Structural & Functional Characterization of the Amyloid-like Glycoprotein Adhesin Als5p of *Candida albicans*

By

HENRY NUNOO OTOO

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of requirements for the degree of Doctor of Philosophy, The City University of New York.

2007

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This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

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Candida albicans is the third most common nosocomial agent in the United States. The fungus expresses a family of cell wall proteins called Als (Agglutinin-Like Sequence proteins). Als proteins share a similar immunoglobulin-like (Ig), conserved Threonine-rich (T), variable Tandem repeats (TR), and glycosylated C-terminal regions. Als5p mediates adhesion to host and self aggregation.

With increasing TR number, binding of Als5p to ligands is enhanced (Rauceo et al., 2006). Biochemical studies done investigated the structural basis for this region's influence on aggregation (TR). Als5p$^{1\text{-}431}$ (Ig-T) and Als5p$^{1\text{-}664}$ (Ig-T-TR) were successfully purified in good quantities (4-10ml of 0.1-0.4mg/ml). Circular Dichroism indicates Als5p$^{1\text{-}664}$ fragment has high beta sheet content and that the TR-region influences the conformation of Als5p$^{1\text{-}664}$. The TR is shown to give Als5p$^{1\text{-}664}$ conformational flexibility.

Als5p mediates aggregation at pH 2-10 and temperatures 5°C-65°C. Soluble Als5p aggregates at low concentrations (<0.5mg/ml) into multimers that are resistant to GuHCl and SDS dissolution. The soluble protein fragment is stable within the pH range 3-9, refolds from 80°C, retains secondary structure at 90°C, and has multiple temperature transitions that are

less cooperative (the unfolding of the regions is not coordinated). The high beta content, ease of aggregation, high pH and temperature stabilities, and the resistance of aggregates to SDS and GuHCl treatment resembles the behavior of an amyloid protein.

Als5p can form amyloids. The computation program TANGO identified a short sequence (IVIVATT) in the T-region with 93% beta aggregation propensity. A 13mer peptide (SNG-IVIVATT-RTV) of this sequence aggregated into a gel, tested positive for amyloid by increased absorbance when bound to Congo red, increased Thioflavin T fluorescence, and formed amyloid-like fibrils. Purified Ig-T and Ig-T-TR aggregates bound Congo red with increased absorbance and formed amyloid-like fibrils.

Thus in these investigation we show a TR region that is functional by increasing Als5p adherence to ligands and gives conformational flexibility to Als5p. Also we have shown a new property of Als5p to form amyloids and this amyloidicity will increase homotypic (Als5p-Als5p) association and therefore increase cell-cell aggregation.

ACKNOWLEDGEMENTS

My utmost gratitude goes to my supportive wife (Mrs. Cynthia Naa Ankamnoa Otoo) for bearing all the burden and frustrations with me.

To the following people for their help: Dr. Peter Kahn of Rutgers (The State University of New Jersey); Michael Baxter of Lehman College; Dr. Lesley Davenport, Dr. Rafael Ovalle, Dr. Richard Magliozzo and Dr. Laura Juszczak all of Brooklyn College; Dr. Yujia Xu, Dr. Dixie Goss, and Dr. Sibnath Ray all of Hunter College; and Dr. Kyeng Gea Lee of the Bronx Community College.

To all my committee members: Dr. Ray Gavin, Dr. Derrick Brazill, Dr. Maria Figueiredo-Pereira, Dr. Dixie Goss, Dr. Anne Dranginis and especially Dr. Lesley Davenport for her advice and rare encouragement before and after I joined the Lipke lab in Fall of 2004, and my mentor Dr. Peter Lipke for providing the funds and environment that made this work possible.

Last but of great importance is my family that nurtured me with their love, guidance, encouragement, prayers and support.

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CHAPTER I

Introduction & Background

A. What is *Candida albicans*?

Candida albicans is a dimorphic yeast. Yeasts are unicellular symbiotic or parasitic fungi that are naturally found in places like plants, soil, water, skin surfaces and warm-blooded animals. Some yeasts such as *Saccharomyces cerevisiae* and related species are employed industrially for fermentation purposes to produce ethanol and for baking. *Saccharomyces cerevisiae* has been used as a model system for much of molecular genetic research because the basic cellular mechanics of replication, recombination, cell division and metabolism are generally conserved between yeast and larger eukaryotes, including mammals. However some yeast like *Candida* species can become pathogenic and inflict harm to other living organisms.

B. Why study Candida infections?

Candidiasis is an infection caused by *Candida* fungi, especially *Candida albicans*. *Candida* species are ubiquitous in the environment, living mostly as commensals in the mouth, gut and vagina together with other normal flora of the host (Odds, 1998). Under conditions that compromise the host immune system, *Candida* species can become pathogenic, causing problems ranging from superficial infections to life threatening and fatal infections (Calderone 2002; Okawa et al, 2004). Usually, *Candida* is kept under control by the native bacteria and by the host's immune defenses. However, the broad spectrum and prolonged use of antibiotics, the reduction in the body’s immune defense in people with diseases like AIDS and diabetes, chemotherapy and extensive surgery often lead to *Candida* pathogenesis (Calderone 2002). Malnutrition and the use of certain medications such as corticosteroids or anticancer drugs also have been related to an

increase in susceptibility to pathogenic *Candida* invasion. *Candida* sp is also able to infect healthy individuals occasionally as in the case of mouth thrush (http://www.intelihealth.com/).

Candida albicans had been the predominant *Candida* species isolated from hospital specimen, however, recently other non-albicans species such as *C. glabrata, C. guillermondi, C. tropicalis, C. parapsilosis*, and *C. formata* have been responsible for fungemia (Barberino et al., 2006). The main risk factors that were reported by Barberino et al., 2006 include the presence of a central venous catheter, use of parenteral nutrition support, chronic renal failure and previous use of antibiotics.

Types of Candidiasis: There are various forms of Candida infections and mostly named according to the site of infection. Some examples are:

1) Thrush which is infection of the mouth (tongue, palate and lips);

2) Esophagitis which occurs when *Candida* infections of the mouth spread to the esophagus;

3) Cutaneous candidiasis which is a skin infection;

4) Vaginal yeast infections which occur in 75% of all women at least once in their life time (Cheng et al., 2005); and

5) Deep candidiasis which occurs when *Candida* accesses the bloodstream causing fever, shock or multiple organ failure (*http://www.intelihealth.com/*).

It should be mentioned that the first documented case of deep organ Candidiasis (brain) was in 1861. The increased use of antibiotics in the 1940s greatly increases the incidence of blood stream Candidiasis (Barberino et al., 2006). Although there is an

increase in the diversity of pathogenic fungi isolated from clinical samples, *C. albicans* is still the predominant cause of fungal infections. Eloy et al. 2006, attest that *C. albicans* still accounts for 55% of yeast bloodstream infections. *C. albicans* is a member of the commensal flora, yet an opportunistic pathogen because it is able to cause disease under depressed host defenses due to AIDS and organ transplantations (Navarro-Garcia et al., 2001).

C. *Biological studies on Candida albicans*

(a) Incidence and factors that aid the pathogenecity of *C. albicans*: The ability to switch from a unicellular to a multi-cellular hyphal mode of growth called dimorphism is a characteristic of the yeast. Dimorphism in *C. albicans* has been shown to be triggered in response to certain environmental conditions such as temperature and pH. Dimorphism has been linked to tissue invasion and virulence, as hyphae forms of *C. albicans* are more pathogenic than the yeast forms (Navarro-Garcia et al., 2001).

Among the factors that aid *C. albicans* to colonize and become pathogenic are the abilities to undergo phenotypic switching, adherence to a wide variety of substrates (from plastics, variety of host cells, to self), hyphae formation and secretion of hydrolases (Calderone 2002; Okawa et al, 2003; Chandra et al, 2001). The Agglutinin-like sequence (*ALS)*, secreted aspartyl proteinase (*SAP*) and lipase gene families are very important in the pathogenesis of *C. albicans* (Cheng et al., 2005).

Extensive work has been done at the gene level to study the yeast for a better understanding of how to combat the disease. Several genes have been defined with roles in virulence. Some are discussed below:

Cell wall genes: The thick and rigid cell wall of *C. albicans* acts as a first defense against host elimination factors such as the immune system and lysing of the cell. The expression of adhesins such as Als proteins and Hwp1 helps with the establishment of the yeast in host (Calderone 2002; Li & Palecek 2003). The hydrophobic proteins expressed on the cell wall surface also aid the yeast to defend itself against phagocytosis. Some cell wall genes like *CHS* (chitin synthase), *MNT1* (mannosyl transferase), *HWP1* (hyphal wall protein), *PMT1* and *PMT6* (mannosyl transferase) have been linked to adherence or virulence. For example, in *C. albicans,* disruption of *PMT1* has been reported to lead to strains that are defective in mannosylation of substrates, aggregate easily, are less adhesive, are less virulent, and have longer duplication time (Navarro-Garcia et al., 2001; Calderone, 2002). The absence of the cell wall in mammalian cells and its accessibility makes it an ideal target for drug development (Cossone, 1989; Odds, 1988; Calderone, 2002; Alberti-Segui et al., 2004).

Metabolic genes: Iron, carbon, purine and lipid metabolism have been shown to play important roles in *C. albicans* virulence (Navarro-Garcia et al., 2001).

Secreted factors genes: In *C. albicans* the Sap proteases which are the best characterized of the secreted factors have at least nine (9) members and have been shown to be differentially expressed depending on the growth conditions. For example, *SAP2* is expressed at acidic pH while *SAP4-6* are expressed at neutral pH. Navarro-

Garcia et al., 2001 also proposed a complementation action by the *SAP* genes where each SAP gene contributes to the virulence of the yeast.

(b) The pathogenicity of *C. albicans:* Pathogenicity of the yeast has been demonstrated to depend not only on yeast virulent factors but also on the host's influence on the microbial environment. The temperature, pH, and osmotic concentration of the micro-environment created by the host have also been reported to play a significant role in the response elicited by the microbe. Thus many factors seem to contribute to the pathogenecity of *C. albicans* (Navarro-Garcia et al., 2001).

(c) Cell Surface Hydrophobicity

Cell surface hydrophobicity plays an important role in *C. albicans* in that, hydrophobicity on the cell surface has been linked to increased adherence, pathogenesis, and invasion (Masuoka and Hazen, 2004, Masuoka et al., 2002). It has been shown that hydrophobic *C. albicans* cells are more adhering than hydrophilic *C. albicans* cells to a variety of host tissues, and also that hydrophobic *C. albicans* cells are more resistant to phagocytic killing than hydrophilic *C. albicans* cells. Hydrophobic proteins on the cell surface of *C. albicans* that become exposed through changes in conformation of the surface fibers have been shown to be responsible for the hydrophobicity of the cell surface, and *C. albicans* is a pathogenic fungus shown to be able to regulate its cell surface hydrophobicity status under different environmental conditions. This cell surface hydrophobicity has been also shown to be linked to the character of the cell wall architecture in *C. albicans* (Hazen and Hazen, 1992 & 1993;

Masuoka et al., 2002; Masuoka and Hazen, 2004). Fibrils on hydrophobic cell surfaces are short, aggregated and variable; whereas those on hydrophilic cell surfaces are longer, evenly spaced and radiating (Masuoka and Hazen, 2004). It is also interesting that more hydrophobic proteins are found on surfaces of the more virulent hyphae forms than in yeast forms that are less virulent. Even more informative is the expression of the hydrophobic proteins during human disease. Of equal importance are the findings that the proteins assigned adhesive functions, such as Als proteins, fall into the hydrophobic class of proteins (Calderone, 2002).

(d) Cell wall mannoproteins

The fibrils seen on the yeast cell surface have been predicted to be manno-proteins (Masuoka and Hazen, 2004). Mannoproteins are found as either soluble proteins (e.g. invertase and acid phosphatase) or attached to the cell wall (e.g. glucanases and adhesion proteins) of the yeast. It is known that ~40% of the cell wall consists of mannoproteins that are of two kinds: SDS and non-SDS extractable mannoproteins (Valentin et al., 1984). These mannoproteins are synthesized intracellularly, glycosylated, and with the help of a secretion signal, secreted into the cell wall (Sanz et al., 1987; Hoyer 2001; Rauceo et al., 2004, 2006).

The cell wall consists predominantly of carbohydrates (~90%), few proteins (~5-10%), and some lipids. The carbohydrate moiety is made of glucan and mannan (Calib et al., 1982). The glucan, which is localized predominantly in the inside of the cell wall is masked by the mannoproteins that are located at the exterior of the cell (Horisberger and Clerc, 1987). The glucan is believed to be part of a network of fibrils

which has been seen on the cell wall by electron microscopy studies (Jelsma and Kreger, 1987; Calib et al., 1988) together with the mannoproteins, which have been shown to consist of high percentage of serine and threonine residues, determines the porosity of the cell wall (Zlotnik et al., 1984; Frevert and Ballou, 1985; Jentoft, 1990). Thus apart from the glucans that give strength to the cell wall, the mannoproteins also help maintain the cell wall integrity.

