecting the freedom of motion of the electrons in the solvent molecules and the dipole moment of the solvent molecules. Specific solvent effects arise from direct chemical interaction between the solvent and fluorophore. Important chemical reactions include hydrogen bonding, acid-base chemistry, and the formation of charge-transfer interactions where an electron in the fluorophore is transferred to another group on excitation [44, 46]. Example would be the emission spectra of 2-anilinonaphthalene in hexane containing small amount (1-2%) of ethanol. The trace amount of ethanol added to a cyclohexane bulk solvent give rise to a new fluorescent band [44]. As the solvent polarity is increased, the emission spectra continue to shift to longer wavelength.

This amount of alcohol is too small to affect the refractive index or dielectric constant of the solvent, therefore, this shift is a result of specific solvent effects which is presumably due to the 2-anilinonaphthalene-ethanol hydrogen-bonded interactions [44].

1.6 AIMS AND OBJECTIVES

The interior of a living cell is a highly concentrated mixture of molecules where proteins and nucleic acids occupy a significant fraction of the total cell volume. The activity of a given macromolecule in this crowded and confined environment may differ significantly from its activity in a dilute solution. The sol-gel method of encapsulating proteins in a silica matrix was investigated as a potential experimental system for testing the effects of molecular confinement on the structure and stability of proteins. Using this method, Eggers and Valentine observed differences between the structural properties of proteins in solution and in the silica matrix [22]. Their results showed that the α -helical content of apomyoglobin is diminished from 60% in solution to 20-25% in the sol-gel glass. The authors hypothesized that protein conformation is influenced directly by the properties of confined water in the pores of the silica.

In this study, we expand the use of sol-gel glasses to characterize the structural and conformational effects of encapsulation on fish type I and type III AFPs. Since these proteins are believed to bind to ice via periodic H-bonds, we hypothesized that AFPs may bind to the oxygen atoms of silica, leading to interesting structural features.

CHAPTER 2:

EXPERIMENTAL PROCEDURES

2.1 MATERIALS AND METHODS

Type I and type III fish AFPs were purchased from A/F Protein Inc. Both proteins were purified from the plasma of cold ocean fish, a natural source. Type I and type III AFPs were obtained from winter flounder and from ocean pout, respectively. Sephadex G-25 semi-solid polymeric beads were purchased from Pharmacia Fine Chemicals for size exclusion chromatography. Tetramethoxysilane and propyltrimethoxysilane were purchased from Aldrich. The probe 5-(and -6)-carboxytetramethylrhodamine succinimidyl ester (5(6)-TAMRA, SE), catalog number C-1171, was purchased from Molecular Probes (Invitrogen). Plastic cassettes for casting sol-gel glasses were purchased from Invitrogen. Purified water was obtained from a MilliQ filtering system (Millipore).

2.2 SOL-GEL PREPARATION

Four different kinds of sol-gel glasses were made (three wet-aged and one dry-aged) using the detailed protocols in this section.

2.2.1 Standard glass

Silica sol was made by mixing 1.0 mL TMOS, 214 μ L purified (MilliQ) water, and 30 μ L of 0.04 N HCl in 3 x 15 mL polypropylene centrifuge tubes and by sonicating

the tubes in contact with an ice-water bath for 20 minutes. A 3.10 mL volume of the sonicated sol was mixed with 4.65 mL of protein solution (type I or type III AFP) in 5 mM potassium phosphate buffer at pH 7.0. Immediately after mixing the silica sol and aqueous protein, the solution was poured between the plates of a plastic cassette using a plastic serological pipette. Cassettes of 1.0 mm plate spacing were used for all wet-aged glasses. Gelation occurred within 15-30 min. After one day of aging at 4°C, the sol-gel samples were layered with a few milliliters of water, covered with parafilm to prevent drying, and returned to the coldbox. Samples were allowed to age for a minimum of two weeks. Typically, the glass sample was found to shrink to 85-90% of the original size, as defined by the dimensions of the cassette. The plastic cassette was then removed by prying the sides away with a spatula, and the silica sheet was cut with a razor blade into rectangular shapes of about 8 mm width x 15 mm length for analysis in a 2 mm pathlength cuvette. Each silica sample was equilibrated with 3.0 mL of the desired buffer solution in a disposable cuvette and stored at an angle to avoid warping of the glass at room temperature. All glass samples were immersed in the same equilibration buffer during analysis of the spectra. Type I and type III AFP samples were equilibrated in 10 mM potassium phosphate, 1 M potassium phosphate, 60% EtOH in water, 1 M KCl, 1 M GuHCl, and 50% TFE buffers at pH 7.0.

2.2.2 Propyl-modified glass

The propyl-modified glass was made by mixing 1.0 mL propyl-TMOS mixture (10% propyltrimethoxysilane plus 90% tetramethoxysilane, based on molar content of

silane), 214 μL purified (MilliQ) water, and 30 μL of 0.04 N HCl in 3 x 15 mL polypropylene centrifuge tubes and by sonicating the tubes in contact with an ice-water bath for 20 min. A 3.10 mL volume of the sonicated sol was mixed with 4.65 mL of protein solution in 5 mM potassium phosphate buffer at pH 7.0. Glass formation and sample preparation steps were the same as that for the standard glass, section 2.2.1.