(i) <u>The *ALS* gene family</u>

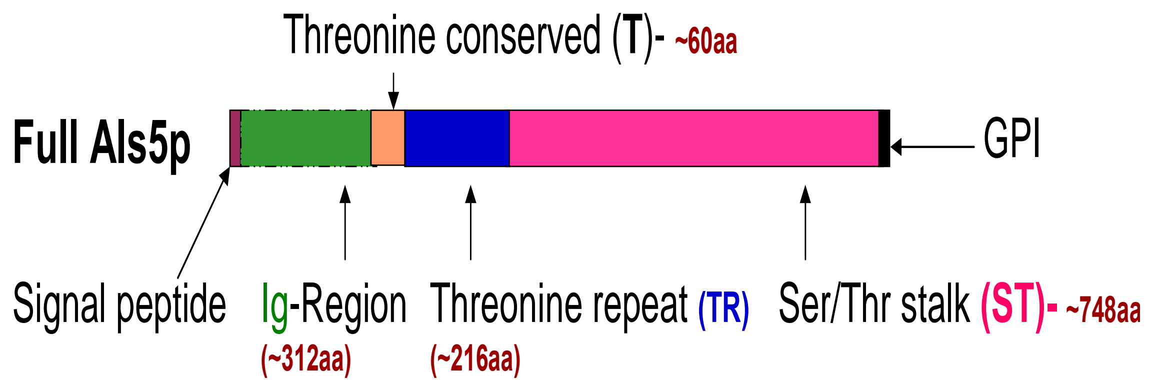
A family of well characterized cell wall mannoproteins (glycoproteins) is the *ALS* (agglutinin-like sequence) family. The *ALS* genes were first described in *Candida albicans.* They encode eight large, cell-surface glycoproteins, some of which have been implicated in adhesion and biofilm formation in hosts (Hoyer 2001; Hoyer and Hecht, 2001; Nobile and Mitchell, 2006). The *ALS* gene family is found in other Candida species like *C. dubliniensis* and *C. tropicalis* also. *ALS1* was the first *ALS* to be characterized and was found to have sequence similarities with the cell-surface adhesion glycoprotein α-agglutinin encoded by *Saccharomyces cerevisiae* (Lipke et al., 1989; Hoyer et al., 1995, 1998, 1999; Gaur and Klotz, 1997; Hoyer and Hecht, 2000, 2001). The sequence similarities between Als1p and Agα1p led to the initial association of Als proteins to adhesion (Hoyer et al., 1995; Lipke et al., 1989). Gaur et al. (1997) first demonstrated the adhesive function of Als5p (formerly known as Ala1p) from a screen that selected transformants able to bind to the highly hydrophobic extracellular matrix protein fibronectin. Als proteins have been shown to be evenly distributed across the cellular surface rather than focally clumped (Hoyer, 2001). Variability in *ALS* gene

structure and expressions, including strain- and allele-specific size differences for the same gene, the absence of particular *ALS* genes in certain have been found (Hoyer, 2001).

In *C. albicans* there are eight *ALS* genes: *ALS1-7*, and *ALS9*. Each *ALS* gene has a similar three domain structure: an N-terminal immunoglobulin-like domain (55-90% identical across the family); a central 36 amino acid tandem repeat domain (with variable number of repeats across the family and glycosylated); and a C-terminal domain also called the 'stalk' (that is heavily glycosylated and varies in length across the family) (FigureI 1). There is a signal sequence and GPI (glycosyl-phosphatidyl-inositol) anchor that localizes the Als protein to the cell surface (Hoyer 2001). The glycosylation of the protein causes Als proteins to migrate slower on gels and thus show higher molecular weights than expected from computational estimations (Hoyer, 2001, Rauceo et al., 2006). Among the *ALS* genes, *ALS7* has been found to have the longest C-terminal and also the most differences from other Als proteins, with 45-50% amino acid identity with other *ALS* genes in the Ig-region (Hoyer 2001; Sheppard et al., 2004; Rauceo et al., 2006). *ALS1, ALS2, ALS4, ALS5* and *ALS9* are located on chromosome 6; *ALS6* and *ALS7* on chromosome 3, and *ALS3* is located on chromosome R in *C. albicans* (Hoyer, 2001). Expression of *ALS* genes in *C. albicans* is affected by growth medium changes (*ALS1*), dimorphism (*ALS3*) and stage of growth (*ALS4*).

Circular dichroism studies of Als5p demonstrate that it is rich in beta-sheet consistent with an immunoglobulin fold structure (Sheppard et al., 2004; Rauceo et al., 2006; Hoyer 2001). The heavy glycosylation of the tandem repeat and C-terminal domains have been predicted to likely confer an extended conformation on the protein

and thus function by elevating the binding Ig-region above the cell surface for ligand presentation (Hoyer, 2001; Jentoft, 1990). One significant difference shown in the binding region of Als proteins by Hoyer (2001), is the presence of N-glycosylation within the Ig-region of Als2p, Als6p and Als9p and not in the other Als proteins. The amino-terminal (binding region) is highly hydrophobic, as demonstrated by its binding to phenyl Sepharose (Hoyer and Hetch, 2001), Congo red and 8-anilino sulfonic acid (Rauceo et al., 2004).



FigureI 1. A cartoon representation of the domains of Als proteins.

(ii) Binding properties of Als1p and Als5p

Both *S. cerevisiae* expressing Als1p/Als5p and *Candida albicans* bind to immobilized peptides and proteins with a flexible accessible backbone as well as the amino acid sequence motif τφ+; where τ represents amino acids with turn propensity (D, K, A, S, N, G, P); φ represents amino acids with bulky hydrophobic or aromatic residues (F, H, I, L, M, T, Y, V, W); and + represents positive amino acids (basic), K and R (Klotz et al., 2004; Gaur & Klotz, 2004). It is however established that the binding properties of Als5p are different from that of Als1p, although both mediate adherence to human cells and extra-cellular matrix components when expressed on the surface of *Saccharomyces cerevisiae*. The adherence behavior of Als5p and Als1p expressing cells have been shown to mimic that of clinical isolates and also *C. albicans* with Als1p knockouts adhere less in an oral pharyngeal Candidiasis model experiment (Kamai et al. 2002; Gaur et al. 1999). Als5p mediated binding has been shown to be strong and occurs within minutes, with the adherence being inhibited by formamide, high pH, and urea (Gaur et al., 1999).

According to Klotz et al. (2004), there may be a degenerate peptide and protein recognition by *C. albicans* as other non-specific sequence or class of proteins and peptides are recognized by *C. albicans*. This degenerate property, according to Klotz et al. (2004), may be due to additional binding specificity manifested by Als proteins in addition to the amino acid sequence and motif found on a targeted protein or peptide. It must be mentioned that they found that a scrambled version of an adherent ligand from a peptide library did not have any adherence (Klotz et al., 2004).

(iii) <u>Other yeast glycoprotein adhesins related to *ALS*</u>

(α) α-agglutinin: The yeast cell adhesin α-agglutinin is a glycoprotein expressed on the cell surface of *Saccharomyces cerevisiae* α-Cells mate with the a-cell types of *Saccharomyces cerevisiae* that express the ligand a-agglutinin. Both cells are haploid and non-pathogenic (Zhao et. al., 2001; Hauser and Tanner 1989; Lipke and Kurjan, 1992; Lu et. al., 1994, Wojciechowicz et. al., 1993; Kapteyn et. al., 1994; Chen et. al., 1995; Lipke et. al., 1995; Grigorescu et. al, 2000). Like the Als super-family proteins, the carboxy-terminal end of α-agglutinin is anchored to the cell wall of the yeast while the amino-terminal is the binding region. Other similarities that exist between Als family and α-agglutinin include the presence of high percentage of beta-sheet structure, the presence of disulfide bond cys residues in the binding region and also presence of three tandem Ig-like domains in the amino-terminal region of the proteins.

In α-agglutinin, although domain III of the Ig-like region is said to be the functional region, domains I & II are also functional since domain III alone does not show measurable activity and partial deletions of domains I & II inactivates the protein (Zhao et. al., 2001).

Some differences have been encountered between Als proteins and α-agglutinin in terms of activity at homeostatic pH and temperature. In comparison to most of the Als proteins, α-agglutinin is less active at the homeostatic conditions of pH 7 and temperature 37°C but rather pH 5-6 and a lower temperature up to 15°C (Terrance and Lipke, 1981; Zhao et. al., 2001).

(β) Flo11 protein: Under various environmental conditions *Saccharomyces cerevisiae* adheres, flocculates, filaments, invades, or forms biofilms. A family of flocculins called Flo proteins (Flo1p, Flo5, Flo10, and Flo11) have been shown to mediate aggregation of *Saccharomyces cerevisiae* (Lo and Dranginis, 1996; Fidalgo et al, 2006) like the Als family of proteins. Unlike the Als proteins that do not require Ca^{2+} for aggregation, the Flo proteins require Ca^{2+} for aggregation. However, structurally, Flo proteins especially Flo11 share structural similarities with both Als proteins and α-agglutinin protein. They all share a hydrophobic N-terminal region with a short signal sequence, a central domain repeat region with threonine rich sequence that is gycosylated and a C-terminal region generally thought to elevate the glycoprotein for presentation to interact with ligands and linked to the cell wall through the GPI sequence anchorage expressed at the C-terminal end of the protein (Fidalgo et al., 2006; Hoyer 2001, Rauceo et al., 2004, 2006). Flo11 has three repeat regions (Lo & Dranginis, 1996).

S. cerevisiae is uniquely able to form a buoyant biofilm called flor which is not characterized by the usual carbohydrate and protein matrix found in other microbial biofilms (Ishigami et al, 2004 & 2005). Flor is formed on the surface of wine after ethanolic fermentation that allows the yeast to respire using oxygen and its buoyancy has been attributed to the surface hydrophobicity of the yeast which has been established to be conferred upon by Flo11 proteins on the yeast cell surface. The Flo11p in flor has been shown to be over expressed and mutated (Palecek et al, 2000; Ishigami et al, 2005; Fidalgo et al., 2006).

Palecek et al. (2000) demonstrated that up-regulation of Flo11p is sufficient to induce both invasive and filamentous growth in *S. cerevisiae* and also flo11Δ mutant

cells do not form chains or invade agar (Lambrechts et al., 1996; Lo and Dranginis 1998). It has been speculated by Ishigami et al., 2005 that the hydrophobicity of Flo11p may be due to conformational change of the protein in the presence of ethanol but not glucose, an analogy to the conformational change that occurs in Als5p on adherence (Rauceo et al., 2004).

(e) <u>Problems associated with treatment of candidiasis</u>

(i) Biofilm formation in *Candida albicans*: Biofilms are a collection of micro-organisms (both bacteria and fungi) and non-microbial particles that form well-defined cellular communities encased in a polysaccharide matrix (Chandra et al., 2001). Biofilms as protective niches act as barriers to medications and outside stresses for the microorganisms, where they can create a source of persistent infection by being a 'safe supply store house'. Biofilms are the most common mode of bacterial growth in nature and are also important in clinical infections, especially due to the high antibiotic resistance associated with them. Chandra et al. (2001) showed that antifungal resistance of biofilm-grown cells increased in conjunction with biofilm formation, and that expression of agglutinin-like (*ALS*) genes was differentially regulated between planktonic and biofilm-grown cells. The *ALS* family has specifically been implicated in biofilm formation through the finding that expression of *ALS1* and other family members increases during biofilm development in vivo (Nobile and Mitchell, 2006). It has been shown that hyphal formation is important in *C. albicans* biofilm formation, based on ultrastructure, mutant analysis and expression profiling; and *ALS3* is also found to be up-regulated during hyphal morphogenesis (Nobile and Mitchell, 2006).

Biofilm formation in *C. albicans* proceeds through three distinct developmental phases, namely attachment and colonization; growth and proliferation to form anchorage; and hyphae formation together with extracellular matrix formation (Nobile and Mitchell, 2006, Chandra et al. 2001).

Candida biofilms are associated with indwelling medical devices (e.g., dental implants, catheters, heart valves, vascular bypass grafts, ocular lenses, artificial joints, and central nervous system shunts), which can act as substrates for biofilm growth. In a multi-center study of 427 consecutive patients with candidemia, the mortality rate for patients with catheter-related candidemia was found to be 41%, and 40% of the patients with microbial colonization of intravenous catheters were found to develop acute fungal infections, with consequences ranging from focal disease to severe sepsis and death (Nguyen et al., 1995).

Using the polymethylmethacrylate biofilm model, Chandra et al. (2001) showed that biofilm-grown *C. albicans* cells are highly resistant to antifungal agents such as fluconazole, nystatin, amphotericin B, and chlorhexidine, similar to reports for catheter-associated *C. albicans* biofilms.

(ii) <u>Resistance to antifungal drugs:</u> Clinical resistance is defined as persistence or progression of an infection despite appropriate antimicrobial therapy (White et al., 1998). There has been increase in resistance to anti-fungals due to increase in use of anti-fungals. The antifungals commonly used are the polyenes, azoles and morphines (Calderone, 2002). Nystatin is the first antifungal agent identified (1950 by Hazen and

Brown) and led to the finding of other polyenes such as amphotericin B, the gold standard to which all antifungals are compared (Calderone, 2002).

Except for 5-flucytosine, the antifungal drugs commonly used are directed directly or indirectly towards ergosterol in the fungal membrane which is involved in various cellular functions. Ergosterol is said to be important for the fluidity and integrity of the membrane and also for the proper function of many membrane-bound enzymes, including chitin synthase, which is important for cell division and growth (White et al., 1998). Polyenes such as amphotericin B and nystatin, are a class of amphipathic antifungal drugs that target membranes containing ergosterol, but not cholesterol containing membranes. These drugs are able to cause a leak in the membrane for ions such as potassium and thereby destroy the proton gradient across the membrane (White et al., 1998). Other drugs that interact with enzymes involve in the synthesis of ergosterol from squalene to mevalonate include the azoles, thiocarbamates, allylamines, and morphines. The azoles include imidazoles and triazoles that are directed against lanosterol demethylase. The pyridines and pyrimidines also inhibit lanosterol demethylase but used in agriculture and not medicine (White et al., 1998). The first azole, miconazole, was developed by Janssen and later fluconazole (Pfizer) and itraconazole (Janssen pharmaceutical Inc.) (Calderone, 2002).

Some factors that have been reported to be associated with resistance include: intrinsic resistance of endogenous strains, replacement with a more resistant strain/species, transient gene expression that renders an endogenous strain temporarily more resistant, genetic alterations that render an endogenous strain resistant, size and variation of a population, and phenotypic switching of cell types from yeast to hypha

(White et al., 1998). Many different types of mechanism are known to contribute to a drug-resistant phenotype in eukaryotes. The most frequent resistance mechanisms reported include reduction in the import of the drug into the cell, modification or degradation of the drug once it is inside the cell, changes in the interaction of the drug with the target enzymatic pathway, and an increased efflux of the drug from the cell (White et al., 1998; Calderone, 2002).

Although *C. albicans* biofilms, like bacterial biofilms, display extreme resistance to several antimicrobial agents, especially the azoles, both echinocandins and lipid formulations of amphotericin B are said to be effective against the *C. albicans* biofilms (Nobile and Mitchell, 2006).

(D) Amyloid Proteins

Amyloids are very self adhering and stable protein aggregates that bind Congo red dye (Cherny *et al*, 2005; Barhart and Chapman, 2006; Stathospulos *et al.*, 2004). There are ~20 proteins known to date that form amyloid protein aggregates and cause disease in mammals. Some of the diseases include Alzheimer's mediated by the Aβ protein, transmissible spongiform encephalopathies mediated by the prion protein, maturity onset diabetes mediated by amylin protein, Huntington's disease mediated by huntingtin protein, familial amyloidosis mediated by transthyretin, familial British dementia mediated by ABri amyloid peptide, and Parkinson's disease mediated by α-synuclein (Srinivasan et. al., 2003). In all these diseases there are no common amino acid sequence reported to be found in the causative protein or peptide, but there may be a common structural intermediate involved in the development of disease. These amyloids have

cross β-pleated sheets with the long axis of their fibrils being parallel to their helical axis and perpendicular to the β-strands (Srinivasan et. al., 2003; Nilsson, 2004). However, both parallel and anti-parallel β-sheet orientations have been found in amyloid fibrils. Typically amyloid fibrils are un-branched and bind diagnostic dyes such as Thioflavin T and Congo red (Eisert et al., 2006; Nilsson, 2004). The maturation of the fibrils is encouraged by the presence of 'seeds' or nucleate amyloid forms which are soluble β-aggregates (Srinivasan et. al., 2003). Seeds for Aβ, β2-microglobulin, and islet amyloid peptide have been reported to be able to form membrane pores as well (Srinivasan et. al., 2003).

There are some environmental conditions known to favor the formation of amyloid by proteins. Some of these factors are pH and concentration dependence which have been reported to play very significant roles in amyloid formations (Srinivasan et. al., 2003).

To ascertain that fibrils formed by a peptide or protein are amyloid and folded into the native β-sheet structure, the well established Thioflavin T and Congo Red binding assays are employed (Levine, H., 1995; Nilsson, 2004; Klunk et. al., 1999). When bound to the β-sheet structure network of amyloids, Congo Red shows an increase in its absorbance spectrum while Thioflavin T undergoes a red shift of its excitation spectrum that is at 450 nm and results in fluorescence emission at 482 nm (Nilsson, 2004; Klunk et. al., 1999).

In terms of morphological forms, four distinct morphological fibril forms are typically found formed by amyloid proteins in terms of the height, length and periodicity. These are small aggregated species, protofibrils, type I mature fibrils and type II mature

fibrils (Srinivasan et. al., 2003; Ding et. al., 1999; Stine et. al., 2003). The type I fibrils are known to have greater heights and more distinct periodicities than the type II which are smaller in height and segmented rather than having beaded appearance with less regular dislocations.

(a) **Biological relevance of amyloid proteins**: Although amyloid proteins have been associated with many disease conditions, some recent findings show that it is also an important physiological process that mediates survival of some organisms. Examples are the bacteria proteins curli and chaplin involved in biofilm formation, and in mammalian melanocytes, amyloid formation has been said to play an important role during the process of melanosome biogenesis (Berson et al., 2003; Cherny et al., 2005; Elliot et al, 2003).

(i) Curli proteins: Curli proteins are extra cellular proteins produced in gram negative *Escherichia coli* that has been demonstrated to form amyloid fibrils and function by mediating host adhesion, biofilm formation and cell invasion (Cherny et al., 2005; Wang et al., 2006; Elliot et al., 2003; Chapman et al., 2002). Curli fibers have been demonstrated to mediate internalization of bacteria by eukaryotic cells (Gophna et al., 2001).

(ii) Chaplin proteins: Chaplins are a group of eight (chpA - chpH) cell surface proteins expressed by *Streptomyces coelicolor* which is a filamentous bacterium known to differentiate by forming specialized spore-bearing aerial hyphae that grows into the air.

The chaplins have been demonstrated to be important in the formation of the aerial mycelium (Elliot et al., 2003). The chaplins have been shown to be amyloid proteins that resemble the hydrophobins of the filamentous fungi that are expressed during the formation of aerial hyphae and fruiting bodies (Elliot et al., 2003; Claessen et al., 2003).

(E) Circular Dichroism Spectroscopy

Circular Dichroism (CD) spectra are very sensitive to secondary structure of proteins and peptides, and are therefore employed to determine the fraction of alpha helix, beta sheet, turn, and un-ordered structures. The spectral wavelength is normally between 260nm to 180nm. CD spectroscopy measures the difference in absorbance of right- and left-handed circularly polarized light (Tamburro et al., 2004). Proteins/peptides are asymmetric or in asymmetric environments causing the circularly-polarized light rays to travel through the optically active medium with different velocities due to the different indices of refraction for right- and left-circularly polarized light called optical rotation. The chromophore is the peptide bond in this area.

(i) Importance of Circular dichroism: CD is employed for various uses including: a) ascertaining if a protein is well folded into secondary and tertiary structures. Each secondary structure gives rise to a characteristic shape and magnitude of CD signal; b) study the conformational stability of a protein under the environmental forces of heat, pH, and denaturants such as 2,2,2-trifluoro-ethanol, guanidine hydrochloride and urea; and c) to study the conformational changes that accompany protein-protein interactions (Wilson and Walker, 1996; Creighton, 1993).

To determine the secondary structure of a protein or peptide, the protein or peptide solution is scanned in the spectral wavelength region of 180-260nm, which is the far ultraviolet region. The signals observed in this region are due to the absorbance of the peptide bonds when they are regularly folded. Because of noise generation by sodium azide, imidazole, and DTT, they are not used in the Far-UV region. The approximate percentage contributed by each secondary structure to a CD spectrum can be obtained by various algorithm calculations the most widely used being the self consistent method, SELCON (Sreerama and Woody, 1993).

In studying the structure of proteins, the presence of different solvent systems do provide information about the structure of the protein molecule and also the roles of various stabilizing and destabilizing forces (Sundd *et al*., 2004, Timasheff, 1993). Organic solvents are known to denature proteins, and the products resulting from these interactions are not completely unfolded but possess a measure of conformation. By changing the solvent system employed, the protein structure can be manipulated to be more ordered or disordered relative to the native state. In general, partially unfolded states arise due to the various contributions of hydrophobic interactions, electrostatic interactions, and hydrogen bonds of the protein molecule and the solvent system. Although there are a wide variety of organic solvents that denature proteins, alcohols are the most widely used because of their good miscibilities with water. Alcohols in general have three distinct effects upon proteins and polypeptides: (i) the destruction of the rigid native protein structure, (ii) the induction or enhancement of helixes, (iii) and the dissolution of peptide aggregates (Sundd *et al*., 2004). Alcohols are known to stabilize helical structures (Nelson and Kallenbach, 1989) and that in turn destabilizes tertiary

structure (Fink and Painter, 1987). The ability of an alcohol to disrupt the internal folding of a protein increases with its chain length and hydrocarbon content, and follows the order trifluoroethanol > propanol > ethanol > methanol (Sundd *et al*., 2004). The destabilization of tertiary structure and stabilization of secondary structure may induce partial protein unfolding and result in intermediates referred to as O-states. An O-state is a stable intermediate on or off the normal pathway of protein folding. In some ways it is said to resemble a "molten globule-like" state of many proteins (Sundd *et al*., 2004). A molten globule-like state is a term used for a partially folded protein with considerable secondary structure, but with few or no fixed tertiary structural contacts. Studies involving such perturbations help us understand the protein-folding problems and also the nature and role of equilibrium intermediates (Sundd *et al*., 2004).

Thermal stability of a protein can be assessed using CD by following changes in the spectrum with increasing temperature. There are two ways of achieving this aim. The entire spectrum in the far- or near-UV CD region can be followed at a number of temperatures, or a single wavelength can be chosen, and the signal at that wavelength is then recorded continuously as the temperature is gradually increased. CD is often used to assess the degree to which solution pH, buffers, and additives such as sugars, amino acids or salts alter the thermal stability of proteins. Many proteins aggregate or precipitate quickly after they are unfolded ("melted"), making unfolding irreversible. If (and only if) the melting is fully reversible, then the melting temperature is directly related to the conformational stability of the protein, and the thermodynamic parameters of the protein folding can be extracted from the data (http;//www.ap-lab.com/Circular-dichroism.htm). A highly co-operative unfolding reaction indicates that the protein existed initially as a

compact, well-folded structure, while a very gradual, non-cooperative melting reaction indicates that the protein existed initially as a very flexible, partially unfolded protein or as a heterogeneous population of folded structures.

CD information in the far-UV can be used to determine if a protein is losing all of its secondary structure, part of its secondary structure, or simply undergoes a conformational change of the secondary structure at high temperatures. Sometimes, a protein that is unfolded can have a defined but completely different secondary structure from the native protein (http;//www.ap-lab.com/Circular-dichroism.htm).

The aromatic amino acids and disulfide bonds are responsible for signals used in determining the tertiary structure of a protein in the near ultraviolet region, 250-350nm. The three aromatic amino acids absorb differently in this region with phenylalanine absorbing between 250-270nm, tyrosine between 270-290nm, and tryptophan between 280-300nm. The absorbance due to disulfides occurs between 250-350nm but is weaker and shows a broader band than the aromatics. Just like the secondary structural signals, the absence of signal is an indication of an undefined tertiary structure. The effect of solvents like TFE and protein-protein interactions can also be studied in the near UV region, although signals in this region is less sensitive compared to that of the Far UV region.

(ii) Importance of studying Conformational switching: Changes in conformation of proteins have been shown to play important roles in various micro-organisms and protein function. These conformational changes can be monitored by circular dichroism. The transition from non-helical to helical structures in viral fusion proteins for example have

been found to promote fusion of the virus and their targeted cell membranes. Also helix to beta-strand switching in the amyloid and prion proteins have been implicated in Alzheimer's, scrapie and bovine spongiform encephalitis diseases (Worobee et. al., 1988, Carr and Kin, 1993; Bullough et. al., 1994; Yang et. al, 1994; Molinari, 1996; Chan et. al, 1997; Weissenhorn et. al., 1997; Baskakov et. al., 2000).

α-agglutinin has also been shown to undergo conformational switching which is related to activity. The switching of the protein is not reversible in the amyloid disease related proteins like Alzheimer's but is reversible in the case of α-agglutinin under certain biological conditions (Zhao et. al., 2001; Jackson et. al., 1999; Prusiner, 1991).