2.2.3 Methanol-free glass

MeOH-free silica gel was made using a 645 μL volume of purified (MilliQ) water, 40 μL of 0.62 N HCl, and 1.23 mL TMOS. The reagents were mixed and sonicated in 3 x 15 mL polypropylene centrifuge tubes in contact with an ice-water bath for 20 min. The cold sol was then transferred to a 500 mL rotavapor flask containing 5.50 mL of water. The flask containing the sol and water was weighed, and the solution was evaporated under vacuum for 5 minutes. After 5 minutes, the flask was weighed again, indicating that 4.18 g mass had been removed. A 3.5 mL volume of the MeOH-free sol was mixed with 3.5 mL of protein (type I AFP) in 20 mM potassium phosphate buffer at pH 7.0. The solution was transferred to a plastic cassette of 1.0 mm spacing. Gelation occurred after 14 hours. After one day of aging at 4 °C, the sol-gel sample was layered with a few milliliters of water and covered with parafilm to prevent drying. The glass sheet was allowed to age for a minimum of two weeks. Smaller samples for experiments were prepared using the procedure of the standard glass (Section 2.2.1).

2.2.4 Xerogel

The protocol to make a xerogel glass is the same as that of the standard glass (Section 2.2.1) except for the aging and drying of the sol and for the choice of cassette. Once the sol is made and transferred to a plastic cassette with 1.5 mm spacing, the reaction was sealed with parafilm. A wider cassette spacing is necessary since xerogels may shrink to half of their original thickness. Upon gelation of the glass within a few hours at room temperature, 13-15 holes were made in the parafilm with a hypodermic syringe needle to allow slow evaporation of the solvents and reaction by-products. Fast drying can cause severe cracking and fracturing of the large glass sheets. The parafilm was removed after 24 hours, and the sample was allowed to dry for a minimum of two weeks at room temperature. After two weeks, the volume of the xerogel was approximately one-eighth of the original volume. In this experiment, the resulting sheet had many cracks that were caused by solvent loss. To prepare samples for analysis, the plastic cassette was removed, and the silica pieces were stored in an airtight container with wet Kimwipes to slowly re-hydrate the silica. After 14-16 hours, the larger pieces were cut into rectangular shapes and placed in contact with equilibration buffers.

2.3 CIRCULAR DICHROISM

CD spectra were recorded on an AVIV model 215 spectrometer with a thermoelectric cell for temperature control. Measurements of CD spectra from 200 to 260 nm were made at 5 and 25 °C for both type I and type III AFPs. For solutions containing free peptides, spectra were recorded in a 2-mm pathlength cell. The

concentrations of sol-gel encapsulated proteins were calculated based on the final dilution of the known protein stock concentrations. For type I AFP, 65 μ L of a stock solution (10 mg/mL) was used to make a concentration of 0.084 mg/mL of glass volume, following the standard glass protocol. For type III AFP, 0.35 mL of stock solution (10 mg/mL) was used to make a concentration of 0.452 mg/mL of glass volume. Because these concentrations are approximations based on dry weight, the CD signal was not converted to molar ellipticity; the raw data is reported in units of millidegrees. For some analyses in the far-UV region, spectra were truncated before 200 nm due to high total absorbance. Also the spectra were averaged over three runs. All spectra were corrected by subtracting the buffer baseline signal.

2.4 FLUORESCENT LABELING

A mass of 10.2 mg of type I AFP was dissolved in 1.0 mL of NaHCO₃, pH 8.6. A 1.3 mg mass of the amine-reactive probe 5-(and-6)-carboxytetramethylrhodamine succinimidyl ester, 5(6)-TAMRA, SE, was dissolved in 100 μ L DMSO. The two solutions were mixed and stirred for 1 hour at room temperature. The reaction was then quenched with 150 μ L of solution containing 0.5 M tris and 0.5 M glycine. The mixture was then purified using size exclusion chromatography to separate unreacted probe from the labeled protein. The column was prepared using 1.5 g of Sephadex G-25 semi-solid beads. The protein eluted first, using 10 mM potassium phosphate buffer as the mobile phase. Fractions that were concentrated in protein or unreacted probe could be distinguished by eye. The protein-containing samples were pooled and stored at 4 °C.

2.5 FLUORESCENCE SPECTROSCOPY

Fluorescence measurements were carried out on a DM 3000 fluorescence spectrofluorometer for samples in solution, standard glass and propyl-modified glasses. A 1-nm slit width setting for both excitation and emission and a triangular cuvette with 1-cm sides were used. The glass wafer was placed against the hypotenuse side of the cuvette and positioned in the cell holder at a 45° angle relative to the incident light. Emission was measured at a right angle relative to the excitation source. For the xerogel samples, a Varian Cary Eclipse fluorescence spectrofluorometer was used with 5-nm slit width setting for both excitation and emission, and a square cuvette with 2 mm pathlength was employed. Excitation spectra for both instruments were made from 450 nm to 570 nm. The emission wavelength was chosen at 590 nm and the wavelength of maximum excitation was observed near 550 nm. The fluorescence measurement was made for TAMRA-labelled type I AFP only, and samples were analyzed in different buffers, both in solution and sol-gel glasses. Concentrations of labeled-AFP for fluorescence measurements in glasses were determined by the maximum signals obtained in dilute solution; glass samples were prepared to keep the maximum emission below $\sim 5 \times 10^5$ counts per second (cps). Only 5 μL of the stock solution was encapsulated for each glass listed in the results section (3.2).