F. Objectives of Dissertation Research

Most of the studies involving adhesive functions of Als proteins have been at the gene level and few studies have been done on the purified protein mainly due to the difficulty of producing the proteins and loss of protein due to aggregation. Hoyer, 2001 and Sheppard et al., 2004 demonstrated the secondary structure of Als5p Ig and Ig-T, respectively. Since then no other work has been done on the structure of the protein. These investigations were aimed at first and foremost producing purified fragments of Als5 proteins (Ig-T & Ig-T-TR). Secondly, to study the secondary structure and function of the Tandem repeat region (TR). Thirdly, the environmental effects of pH, temperature, alcohol (2, 2, 2-tri-fluoroethanol) and salt (sodium chloride) on the conformational structure of the soluble purified Ig-T-TR proteins were studied to gain more insight into the possible structure, function and conformational behavior of the Als5 protein. Finally, the amyloid forming properties of Als5p were explored and the amyloid forming region was determined.

CHAPTER II

Purification and Biochemical characterization of Als5p,

& Structure and Function of the Repeat Region

ABSTRACT

Candida albicans colonizes sites with different pH values in the body: mouth (about 7), large intestines (about 8), vagina (about 4), and is able to survive under harsh pH conditions like pH 2 in the stomach. The Als family of cell wall proteins have been implicated in adhesion and pathogenesis. Als5p both mediates adhesion to ligands and fungal aggregation when expressed on the surface of the non-flocculent *Saccharomyces cerevisiae* cells at room temperature. Aggregation experiments using Als5p-expressing non-flocculent *S. cerevisiae* cells indicate that aggregation occurs over a broad pH range of 2-10 and temperatures 5^oC-65^oC. Consistent with these observations, soluble Als5 proteins aggregate at very low concentrations into multimers that are resistant to dissolution by pH, temperature, and to SDS and GuHCl treatments.

$Als5p^{1\text{-}431}$ and $Als5p^{1\text{-}664}$ were expressed in *S. cerevisiae*, and purified from cultured medium. The final concentration of the protein solution was kept below 0.5mg/ml in order to produce enough protein for these studies. Circular dichroism (CD) Far UV spectroscopy experiments on purified $Als5p^{1\text{-}664}$ protein in solution shows that the protein folded within a broad pH range of 3-9, not completely unfolded at 90^oC, refolded from 80^oC, and had multiple temperature transitions that were non-cooperative in nature. The high beta content, ease of aggregation, high temperature stability, ability to withstand harsh pH, and the resistance of aggregates to SDS and GuHCl treatments resembles the behavior of amyloid proteins. Also comparison of purified $Als5p^{1\text{-}431}$ and $Als5p^{1\text{-}664}$ led to deduction of the structure the repeat region (amino acid 432-664). The repeat region influences the structure of Als5p, adds flexibility to the protein ($Als5p^{1\text{-}664}$), and its presence increases Als5p binding to fibronectin coated beads based on cellular

aggregation experiments. Thus the repeat region of Als5p (TR) aids binding and gives conformational flexibility to the Als5 protein. The solution studies on Als5p$^{1\text{-}664}$ also show a protein with properties of an amyloid protein.

INTRODUCTION

Not all of the eight members of the *ALS* family exhibit adhesive function and the degree of adhesion also varies; for instance, loss of Als3p has been reported to affect *C. albicans* adhesion more than loss of Als1p (Hoyer, 2001; Zhao *et al*., 2001; Gaur and Klotz, 1997). Since colonization by *C. albicans* is initiated by the adhesion of the fungal cell surface to host tissue, it is important to characterize these adhesins that mediate the adhesion to host and other surfaces. Adhesion is very important also because it is the first step in fungal pathogenesis (Calderone, 2002; Alberti-Segui *et al*., 2004).

After the initial binding of the N-terminus of Als protein to a ligand such as fibronectin, the cells aggregate. Adhesion of the protein to a ligand triggers a change in the conformation of Als5p that propagates around the cell surface, forming ordered aggregation-competent regions (Rauceo *et al.*, 2004). These findings led to the hypothesis that the repeat region may be the major region were the conformational shift occurs, because the Ig-like region, known to be the substrate binding region (Sheppard et al., 2004), are globular with high probability of disulfide bonds. Thus the Ig-like region will have a less flexible structure to change conformation. There is a high probability of a change in the TR-region that will allow the whole protein to adapt a more hydrophobic conformation upon ligand induction that will increase adhesion. Also, repeat regions occur in all eight *ALS* genes, and Als1p, with 20 repeats, shows visible aggregates in culture while Als5p, with only 6 repeats, does not show such visible aggregates. This suggests that the repeat region may be playing a significant role in aggregation or adhesion and does not only increase the length of the protein to help present the Ig-like region for binding (Sheppard et al., 2004).

Saccharomyces cerevisiae expressing Als5p cells aggregate within a wide pH 2-10 (Gaur *et al.,* 1999) in the presence of ligand coated beads. Thus the adhesin must be stable (not denatured or destroyed) at those pH values, temperature, and salt concentration in order to function properly. To this end in these investigations, the environmental effect of pH, TFE, salt, and temperature on the conformational behavior of purified Als5p$^{1-664}$ was studied using far UV Circular Dichroism spectroscopy.

Protein conformational changes are important for certain biological activities to occur. For example, in viral fusion proteins transition from a non-helical to helical structure favors membrane fusion. Likewise α-helical to β-strand transformation in amyloid and prion proteins is associated with the diseases Alzheimer's and scrapie (Zhao *et al.*, 2001). CD can be employed to study the changes in secondary structure of a protein in response to the environmental effects of temperature, pH, solvent and salt (Tamburro *et al*., 2004). Far-UV can be used to determine if a protein is losing all of its secondary structure, part of its secondary structure, or simply undergoes a conformational change of the secondary structure at high temperatures.

Saccharomyces cerevisiae has been used often as a host for the production and secretion into liquid media of heterologous proteins. Examples of such proteins are cellobiohydrolase (molecular weight 200kDa) and haemagglutinin (molecular weight ~250kDa) (Jabbar and Nayak, 1987; Penttila et. al., 1988).

These investigations aimed to study the structure and role of the TR on Als5p binding as well as the solution behavior of Als5p$^{1-664}$. The secondary structure of the protein was determined by CD and function of the repeat region deduced from whole cell aggregation in the presence of ligand coated magnetic beads. These investigations

overcame the problem of purified protein aggregation. Having enough quantities of protein allowed solution studies of the repeat region (TR) to investigate the influence of the TR on the secondary structural conformation of Als5p$^{1-431}$. In studying the function of the repeat region, truncated versions of soluble Als5p (Als5p$^{1-664}$ and Als5p$^{1-431}$), and cell surface bound full length Als5p (with 0, 2, & 4 repeats) were employed (Rauceo et al., 2006).

MATERIALS & METHOD

Materials: Trifluoroethanol was purchased from Fluka. Sodium phosphate mono- and di-basics, ammonium sulfate and sodium chloride were purchased from Fisher. Dialysis tubing was purchased from Spectrum laboratories. Pre-cast gels were purchased from Cambrex. Ni-NTA resins were purchased from QIAGEN. Anti-V5 and Anti-His6x-peroxidase-conjugated antibodies were purchased from Invitrogen. Super Signal West Pico Stable Peroxide and Super Signal West Pico Lumino/Enhancer Solutions were purchased from Pierce. Ethanol and acetic acid were purchased from ARCO. CSM-ura, yeast nitrogen base, and peptone were purchased from BIO 101 systems. Quartz cuvettes were purchased from HELLMA and the AVIV spectropolarimeter model 215 instrument was used.

Als5p fragment purifications: The method is discussed in Rauceo *et al*., 2006. Briefly, non-flocculent *S. cerevisiae* cells transformed with the p-YES2.1/V5-His-TOPO vector containing the correct insert of *ALSS* sequence were induced by growth in csm-ura medium with galactose as the carbon source to grow. Cultures were harvested after four days at 30°C, and rpm of 175, centrifuged and supernatant kept on ice. The supernatant was filtered through a 0.22µm filter, and then concentrated 10-fold using a Millipore filtration apparatus with a molecular cutoff of 30kDa. The pH of the protein solution was adjusted using tris-base to pH7. The protein was bound to a pre-washed and equilibrated Ni-NTA column with wash buffer (50mM sodium phosphate, 300mM NaCl, 20mM imidazole, pH 7). Non-specific proteins and substances were removed from the column by washing again with wash buffer and then protein eluted with high concentration

imidazole elution buffer (50mM sodium phosphate, 300mM NaCl, 500mM imidazole, pH 7). Eluted fractions with tagged proteins were identified using dot-blot analysis, then pooled and dialysed exhaustively into 20mM sodium phosphate buffer pH 6.2 at 5^oC with stirring. The dialysis membrane had a molecular cutoff of 8,000 kDa. The pure protein solution was further concentrated by dialysis in the 20mM sodium phosphate buffer pH 6.2 with 10% polyethylene glycol with molecular weight of 35,000 (Sigma). The concentration of the protein was determined by A_{280nm}, were an OD of 1.00 corresponds to 1mg/ml protein (Rauceo et al, 2006).

Dot Blot Analysis of proteins: The method is discussed in Rauceo *et al.*, 2006. Briefly, protein samples were spotted onto nitrocellulose membranes and allowed to dry. The membranes were blocked with 1x Phosphate Buffered Saline (PBS; 10mM sodium phosphate, 137mM NaCl, 1.8mM potassium phosphate, 2.7mM KCl, pH7) with 5% milk for an hour. The membrane was then washed three times with PBS+0.1%Tween-20. The membranes were then probed with Anti-V5 or Anti-His6x-peroxidase-conjugated antibodies at a working concentration of 1:5000 for an hour and washed three times with PBS+0.1%Tween-20. The presence of protein was detected by incubating the membrane with equal amounts of Super Signal West Pico Stable Peroxide and Super Signal West Pico Lumino/Enhancer Solutions. The protein luminescence were detected by exposure to film in the dark and developed with X-OMAT developing apparatus.

Polyacrylamide Gel Electrophoresis: Als5p samples were electrophoresed on 4-20% pre-cast native and non-native gels both at room temperature and in the cold when

needed. Gels were stained with Coomasie Blue dye over-night at room temperature with slow shaking and then de-stained with 5% acetic acid solution with 10% ethanol. The molecular weights of the proteins were determined by running a molecular weight marker along side the proteins.

Western Blot Analysis: After electrophoresis, the proteins were transferred onto a nitrocellulose membrane from the gel. The proteins were then probed using the method described above for Dot-Blots (Rauceo et al., 2006).

Circular Dichroism Spectroscopy: The secondary structures of the proteins were determined by far UV spectra analysis of the purified authentic proteins between a wavelength of 190nm-250nm using a quartz cuvette of 1mm, 2mm or 10mm path length depending on the kind of experiment and the concentration of the protein solution. For lower protein concentrations (< 0.1mg/ml), a longer path length (10mm) is used. The spectra were averaged, baseline subtracted, and the secondary structural percentages obtained by the self-consistent method with the SELCON program (Sreerama & Woody, 1993) a program for secondary structure estimates using the raw data.

The graphs are smoothed using local smoothing polynomial regression and weights computed from the Gaussian density using the Sigma-plot software 2001 version.

Sigmoidal fitting of CD melting curves were computed using the Microcal Origin version 5 software that calculates the Boltzman sigmoidal fit. The formula used is:

$$\mathbf{y = A_1 - A_2 / \{1 + e^{(x_1 - xo)/(dx + A2)}\}}$$

where: x_o = initial x values; x_1 = final x values; dx = change in x

A_1=highest value; A_2= lowest value

Molar Ellipticities were calculated using the formula:

Molar Ellipticity (θ_m) = ellipticity(θ)/ (molar concentration)(aa)(L)(10)

Where: aa = number of amino acids & L = path length of light in cuvette

Adherence Assays: The method is discussed in Rauceo *et al*., 2006. Briefly, non-flocculent *S. cerevisiae* expressing Als5p were grown to late logarithmic phase in Yeast Peptone Galactose (YPGal) media solution at 30°C, rpm 175. Harvested cells from centrifugation were washed three times with Tris-EDTA (TE) buffer, pH7. Re-suspended cells in TE buffer were mixed with fibronectin-coated magnetic beads in a cell-to-bead ratio of 100:1, and incubated at the experimental temperature with gentle shaking for 30mins. The mixture was briefly re-suspended and then cells that were adhered to the magnetic beads were separated from non-adherent ones by using a Dynal magnetic separator. Non-adherent cells were pipetted out and the adherent ones washed three times with TE buffer while still inserted into magnetic separator. The bead-cell aggregates were then re-suspended in the TE buffer and samples placed on microscope slides, sealed, and examined under a Nikon Optiphot-2 microscope equipped with a Sony DK-500 camera.

RESULTS

(A) Als5 protein purification

Problems with purification: Prior to this study we knew that Als5p mediated aggregation at a wide temperature and pH range (FigureII 1) and we wanted to study the solution behavior of the protein to ascertain if it correlates with the surface protein aggregation. However, producing enough purified Als5p for Circular Dichroism studies was a problem. Very little protein was obtained and also the protein aggregated. To solve this problem tens of liters of yeast were cultured during the course of this study. Also, steps were taken to reduce contamination, degradation, and aggregation of the protein solution.

Problem with aggregation: Formation of aggregates by soluble purified Als5p was a major hurdle in these studies. The proteins are so aggregate-prone that concentrations of 0.5 mg/ml cannot be obtained without aggregates forming. The formation of aggregates causes a lot of noise in the CD data acquisition apart from loss of proteins. Because of these aggregates the proteins could not be run successfully on a gel prior to this project.

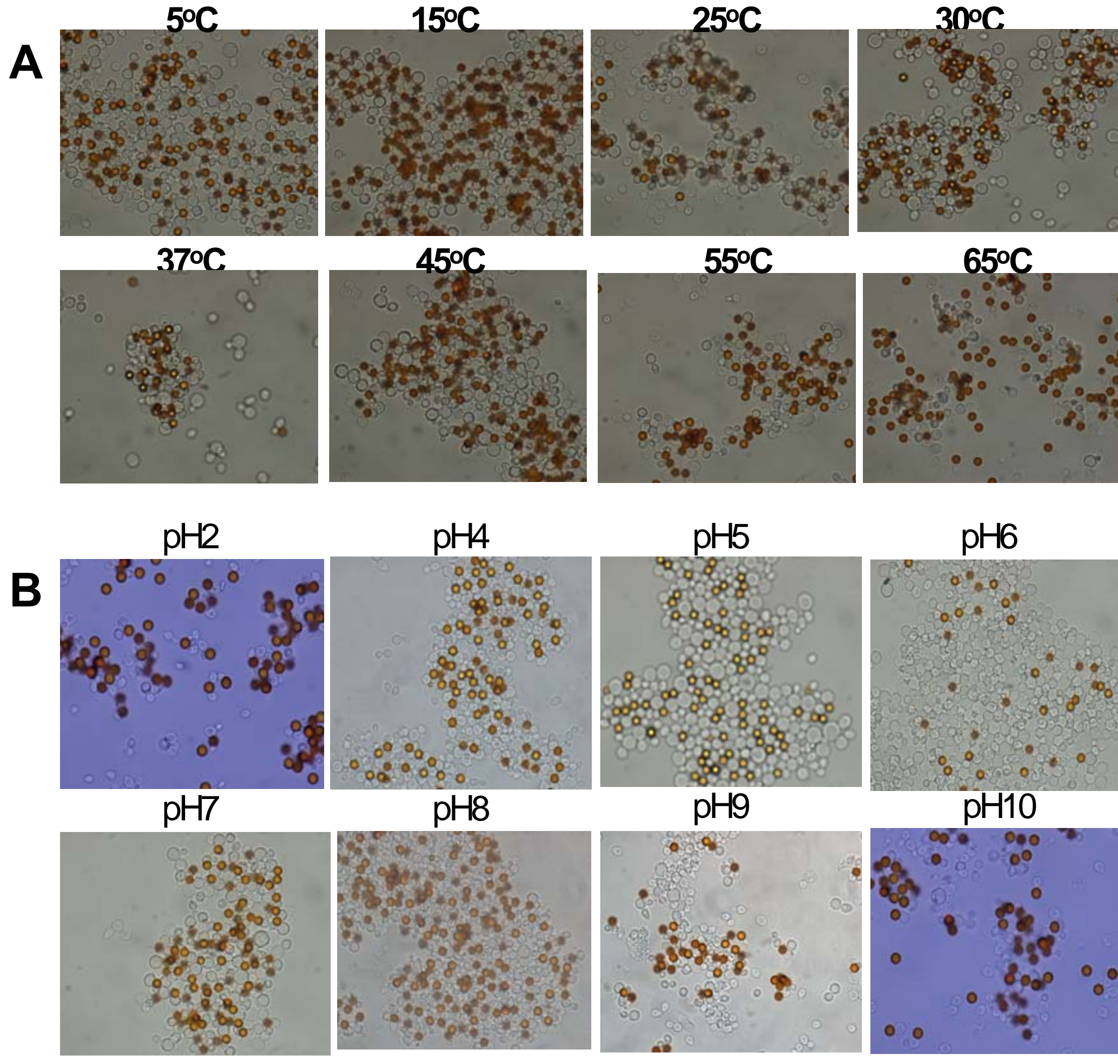
A combination of working on ice, centrifugation, filtration, low protein concentration and timing resulted in preparations at <0.5mg/ml solutions with few aggregates to give required CD signals.

Dynamic light scattering of the protein solution (Ig-T-TR) with aggregates present showed varied sizes and populations (data not shown). The results seemed inconsistent at first, because only one or two populations of aggregate sizes were expected. Later results from Western blot analysis (FigureII 2A) indicated that this is not the case, and that

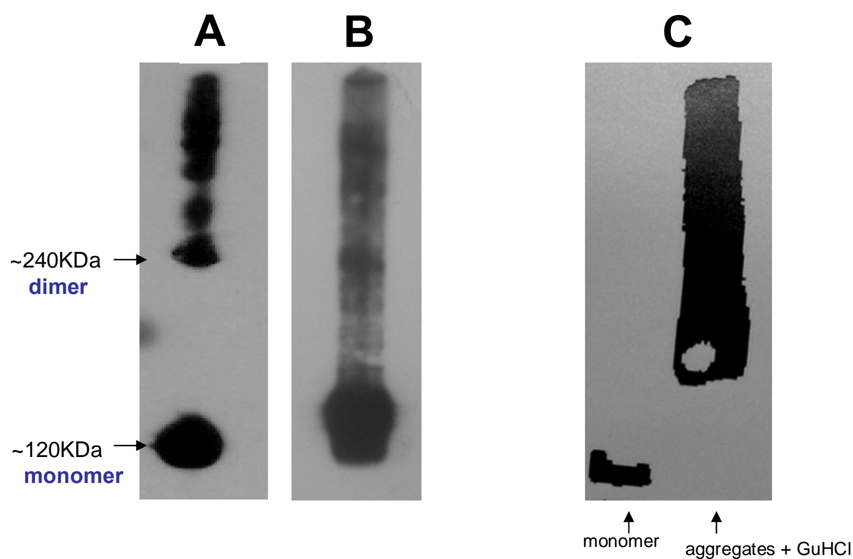
different aggregate populations are formed as multiple bands are observed (monomers and multimers). This was observed for both Ig-T and Ig-T-TR regions.

To determine the molecular sizes of the purified non-aggregated samples of Als5p$^{1-431}$ and Als5p$^{1-664}$ eletrophoresis was employed using a 4-20% SDS gel which was stained with Coomasie blue dye. The stained proteins show sizes of ~120kDa for Als5p$^{1-664}$ and ~60kDa for Als5p$^{1-431}$ (FigureII. 3) respectively. The proteins run at higher molecular weights than the expected calculated sizes of 48kDa for Ig-T and 72kDa for Ig-T-TR due to the presence of hydroxyl groups and the glycosylation of the TR region (Hoyer 2001, Rauceo et al, 2006).

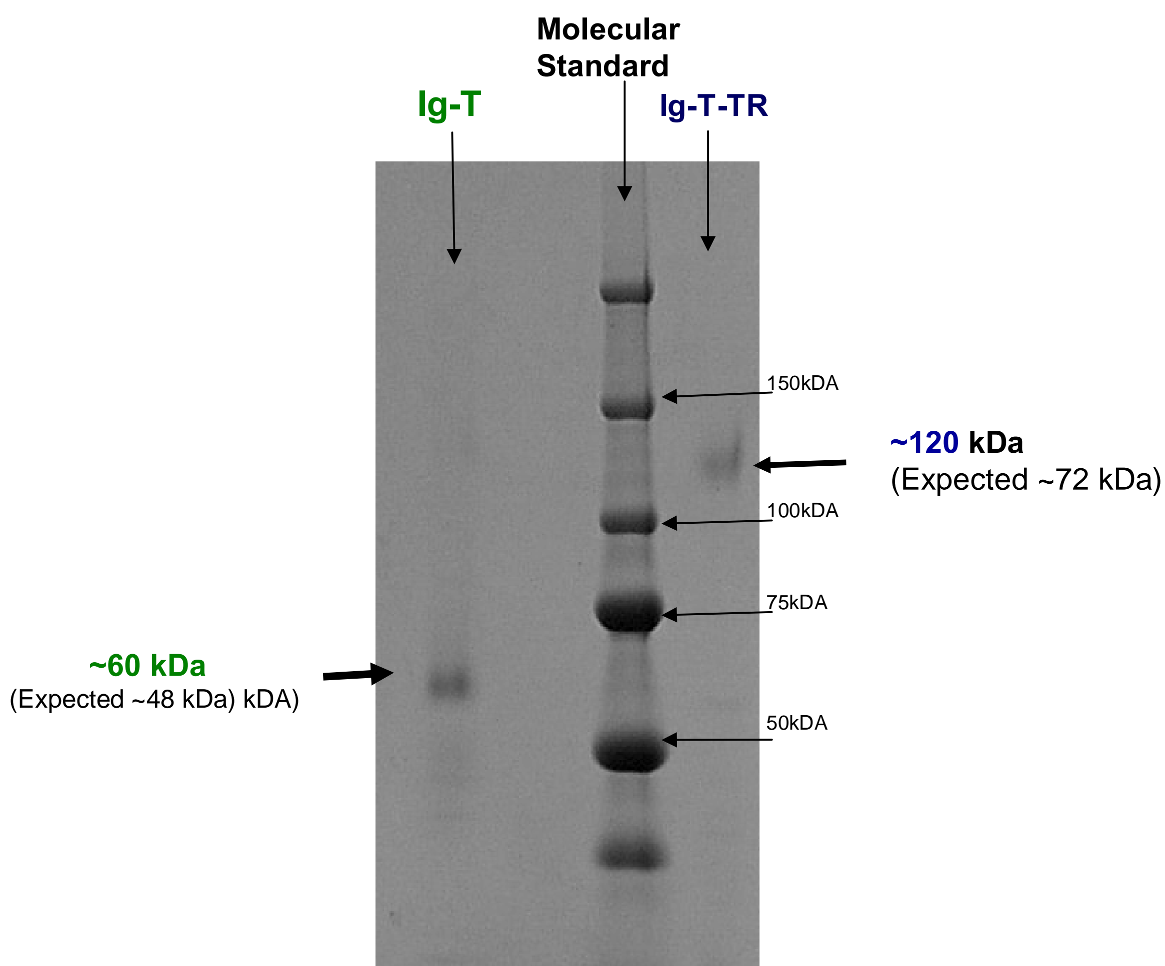
Resistance of aggregated Als5p to dissolution: Soluble purified Als5p$^{1-664}$ aggregates easily from solution at low concentration of ≤ 0.5mg/ml into multimers (FigureII 2A) during the process of protein concentration after purification. The presence of multiple bands means the presence of different sizes of aggregates of the protein. To test whether the aggregates can be dissolved in SDS, the aggregates were soaked in 10% SDS overnight (FigureII 2B). The addition of 10% SDS (FigureII 2B) to the protein aggregate did not dissolve the aggregates as the western blot analysis shows the presence of multiple bands corresponding to different sizes of the protein aggregates. The ease of aggregation and resistance of the aggregates to SDS are properties exhibited by amyloid proteins. The protein was also soaked in 6M GuHCl overnight and that did not dissolve the aggregates either (FigureII 2C).



FigureII 1. Temperature & pH effects on Als5p mediated aggregation: The degree of aggregation of non-flocculent *S. cereviseae*-expressing full length Als5p were studied at different temperatures (A) and pH values (B) in the presence of fibronectin coated beads acting as a ligand to induce aggregation.



FigureII 2. Western Blot analysis of purified Ig-T-TR aggregates: Purified Ig-T-TR (Als5p$^{1-664}$) solutions (>0.4mg/ml) containing aggregates were electrophoresed on (A) 4-20% non-SDS gradient gel; aggregated purified protein was incubated in 10% SDS (B) and 6M GuHCl (C) overnight and electrophoresed on 4-20% SDS gradient gel. Samples were transferred onto nitrocellulose membranes and probed with Anti-V5-HRP antibodies.