CHAPTER 3:

SOL-GEL GLASS ENCAPSULATION OF TYPE I FISH AFP

3.1 BACKGROUND ON TYPE I FISH AFP

The first AFP structure to be identified was that of the winter flounder type I AFP. This monomeric, alanine-rich protein is comprised of 37 amino acids and contains three 11-amino acid repeats (TAANAAAAAAA) [50]. Circular dichroism and crystal structure studies show that the structure of the protein is exclusively α -helical with all polar residues located on one side of the helix [51-52]. Using chemical synthesis, residue replacement experiments have located the ice-binding site and have established the involvement of hydrophilic residues. Based on this study, it was proposed that the spacing of the four threonines provides a good match to the spacing of oxygen atoms of water in the ice lattice [52-53] (Figure 3.1). The match in periodicity seems to support previous suggestions that AFP ice binding is mediated primarily by the formation of multiple hydrogen bonds between the protein and the ice surface.

Figure 3.1: Crystal structure of type I fish AFP. The side-chains of the four threonines are shown in ball and stick form. (Figure courtesy of Dr. Jeffry D. Madura, Center for Computational Sciences, Duquesne University, 2007).

To prove this, several groups have studied the role of the threonines by substituting them with serine, glycine, valine, and allothreonine (threonine with altered chirality at the β -carbon) [51-54]. Based on the hypothesis that AFP/ice binding is mediated by hydrogen bonds, one would expect a mutation of threonine to serine to cause only a relatively small loss in activity, given that the hydrogen bonding potential is still present in the side-chain. Also, one would expect a mutation to valine to have little activity, since methyl groups would not be able to form hydrogen bonds to ice. However, the all-valine mutant had between 30-50% activity relative to the wild-type protein [52], whereas the serine mutant essentially had no activity. These results were completely opposite from the expected results. To ensure that the mutations did not affect the protein structure, the mutant proteins were examined by CD and NMR, and all mutants were found to have similar structure to the wild-type.

The allothreonine and valine mutant studies showed that the hydroxyl group still has some role, most likely in hydrogen bond formation [52, 54]. As previously stated, the valine mutant did not show a considerable loss in activity. However, the allothreonine, which has the same chemical composition as threonine, lost a similar amount of activity as the valine mutant. This would suggest that the location of the hydroxyl group is important to form some type of hydrogen bond with ice. The methyl group also has a role, but it is not clear whether this is a hydrophobic interaction or something else.

If the protein binds to ice via periodic H-bonds, it seems logical that type I AFP may bind to the oxygen atoms of silica through a similar mechanism. In this study, we

used four different types of sol-gel glasses, standard glass, propyl-modified glass, methanol-free glass and xerogel, to encapsulate type I fish antifreeze protein. The structure of the encapsulated protein in each glass was compared to its structure in solution using CD and fluorescence spectroscopy.

3.2 RESULTS AND DISCUSSION

3.2.1 CD results for type I fish AFP in solution

To analyze the secondary structure of type I AFP in solution, CD spectra were collected. The far-UV CD spectra of samples in different solutions were collected at 25 °C and 5 °C to compare any solvent-specific effects on the secondary structure of the protein as a function of temperature.

Figure 3.2: Circular dichroism spectra of type I fish AFP in solution at 25 °C. Far-UV CD spectra are shown for the following solutions: (○) protein in 3 M GuHCl buffer; (x) protein in 1 M potassium phosphate buffer; (—) protein in 10 mM potassium phosphate buffer; (---) protein in 60% EtOH; (Δ) protein in 50% TFE. [AFP] = 0.050 mg/ml in 2 mm pathlength cuvette.

The solution spectra in 60% EtOH, 50% TFE, 1 M potassium phosphate and 10 mM potassium phosphate have similar shapes with ellipticity minima at 208 and 222 nm (Figure 3.2). However, the spectra for protein in 50% TFE and 60% EtOH show more helicity (a more negative ellipticity) than the spectra for protein in 1 M and 10 mM potassium phosphate. Alcohol solutions are known to be helix-inducing agents, and, therefore, the spectra obtained in TFE and EtOH were expected. The spectrum for type I AFP in 3 M GuHCl yields a less negative ellipticity, indicating less helical secondary structure. GuHCl is known for denaturing the structure of proteins, and the results show that the α -helical structure of type I AFP is diminished by this buffer.

The secondary structure of type I AFP was also analyzed by CD at a lower temperature of 5 °C (Figure 3.3).

Figure 3.3: Circular dichroism spectra of type I fish AFP in solution at 5 °C. Far-UV CD spectra are shown for the following solutions: (○) protein in 3 M GuHCl buffer. (x) protein in 1 M potassium phosphate buffer. (—) protein in 10 mM potassium phosphate buffer. (---) protein in 60% EtOH. (Δ) protein in 50% TFE. [AFP] = 0.050 mg/mL in 2 mm pathlength cuvette.

The CD spectra for type I AFP indicate that the helical content is enhanced at 5 °C relative to 25 °C for all solutions tested. The largest enhancement was observed for type I AFP in phosphate buffers for which the ellipticity value at 222 nm changed by 64% (10 mM Kphos) and 57% (1 M Kphos). It seems appropriate that antifreeze proteins should have more stable structures at lower temperature, since their biological role is to bind to ice, and this result supports that view.

3.2.2 CD results for type I AFP in glass

3.2.2.1 Standard glass

Individual wafers of standard glass-entrapped type I fish AFP were equilibrated with three different solutions (1 M and 10 mM potassium phosphate and 1 M guanidine hydrochloride) to determine any solvent-specific effects on the secondary structure of the protein. Changes in conformation were monitored at 25 °C by far-UV CD (Figure 3.4). At this temperature, the spectra indicate a weak α -helical structure, with ellipticity minima at 208 and 222 nm. The magnitude of the ellipticity is significantly weaker than the same amount of protein in solution.

Figure 3.4: Secondary structure of standard glass encapsulated type I fish AFP. Far-UV CD spectra are shown for the following solutions: ($\diamond$) glass wafers containing type I fish AFP were equilibrated with 10 mM potassium phosphate (pH 7.0). (- - -) glass sample containing type I fish AFP were equilibrated with 1 M potassium phosphate. (—) glass sample containing type I AFP equilibrated with 1 M guanidine hydrochloride. Initial [AFP] = 0.084 mg/mL, 1 mm thick glass.

Minor solvent effects were observed for this protein when encapsulated in standard glass. In the presence of 1 M potassium phosphate, the protein shows slightly more helicity than the one in 10 mM potassium phosphate. And in 1 M GuHCl, the spectrum was nearly identical to the one in 1 M potassium phosphate. If the CD signal was weak due to adsorption of the protein to the silica surface, the relatively low concentration of the chaotrope, GuHCl, might have relieved the adsorption and enhanced the helicity of the protein, but this was not observed.

3.2.2.2 Propyl-modified glass

The next approach was to encapsulate type I AFP in the propyl-modified glass. Eggers et al. previously encapsulated apomyoglobin in propyl-modified glass and observed more helicity than encapsulating the same protein in standard glass (accepted for publication, *ChemComm*, 2007). Based on this result, individual wafers of propyl-modified glass-entrapped type I fish AFP were examined after equilibration in two different buffers. Changes in conformation were monitored at both 5 °C and 25 °C by far-UV CD (Figure 3.5).

Figure 3.5: Secondary structure of encapsulated type I fish AFP in propyl-modified glass. Far-UV CD is shown for the following solutions: (—) 10 mM potassium phosphate (pH 7.0) at 25 °C; (- - -) 10 mM potassium phosphate at 5 °C; (○) 1 M potassium phosphate at 25 °C; (+) 1 M potassium phosphate at 5 °C. [AFP] = 0.084 mg/mL, 1 mm thick glass.

There were substantial changes in the spectra collected at the lower temperature.

The encapsulated protein equilibrated with 1 M potassium phosphate at 5 °C shows ellipticity minima at 208 and 222 nm, as observed in solution. The same glass sample equilibrated in the same buffer but at a higher temperature (25 °C) shows less helicity. As seen in the standard glass, the propyl-modified glass equilibrated in 10 mM potassium phosphate shows slightly less secondary structure than in 1 M potassium phosphate. In general, the CD spectra of type I fish AFP in both the standard and propyl-modified glasses are much weaker compared to that obtained in solution, which suggests that the protein is leaching out from the glass. One possibility is that the AFP is diffusing out of the glass immediately following synthesis due to the high content of methanol. Another

possibility is that methanol in the initial sol is causing the protein to aggregate, reducing the molar ellipticity of the final glass.

3.2.2.3 Methanol-free glass

In the first step of sol-gel glass formation from TMOS, hydrolysis leads to the production of methanol [26]. As mentioned above, we thought the methanol in the sol might lead to aggregation or to leaching from the glass. Therefore, a methanol-free glass was made by evaporating the methanol prior to adding the protein for the gelation step. This was done with a rotary evaporator apparatus (see Section 2.2.3 for more detail).

Individual wafers made by the methanol-free protocol were equilibrated with five different solutions (1 M and 10 mM potassium phosphate, 2 M KCl, 1 M GuHCl and 50% TFE) to determine any solvent-specific effects on the secondary structure of the protein. Changes in conformation were monitored at 25 °C by far-UV CD (Figure 3.6).

Figure 3.6: Secondary structure of encapsulated type I fish AFP using the methanol-free protocol. Far-UV CD spectra are shown for the following solutions: (—) glass wafer equilibrated with 10 mM potassium phosphate (pH 7.0); (x) glass sample equilibrated with 1 M potassium phosphate; (◊) glass sample equilibrated with 2 M KCl; (○) glass sample equilibrated with 1 M GuHCl; (- - -) glass sample equilibrated with 50% TFE. [AFP] = 0.086 mg/mL, 1 mm thick glass.

Similar spectra of weak ellipticity were observed for the protein in the presence of all five solutions. Apparently, the methanol-free approach did not prevent leaching. The glass in 2 M KCl yields the best spectrum with ellipticity minima at 208 and 222 nm, as expected. However, the signal is still weak compared to the CD spectra observed in standard and propyl-glasses. Overall, the results suggest that leaching is enhanced in glasses made by the methanol-free technique.

As described above, type I fish AFP encapsulated in standard glass, propyl-modified glass and MeOH-free glass all have weaker CD signals than those generated by

the same amount of protein in solution. These glasses are wet-aged and, therefore, have similar pore sizes. This could be an important factor if the protein is leaching from the glasses.

At this point in the research, it was not clear if the weak signals were due to leaching, aggregation, or unfolding in the glass environment. Thus, we turned to a different analytical technique, fluorescence spectroscopy, to search for further clues.

3.2.3 Fluorescence spectroscopy results for type I fish AFP

The tetramethylrhodamine probe, 5(6)-TAMRA, SE, is one of the most common red fluorescent dyes used to covalently label proteins [49]. During the reaction, the succinimidyl ester group reacts with primary or secondary amines under mild conditions. In this case, the succinimidyl ester reacts with the primary amine of a single lysine residue found in type I fish AFP or the N-terminal free amine (Figure 3.7). Sephadex G-25 size-exclusion chromatography was used to remove the unreacted probe. The conjugated type I AFP eluted first, while the unreacted probe eluted last.