FigureII 3. Coomasie Blue Stained SDS PAGE gel Analysis of Als5 protein domains: Ni-NTA chromatography purified (0.2mg/ml) Als5p$^{1\text{-}431}$ (Ig-T) and Als5p$^{1\text{-}664}$ (Ig-T-TR) were concentrated, spun, and filtered before electrophoresis on 4-20% SDS gradient gel and then stained with coomasie blue for molecular weight determination. The slow migration of the Als5p$^{1\text{-}431}$ may be due to high contents of hydroxy amino acids, and the slower migration of Als5p$^{1\text{-}664}$ due to the presence of O-glycosylations in the repeat region. The expected molecular weights were obtained based on the primary sequence.

(B) Function of the Threonine Repeat Region (TR):

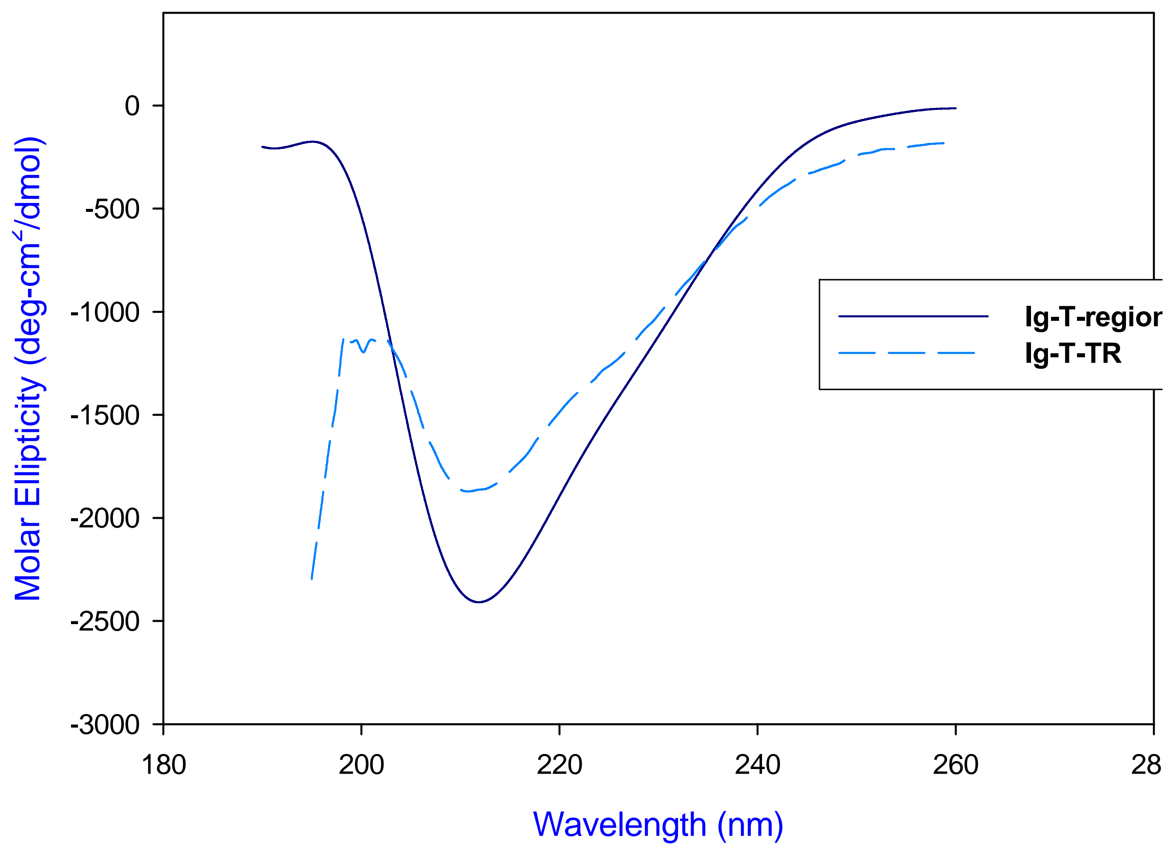
Secondary structure: To determine the secondary structure of the soluble Als5p domains, the far UV CD spectra of purified Als5p$^{1-431}$ (Ig-T) and Als5p$^{1-664}$ (Ig-T-TR) were obtained at 25°C between 195-250nm. Both proteins show minima at ~215nm, and Als5p$^{1-664}$ shows an extra shoulder around 227nm (FigureII 4). The inserted table gives the calculated secondary structural percentages of the proteins. It indicates that there are high percentages of beta sheets (38.1%) and unordered (37%) structures in Ig-T-TR but low helical content of 3.6%. Our results of the Ig-T secondary structure is in agreement with the Ig structure obtained by Hoyer (2001).

Effect of TFE on 36mer Repeat: In studying the structure of proteins, the stabilizing and destabilizing effects of certain solvents, such as the organic solvent TFE (2, 2, 2-trifluoroethanol) provide valuable insight about the forces involved in the structure of the protein (Sundd *et al*., 2003, Timasheff, 1993). Thus the effect of the presence of fluorinated organic solvent like TFE on the structure of Als5p was done to shed further light on the protein structure and conformation.

Since the TR alone could not be successfully expressed from yeast and a 216mer peptide corresponding to the six repeats could not also be synthesized, only one repeat sequence (36mer) was synthesized. The peptide was dissolved in phosphate buffer pH 5.6, and TFE was titrated to concentrations of 10, 20, 30, and 40%. The CD spectra obtained at 25°C shows a minimum for the 0% and 10% TFE peptide solutions at ~198nm. The wavelength of minimum ellipticity was ~200nm for the 20% TFE solution, ~202nm for the 30% TFE solution and ~204nm for the 40% TFE solution (FigureII 5).

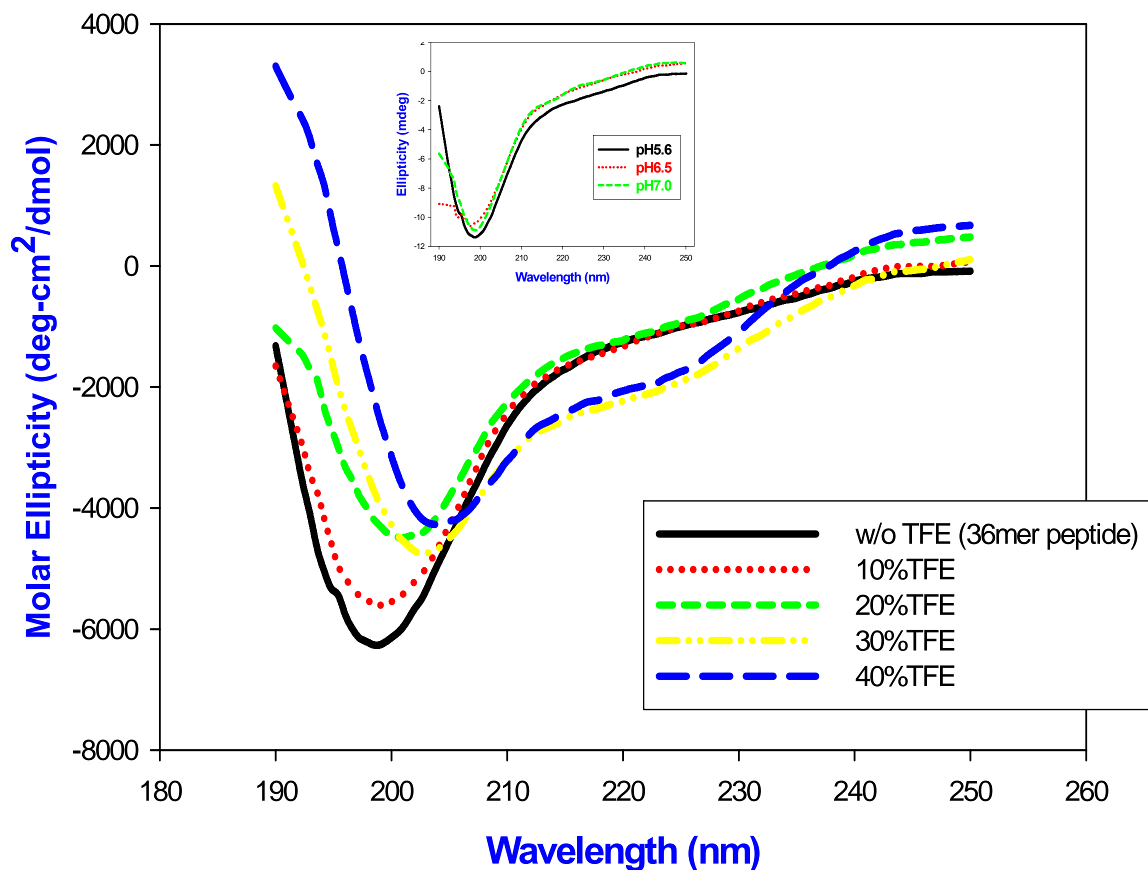
The inserted table shows that helix content increased from 7.7 ± 0.3% to 24.4 ± 0.3%, and beta sheet decreased from 29.5 ± 0.1% to 20 ± 0.3% as the TFE concentration was increased from 0% to 40%. Also the unordered structural content decreased from 39 ± 1.9% to 33.7 ± 0.7% as concentration of TFE was increased from 0% to 40%. Thus the conformation of the peptide was changed and hence is flexible.

FigureII 4. Far UV Circular Dichroism Spectroscopy Spectra of Als5p$^{1\text{-}431}$ (Ig-T) & Als5p$^{1\text{-}664}$ (Ig-T-TR): Spectra of purified proteins of Als5p in 20mM sodium phosphate buffer pH 6.2 were obtained in 1mm cuvettes at 25°C using the AVIV spectroscopy instrument between 260nm and 195nm. The percentages of secondary structure were calculated using the SELCON program on the raw data (Rauceo et al., 2006).



Secondary structure	% α-Helix	% β-sheet	% Turns	% Unordered
Ig (Hoyer, 2001)	4.0	60.0	21.0	23.0
Ig-T (Otoo, 2005)	3.9	40.6	21.9	33.7
Ig-T-TR (Otoo, 2005)	3.4	39.5	20.4	37.3

FigureII 5. Circular Dichroism spectra analysis of One Repeat Domain (36mer) of the TR region of Als5p: 2,2,2-Trifluoroethanol (TFE) was titrated to 36mer peptide in 10mM phosphate buffer pH 5.6 to 10, 20, 30, and 40% concentrations and the complete far UV spectra scan was measured at 25°C. The control had no TFE added. The insert shows the conformation of the protein at pH 5, 6.5 and 7 (no TFE). Secondary structural percentages were calculated using the SELCON program.

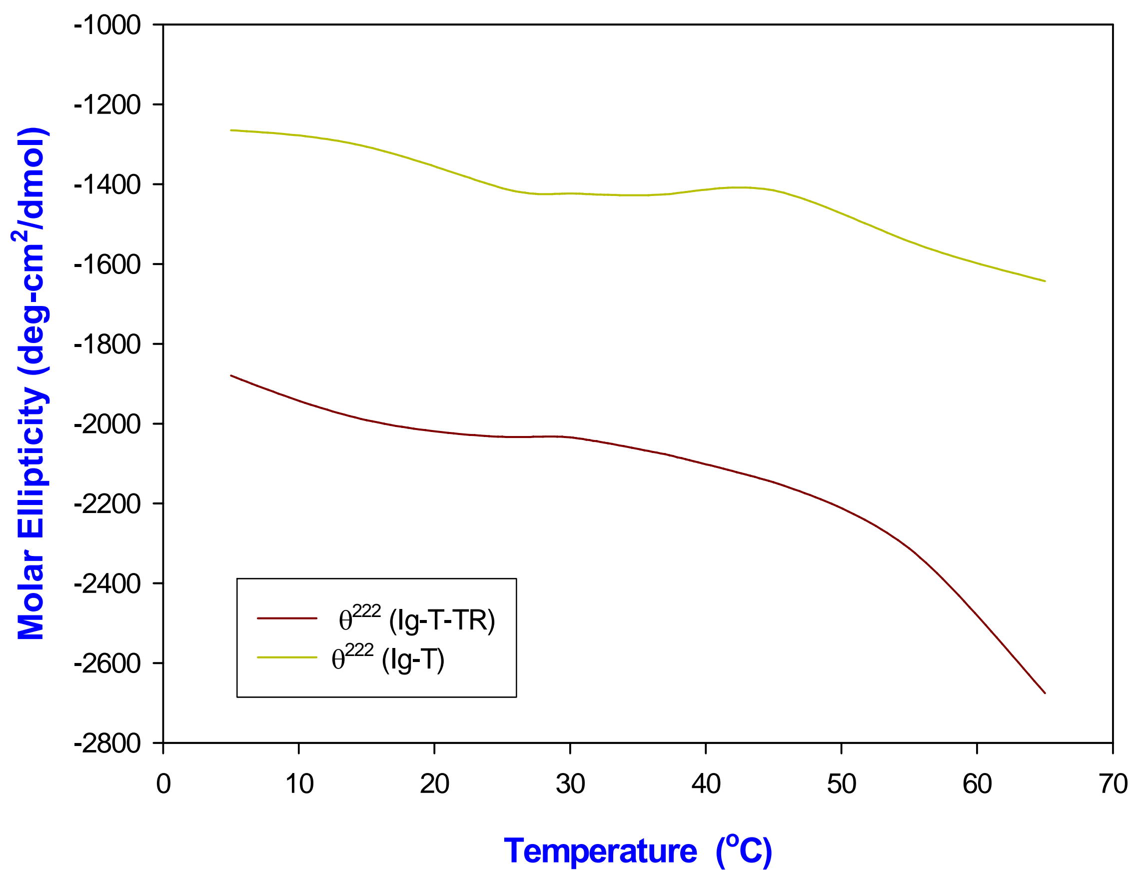


	Alpha-Helix	Beta-Sheets	Turns	Unordered
0% TFE	7.7 ± 0.3	29.5 ± 0.1	26.7 ± 0.9	39 ± 1.9
10% TFE	9.5 ± 0	28.7 ± 0.5	22.9 ± 1.4	37.7 ± 0.2
20%TFE	12.7 ± 1.6	24.1 ± 1.1	23.7 ± 1.7	37.2 ± 1.0
30% TFE	21 ± 0.8	22.3 ± 0.6	23.6 ± 0.2	32.8 ± 0.3
40% TFE	24.4 ± 0.3	20 ± 0.3	19.1 ± 0.1	33.7 ± 0.7