Figure 3.7: 5(6)-TAMRA attached to type I fish AFP via amide linkage to Lys 18.

Individual wafers of standard glass, propyl-modified glass and xerogel entrapped with fluorescently labeled type I fish AFP were equilibrated with four different solutions to determine the emission spectrum of the attached fluorophore. The relative intensity of the fluorescence excitation was measured from 450 to 570 nm. The data were taken at multiple time points after addition of solutions (0, 0.33, 2, 24, 96 hours); only data at $t = 0$ and $t = 96$ hours are presented.

3.2.3.1 Fluorescence spectroscopy of Type I AFP in solution

The fluorescence response to a series of different solutions is shown in Figure 3.8. Samples were analyzed immediately after immersion ($t = 0$). Similar peak intensities were observed for the protein in the presence of 1 M KCl, 1 M potassium phosphate and water ($\sim 6.50 \times 10^5$ cps). However, a higher intensity was observed for the protein conjugate in 60% EtOH (1.17×10^6 cps) at 555 nm.

Figure 3.8: Excitation spectra at 590 nm for 5(6)-TAMRA attached to type I fish AFP in solution. (---) 60% EtOH, (···) 100% water, (-·-) 1 M potassium phosphate and (—) 1 M KCl. 1 M potassium phosphate buffer is used as a protein blank (-·-).

The higher intensity observed for 60% EtOH is due to reduced polarity of this solvent. The tetramethylrhodamine group of TAMRA is an electron donor, and the carbonyl group in Figure 3.7 is strongly electron-withdrawing, resulting in a significant dipole moment in the ground state [46]. In the excited state it is likely that this charge separation increases, resulting in a larger dipole moment than in the ground state. Such an increase in dipole moment could explain the sensitivity of the excitation spectrum of 5(6)-TAMRA to solvent polarity; more polar solvents such as water should partially reduce fluorescence whereas more nonpolar solvents such as ethanol should enhance fluorescence.

3.2.3.2 Fluorescence spectroscopy of type I AFP in standard glass

Individual wafers of TAMRA-labeled type I fish AFP entrapped in standard glass were equilibrated with three different solutions (10 mM potassium phosphate, 100% water and 60% EtOH). The fluorescence responses are shown in Figure 3.9 (A) and (B). At $t = 0$, the glass sample in 10 mM potassium phosphate displays an intensity of 6.86×10^5 cps, while the glass sample in 100% water has an intensity of 4.96×10^5 cps and the intensity for the glass sample in 60% EtOH is 3.85×10^5 cps at 555 nm (Figure 3.9A).

After an equilibration of 96 hours (Figure 3.9B), the intensity of the protein in 10 mM potassium phosphate increased 41%. Meanwhile, the intensity of the glass sample in 100% water decreased about 20%, and the intensity of the protein in 60% EtOH was diminished by 75%. One possible reason for the large decrease in the EtOH solution is the fact that alcohols are known to enhance helical structure in proteins [22]. The ethanol may have induced one continuous helix in the structure of type I fish AFP, and thus caused the protein to leach out more easily from the glass.

Figure 3.9: Standard glass excitation spectra at 590 nm for 5(6)-TAMRA attached to type I fish AFP. (—) 100% water, (—) 10 mM potassium phosphate and (- -) 60% EtOH. 10 mM potassium phosphate buffer was used as a protein blank (— -). (A) Spectra at $t = 0$. (B) Spectra at $t = 96$ hrs (4 days).

3.2.3.3 Fluorescence spectroscopy of type I AFP in propyl-modified glass

The fluorescence responses of TAMRA-labeled AFP in propyl-modified glass wafers are shown in Figure 3.10. Once again, the intensity values were compared at

555 nm. At $t = 0$, the glass sample in 60% EtOH has 3.90×10^5 cps, while the one in 100% water has intensity of 7.54×10^5 cps, and the one in 10 mM potassium phosphate has a higher intensity of 8.40×10^5 cps (Figure 3.10A).

After an incubation period of four days, the intensity of the encapsulated protein changed in a fashion similar to the standard glass results. The signal for the glass sample in 10 mM potassium phosphate increased in intensity by 22%. The intensity for the glass in 100% water was diminished by 56%, and the one in 60% EtOH displayed a drastic reduction of 69% (Figure 3.10B).

Figure 3.10: Propyl-modified glass excitation spectra at 590 nm for encapsulated 5(6)-TAMRA attached to type I fish AFP. (—) 100% water, (—) 10 mM potassium phosphate and (- - -) 60% EtOH. 10 mM potassium phosphate buffer is used as a protein blank (- . -). (A) Spectra at $t = 0$. (B) Spectra at $t = 96$ hrs (4 days).

The results of standard glass and propyl-modified glass yield similar conclusions. The intensities of the signals for glasses in potassium phosphate buffer and 100% water change slightly from $t = 0$ to $t = 96$ hrs. However, the intensity for a glass in 60% EtOH

is greatly diminished by day four. These are wet-aged glasses, and the pores in the glass matrix may be large enough to allow the protein to escape from the glass.

To help prevent the protein from leaching out, xerogels with smaller pore sizes were made. The xerogel process involves the slow evaporation of the liquid phase within the pores and results in considerable weight loss [26]. At this stage, the pores collapse, and there is a decrease in average pore size and total glass volume. The reduced pore size should slow diffusion and reduce the leakage of small proteins from the matrix when the xerogel sample is immersed in excess buffer.