Sequence:
HNPTVT TTEFWS ESYATT ETITNG PEGTDS VIVREP

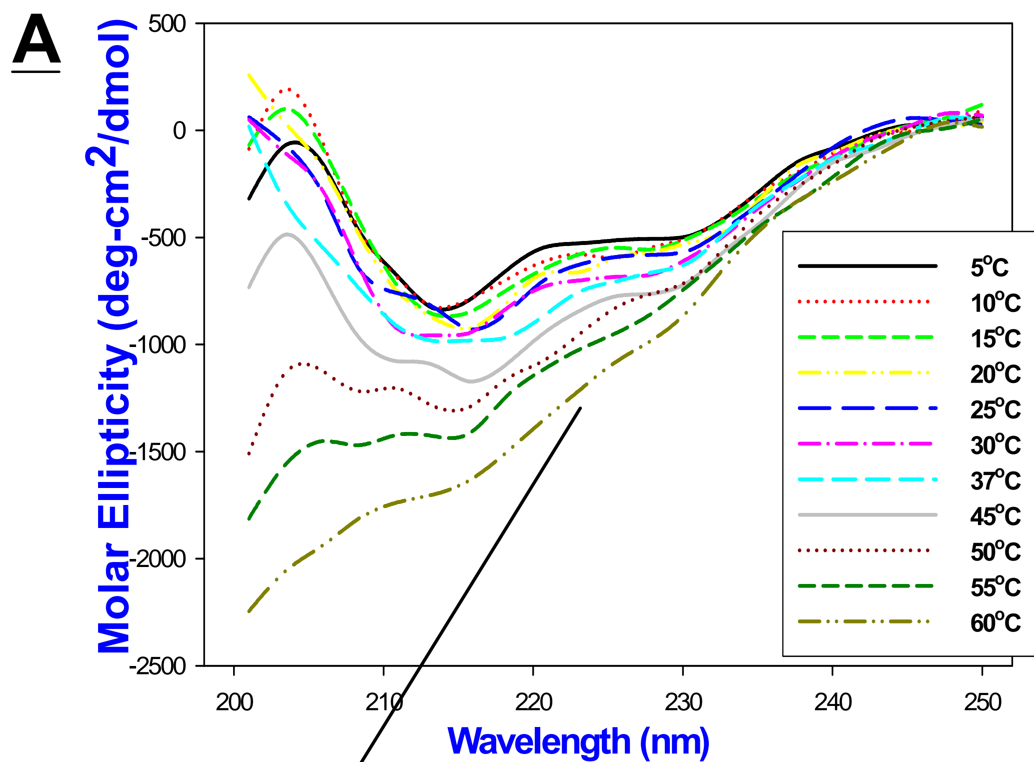
Temperature effect on Als5p$^{1-431}$ and Als5p$^{1-664}$: To determine whether TR adds flexibility to the protein, the far UV CD spectra were obtained for both Als5p$^{1-431}$ and Als5p$^{1-664}$ at 222nm with increasing temperature. FiguresII 6-8 show that the effect of temperature is more pronounced on Als5p$^{1-664}$ than Als5p$^{1-431}$. Thus Als5p$^{1-431}$ has a less flexible conformation than Als5p$^{1-664}$. Melting temperature (Tm) determination for Ig-T-TR shows that the melting of Ig-T-TR has multiple temperature transitions. The Tm values obtained varied from prep to prep except for the first melting transition around 13°C (FigureII 7) and a minor transition near 40°C (neither was present in Ig-T). The effect of temperature on Ig-T (FigureII 8) is minimal as compared to Ig-T-TR (FigureII 7). Thus the TR adds flexibility to the protein and not stability.

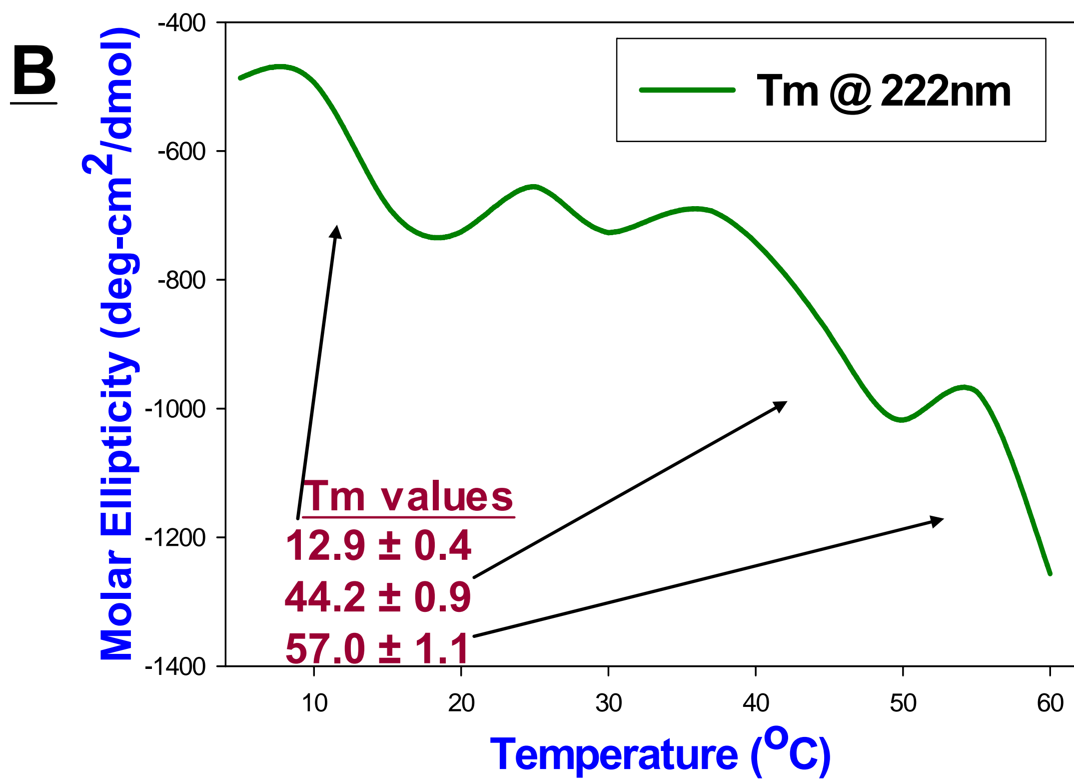
Presence of disulfide bonds in the Ig-T region: Als5p resists complete unfolding at a high temperature. This observation prompted us to ascertain whether the cysteine residues in the Ig-like form disulfide bonds that stabilize the protein. The soluble proteins were incubated in 10mM DTT and probed using electrophoresis (FigureII 9). The results show that the sample without DTT ran faster than the sample with DTT, implying that the protein plus DTT is less folded than the one without DTT. In other words, the protein without the DTT is more compact and that disulfide bonds are broken in the protein with added DTT. The presence of disulfide bonds in the Ig-like region makes it more stable, that is, the Ig-like region has a restricted conformation (FigureII 9). This reinforces our prediction that the TR region is more flexible than the Ig-T region.

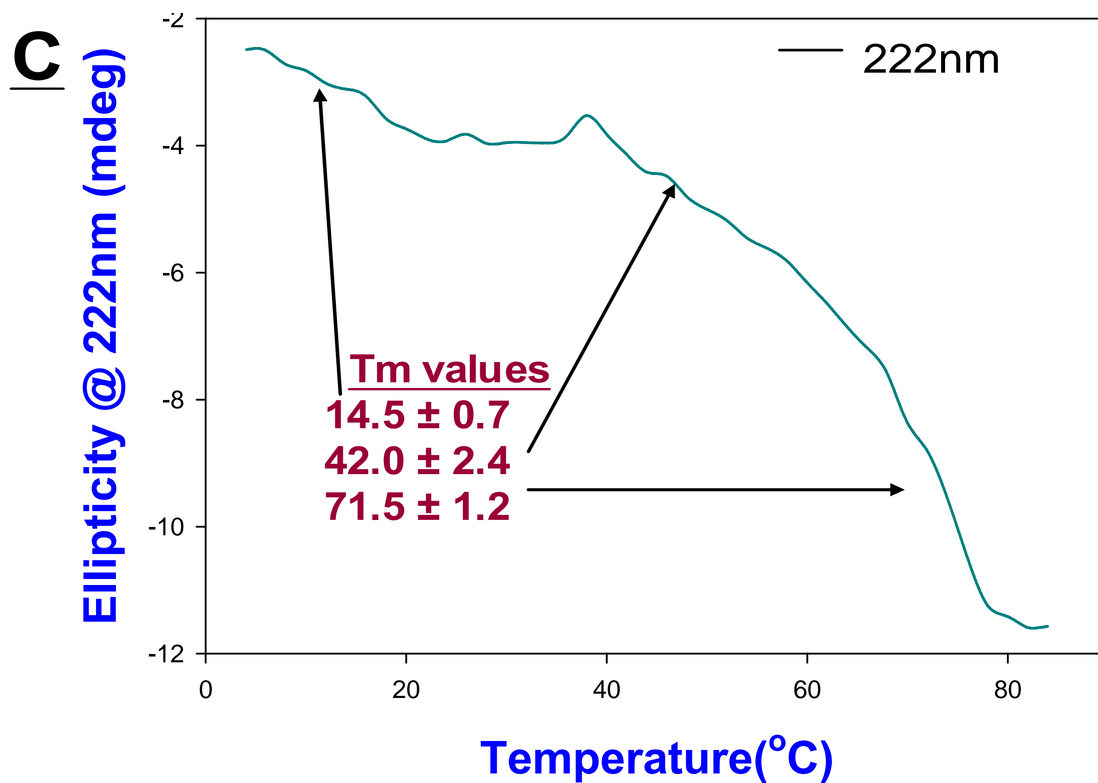


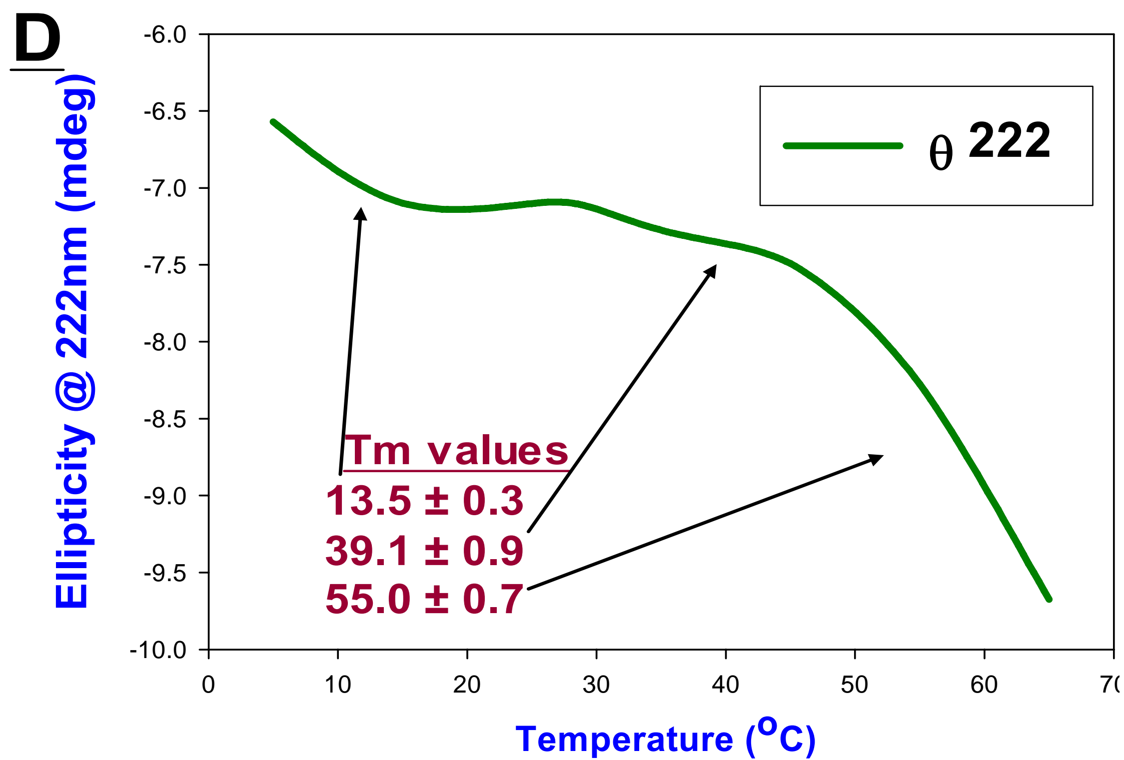
FigureII 6. Effect of Temperature on Als5p$^{1\text{-}431}$ (Ig-T) & Als5p$^{1\text{-}664}$ (Ig-T-TR): Far UV CD ellipticity values for Als5p$^{1\text{-}431}$ & Als5p$^{1\text{-}664}$ were obtained in 20mM sodium phosphate buffer pH 6.2 were acquired at 222nm as the proteins were heated from 5°C to 65°C at 2°C intervals/5min.