3.2.3.4 Fluorescence spectroscopy of type I AFP in xerogel

In Figure 3.11, the emission spectra for 5(6)-TAMRA attached to type I fish AFP encapsulated in a xerogel are shown. At $t = 0$, all solutions (1 M and 10 mM potassium phosphate, 100% water and 60% EtOH) yield a similar profile and intensity. After the xerogels are equilibrated for 96 hours, the intensity of the samples in 1 M and 10 mM potassium phosphate and 100% water are relatively unchanged when compared to $t = 0$ (~ 480 a.u. at 555 nm), but the sample intensity in 60% EtOH is reduced significantly (76.6 a.u. at 555 nm) (Figure 3.11B). Thus, the smaller pores of the xerogel prevented leaching over a 4-day period in the presence of all solutions except 60% EtOH. The ethanol may have induced the helix in the structure of type I fish AFP, and thus even with the smaller pore size glass, caused the protein to leach.

Figure 3.11: Excitation spectra at 590 nm for 5(6)-TAMRA attached to type I fish AFP encapsulated in xerogel. (—) 100% water, (---) 10 mM potassium phosphate, (—) 1 M potassium phosphate, and (-.-) 60% EtOH. (A) Spectra at $t = 0$. (B) Spectra at $t = 96$ hrs (4 days).

3.2.3.5 CD spectra in xerogel

Individual wafers of type I fish AFP in xerogel samples were equilibrated for one day with two different solutions (10 mM potassium phosphate and 60% EtOH/H₂O).

Changes in conformation were monitored at 5 and 25 °C by far-UV CD (Figure 3.12).

For all conditions, the spectra showed ellipticity minima at 208 and 222 nm, distinctive for a helical protein.

Figure 3.12: CD spectra of type I fish AFP in xerogel samples. Far-UV CD spectra are shown for 10 mM potassium phosphate and 60% EtOH at 5 and 25 °C; (—) glass wafer with 10 mM potassium phosphate (pH 7.0) at 25 °C. (- - -) glass sample equilibrated with 10 mM potassium phosphate at 5 °C; (x) glass sample equilibrated with 60% EtOH/H₂O at 25 °C; (o) glass sample equilibrated with 60% EtOH/H₂O at 5 °C. [AFP] = 0.084 mg/mL, 0.75 mm thick glass.

In the presence of 10 mM potassium phosphate, the ellipticity was stronger than that observed in any other glass, approaching -23 mdeg at 222 nm. Ethanol enhances the helical content of sol-gel glass encapsulated type I fish AFP. The ellipticity of the xerogel sample in 60% ethanol approaches that of the solution spectra in phosphate buffers (if compared on a molar basis). Both samples show more helicity at 5 °C than at 25 °C, which is expected. As stated before, antifreeze proteins evolved to function at a temperature near 0 °C, and, therefore, lower temperatures should yield the functionally-active structure for binding to the ice/water interface.

The CD analyses of the xerogel glass samples yielded a stronger signal than all the other glasses. Since the xerogel glass has smaller pore sizes than the wet-aged glasses, it appears that protein leaching was greatly reduced and that leaching was indeed the reason for low CD signals in the wet-aged glasses.

3.2.4 Discussion of type I AFP results

The sol-gel entrapment technique constitutes an alternative method for studying the structure of fish antifreeze protein in a crowded environment. We were able to make thin wet-aged and xerogel glasses of low optical absorbance as required for CD and fluorescence spectroscopies. The spectroscopic data indicate that the structure of the monomeric, 37 amino acid long, type I fish AFP depends on the solvent conditions, temperature, and particular glass encapsulation method.

Through structure characterization using CD, the protein was shown to have an α -helical conformation in solution and to become more helical by reducing temperature or

adding an alcohol. On the other hand, encapsulation of the protein in wet-aged sol-gel glasses resulted in very low ellipticity values under all solution conditions. The fluorescence studies confirmed that the protein was leaching from the wet-aged glasses during equilibration periods with excess solution. However, reducing the pore size by xerogel formation appears to trap the protein in the glass, as first observed by fluorescence. The TFE and 60% EtOH solutions enhance the α -helical structure of the protein, but these reagents may also facilitate leaching in wet-aged glasses. This conclusion is supported by experiments using both CD and fluorescence spectroscopies. The best results were obtained in the xerogel samples after only one day of equilibration time.

CHAPTER 4:

SOL-GEL GLASS ENCAPSULATION OF TYPE III FISH AFP

4.1 BACKGROUND ON TYPE III AFP

Although less extensively studied than type I AFPs, type III AFPs have also been characterized using x-ray crystallography and NMR [55]. These studies indicate that type III AFP is a small globular protein consisting of 66 amino acids. It has eight β -strands that form two sheets of three anti-parallel strands and one sheet of two anti-parallel strands (Figure 4.1). The triple-stranded sheets are packed orthogonally into a β sandwich [55]. Since type III AFP is a globular protein that does not contain a repetitive sequence, the ice lattice model of matching periodic H-bonds does not appear to apply. The three-dimensional structure of type III AFP has been well resolved, and the putative ice-binding residues have been identified and mapped in detail through extensive site-directed mutagenesis of selected residues. Based on structures obtained by high-resolution x-ray crystallography [56], a model was proposed whereby surface adsorption occurs through a hydrogen bond match between the side chains of Gln-9, Asn-14, Thr-15, Thr-18, Gln-44, and the ice prism (Figure 4.1) [56]. Initial mutant studies on type III AFP provide a consistent relationship between the effect of mutations on AFP activity and the loss of a hydrogen bond [57]. Loss of a H-bond causes a decrease of 30-50% activity [56-58]. With the determination of a medium resolution NMR structure, it was found that the mutations that had an effect on activity were clustered onto one face of the protein, the putative ice-binding site of type III AFP [56-59].

Figure 4.1: Crystal structure of type III fish AFP. Yellow and red arrows represent β -strands [56]. (Figure courtesy of Dr. Jeffry D. Madura, Center for Computational Sciences, Duquesne University, 2007).

To further understand the structure in dilute and crowded environments, we used three different types of sol-gel glasses, i.e., standard glass, propyl-modified glass and xerogel, to encapsulate type III fish antifreeze protein. The structure of each encapsulated protein was compared to its structure in solution using CD spectroscopy.

4.2 RESULTS AND DISCUSSION

4.2.1 CD results for type III AFP in solution

The secondary structure of a protein can be determined by CD spectroscopy in the far-UV region (190-250 nm) [41, 60]. At these wavelengths, the chromophore is the peptide bond, and the signal arises when it is located in a regular, folded environment.

Here, the secondary structure of type III AFP in solution is characterized using CD spectra. The spectra of samples in 10 mM and 1 M potassium phosphate buffer collected at 5 °C and 25 °C exhibit a positive value at 220 nm and becomes negative as the wavelength approaches 200 nm, typical for a random coil structure (Figure 4.2).

Figure 4.2: CD spectra of type III fish AFP in solution. The spectrum is the average of three measurements at 5 and 25 °C; (—) protein in 1 M potassium phosphate at 25 °C; (x) protein in 10 mM potassium phosphate at 25 °C; (+) hidden behind blue, protein in 10 mM potassium phosphate at 5 °C. [AFP] = 0.20 mg/mL, 2.0 mm pathlength cuvette.

Similar spectra were observed for type III AFP in 10 mM and 1 M potassium phosphate buffer. Also, the structure of type III AFP was unchanged at 5 and 25 °C in solution. Previously, the structure of this protein was identified using two-dimensional NMR spectroscopy [55]. The CD result in solution is in accordance with the NMR result; the 66-amino acid-long type III AFP has no helical character. However, the random coil result shown here is unexpected since the NMR structure indicates

significant β -structure which should be apparent in the CD spectrum as a minimum near 215 nm [55].

4.2.2 CD results for type III AFP in glass

4.2.2.1 Standard glass

Individual wafers of standard glass-entrapped type III fish AFP were equilibrated with four different solutions (1 M and 10 mM potassium phosphate, 1 M guanidine hydrochloride and 4 M potassium chloride) to determine any solvent specific effects on the secondary structure of the protein which result from its encapsulation in the silica matrix. Changes in conformation were monitored at 25 °C by far-UV CD (Figure 4.3).

Figure 4.3: Standard glass encapsulated type III fish AFP. CD spectra are shown for the following solutions: (—) glass sample equilibrated with 10 mM potassium phosphate (pH 7.0); (- - -) glass sample equilibrated with 1 M potassium phosphate; (x) glass sample equilibrated with 1 M guanidine hydrochloride; (+) glass sample equilibrated with 4 M potassium chloride. [AFP] = 0.452 mg/mL, 1.0 mm glass thickness.

The spectra indicated a major change in structure relative to dilute solutions. All four solutions yield similar CD spectra that suggest a protein with partial α -helical structure. Clearly, encapsulation of the protein changed the secondary structure. The altered structure may be attributed to confinement or crowding effects.

4.2.2.2 Propyl-modified glass

Propyl-modified glass-entrapped type III fish AFP samples were equilibrated with 1 M and 10 mM potassium phosphate buffers to determine whether the protein behaves the same in this glass environment. Changes in conformation were monitored at 25 °C by far-UV CD (Figure 4.4).

Figure 4.4: Propyl-modified glass-encapsulated type III fish AFP. CD spectra are shown for the following buffers; (—) glass wafers equilibrated with 10 mM potassium phosphate (pH 7.0); (- - -) glass sample equilibrated with 1 M potassium phosphate at 25 °C. [AFP] = 0.452 mg/mL, 1.0 mm glass thickness.

Once again the encapsulated type III AFP appears to have enhanced secondary structure relative to dilute solution. The spectra are similar to those obtained with the standard glass indicating partial helical structure in the propyl-modified glass.

4.2.2.3 Xerogel

To further study confinement effects, type III AFP was encapsulated in a xerogel, as performed for type I AFP. Individual wafers of type III fish AFP encapsulated in a xerogel were equilibrated with 1 M potassium phosphate, 10 mM potassium phosphate and 1 M potassium chloride buffers to determine the secondary structure of the protein. Changes in conformation were monitored at 25 °C by far-UV CD (Figure 4.5).

Figure 4.5: CD spectra for type III fish AFP encapsulation in xerogel. (—) glass wafers equilibrated with 10 mM potassium phosphate (pH 7.0). (- - -) glass sample equilibrated with 1 M potassium chloride. (x) glass wafers equilibrated with 1 M potassium phosphate. [AFP] = 0.452 mg/mL, 0.75 mm glass thickness.

As seen above, all three spectra were rather flat and featureless, indicating a lack of protein. Since we do not expect a leaching problem in xerogels, the lack of ellipticity may indicate that the stock solution of protein was degraded or otherwise compromised before making the glass. In the future, this experiment should be repeated with a fresh stock solution of type III AFP.

4.2.3 Discussion of type III AFP results

Previous results using x-ray crystallography and two-dimensional ^1H NMR indicated that the solution structure of type III AFP is globular with a β -pleated sheet fold and no helical character [55]. However, using circular dichroism to examine the secondary structure of type III AFP and by comparing the structure in the glass to that in solution, two surprising observations were made. (1) A random coil structure was indicated in dilute solution, and (2) confinement induced a slight change toward helical structure (but no obvious β -structure).

The CD result is not consistent with the NMR and x-ray crystallography results. In solution the spectra show a random coil structure both at 5 and 25 °C. This result was surprising since the crystal structure indicates a significant amount of β -structure, and the expected CD spectrum would have a minimum near 215 nm (see figure 1.5). X-ray crystallography and NMR also showed that type III AFP lacks helical character in solution. However, encapsulating the protein in silica glass changed the secondary structure of the protein slightly toward an α -helix. The altered structure may be attributed to confinement or crowding effects.

Unlike type I AFP, there was no leaching observed by CD for glass encapsulated type III AFP. The reason for this may be the size and structure of the protein. Type I AFP is small with just 37 residues and adopts a single α -helix. Encapsulating type I AFP in wet-aged glasses under all solution condition allowed the protein to leach out. However, reducing the pore size by xerogel formation trapped the protein in the glass. The size and structure of the protein appear to be responsible for the leaching problem. In the case of type III AFP, this protein has 66 amino acid residues and has a globular structure. It is twice as big as type I AFP, and the structure is apparently too bulky to leach out of the glass.

CHAPTER 5:

SUMMARY OF RESULTS AND CONCLUDING REMARKS

The sol-gel technique of encapsulating proteins in a silica matrix provides a method to simulate cellular crowding *in vitro*. Using the sol-gel technique and characterizing the encapsulated protein by using circular dichroism, we have examined the secondary structure of type I and type III fish AFPs.

Type I AFP has an α -helical structure in solution. The structure is more helical in alcohol solutions (e.g. TFE and 60% EtOH) and at a lower temperature (5 °C).

Encapsulating the protein in wet-aged (standard, propyl-modified and methanol-free) glasses resulted in very low ellipticity values under all solution conditions. Using fluorescence spectroscopy, it was determined that type I AFP leached from the wet-aged glasses. Using a xerogel protocol, the protein was trapped in the glass and showed high ellipticity values.

Type III AFP has a random coil structure in solution. Encapsulating the protein in wet-aged (standard and propyl-modified) glasses resulted in a slight gain in helical structure. The reason could be that protein structures are influenced in crowded environment by factors that are not apparent in dilute solutions. In a crowded environment, non-specific interactions can occur between the protein and the crowding agent, in our case, the silica matrix.

Eggers and Valentine reported that the α -helical content of their model protein, apomyoglobin, was reduced from 60% in solution to 20-25% on encapsulation [22].

When salt solutions containing the anions HPO_4^{2-} , SO_4^{2-} , Cl^- paired with K^+ , or the cations $\text{N}(\text{CH}_3)_4^+$, K^+ and Li^+ paired with Cl^- were used, an ion-dependent change in the α -helical content of encapsulated apomyoglobin was observed. These ions are believed to influence the protein structure indirectly through changes in the hydrogen-bonding properties of water. The physical properties of water are strongly linked to its hydrogen-bonding character, and it is known that the network of hydrogen bonds is interrupted at a solid interface [22]. Therefore, Eggers and Valentine hypothesized that the unusual water structure in the pores of the silica matrix diminishes the hydrophobic effect, a dominant force in protein folding. Presumably, addition of phosphate ions perturbs the unfavorable water structure and reestablishes the hydrophobic effect, allowing the protein to adopt its native conformation.

Our finding for type III AFP is not fully consistent with the results observed by Eggers and Valentine [22]. The change in structure that we observed for encapsulated type III fish AFP was found to be independent of salt. Therefore, we conclude that the enhanced secondary structure observed for encapsulated type III AFP must be due to crowding.

Further work may elucidate whether these crowding effects are linked with changes in the properties of water. Also, for type I AFP, it would be interesting to study the structure with more buffered salt solutions and at even lower temperatures (below 5 degrees). Since the xerogel was successful in trapping the protein, it is now possible to study the structure of type I fish AFP in a crowded environment using other buffers. For type III AFP, the results obtained in the xerogel need to be repeated with a fresh protein

stock solution; the spectrum showed weak ellipticity which might have been caused by a degraded stock solution. In addition, by attaching a fluorescence probe to the N-terminus of type III AFP, this protein could also be studied by fluorescence spectroscopy.

Fluorescence spectroscopy is a useful analytical technique used in this research to identify leaching of type I fish AFP from wet-aged glasses. Using TAMRA-labeled type I fish AFP with polar and non-polar solvents, spectral shifts were detected from the general effect of solvent polarity whereby the energy of the excited state decreases with increasing solvent polarity. Even though fluorescence measurements do not provide detailed structural information, the technique provides changes in the structural and dynamic properties of compounds. This phenomenon is useful for analyzing fluorescent spectra for different biomolecules. In general fluorescence spectroscopy holds great promise for our understanding of antifreeze proteins and their structure-property relations. In a future, using xerogel glass, both type I and type III fish AFP could be studied in various solvents and temperature.

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