FigureII 7. CD spectra of Als5p$^{1-664}$ at different temperatures & Tm determination: (A) Complete Far-UV wavelength scans were obtained for Als5p$^{1-664}$ in 20mM sodium phosphate buffer pH 6.2 at a temperature range 5°C -60°C at 2°C intervals/5min. Solution was equilibrated at each temperature for 5mins. B shows the melting curve from A at 222nm. C & D are two separate melting temperature determinations for Ig-T-TR at 222nm with their estimated Tm values inserted (B, C & D). The melting temperatures were determined using the Boltzman sigmoidal curve fitting using Microcal version 5 software.

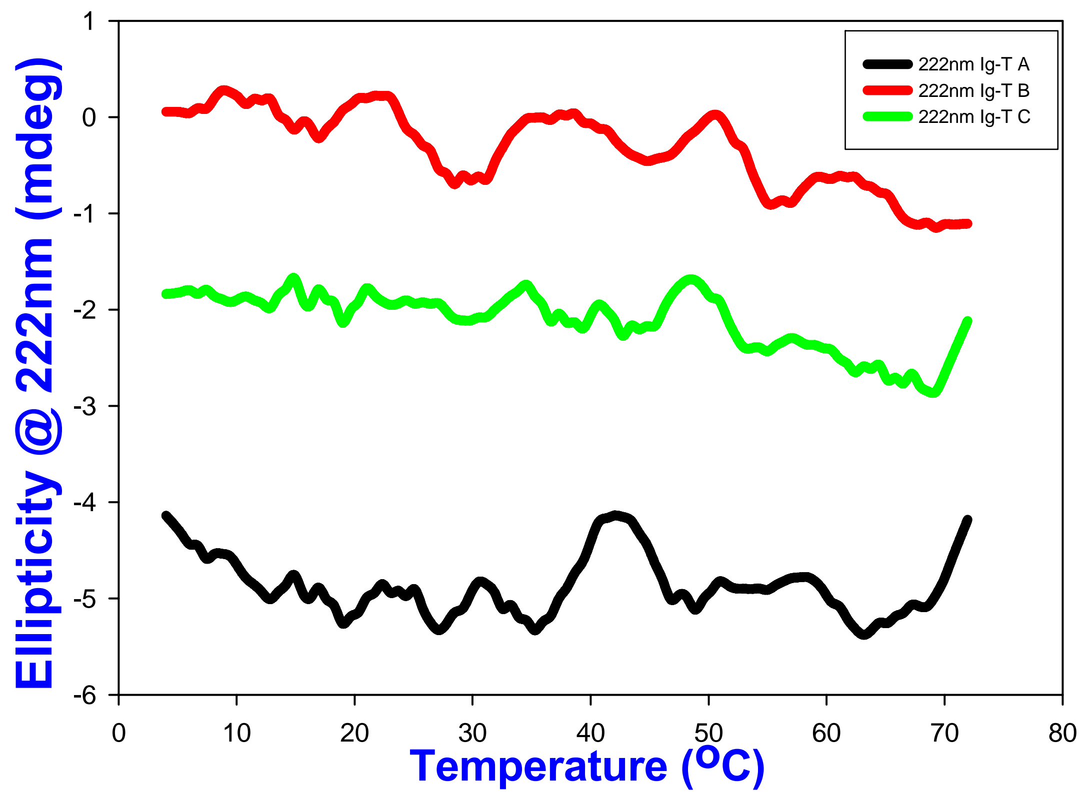

A
Molar Ellipticity (deg-cm^2/dmol)
500
0
-500
-1000
-1500
-2000
-2500
200
210
220
230
240
250
Wavelength (nm)
5°C
10°C
15°C
20°C
25°C
30°C
37°C
45°C
50°C
55°C
60°C

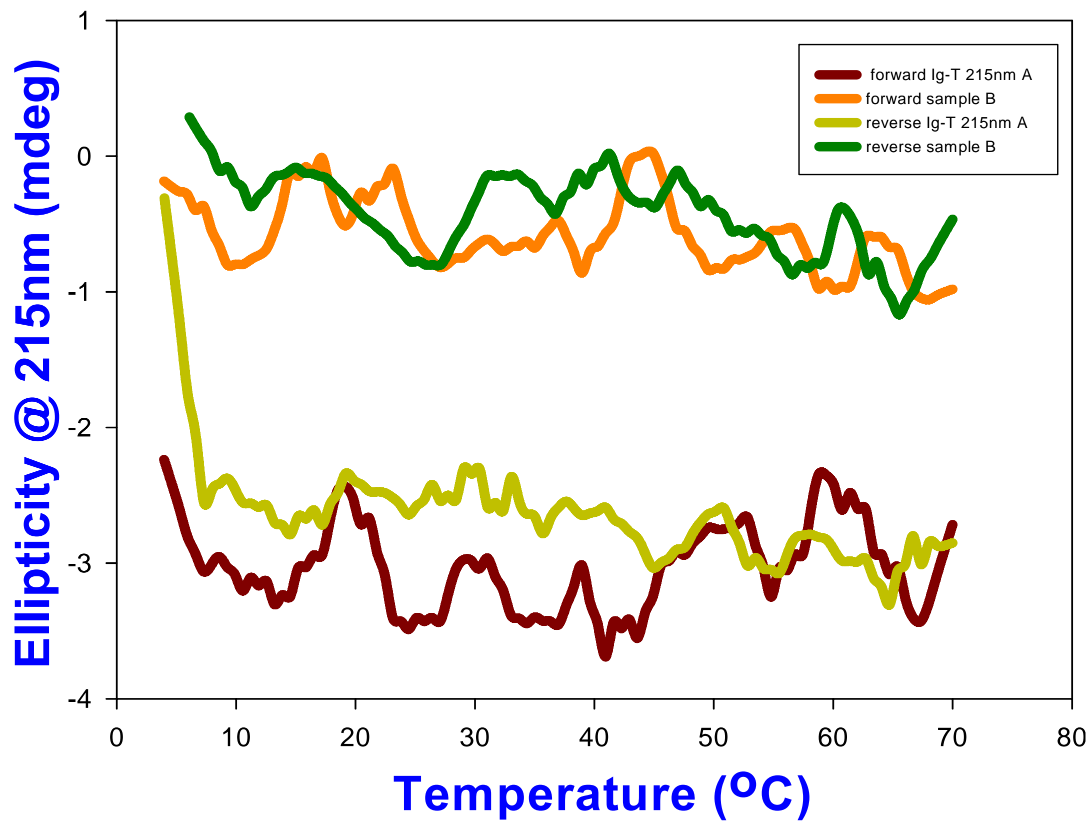

B
Molar Ellipticity (deg-cm^2/dmol)
-400
-600
-800
-1000
-1200
-1400
10
20
30
40
50
60
Temperature (°C)
Tm @ 222nm
Tm values
12.9 ± 0.4
44.2 ± 0.9
57.0 ± 1.1

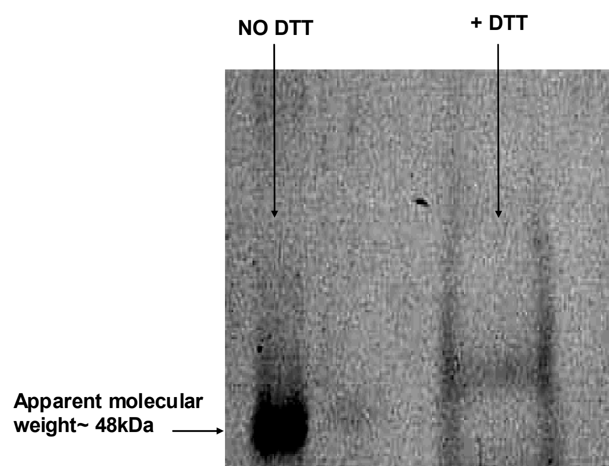

C
Ellipticity @ 222nm (mdeg)
Temperature(°C)
222nm
Tm values
14.5 ± 0.7
42.0 ± 2.4
71.5 ± 1.2
-2
-4
-6
-8
-10
-12
0
20
40
60
80


D
Ellipticity @ 222nm (mdeg)
Temperature (°C)
θ 222
Tm values
13.5 ± 0.3
39.1 ± 0.9
55.0 ± 0.7
-6.0
-6.5
-7.0
-7.5
-8.0
-8.5
-9.0
-9.5
-10.0
0
10
20
30
40
50
60

FigureII 8. Tm determination for Ig-T: The Circular Dichroism spectra for Ig-T were obtained for three different samples at 222nm (A) & for two different samples at 215nm (B) between 4°C to 70°C at 2°C intervals/5min. Samples were purified from different cultures and concentrations are different (0.18mg/ml to 0.22mg/ml).

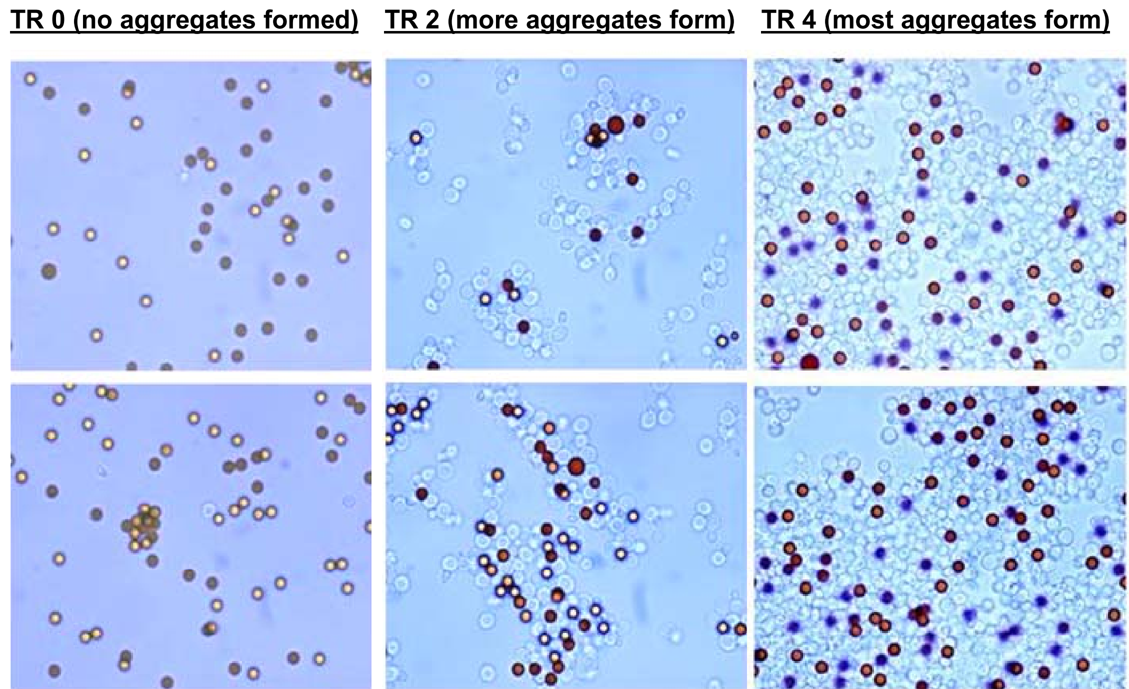

222nm Ig-T A
222nm Ig-T B
222nm Ig-T C
Ellipticity @ 222nm (mdeg)
Temperature (°C)
1
0
-1
-2
-3
-4
-5
-6
0
10
20
30
40
50
60
70
80


forward Ig-T 215nm A
forward sample B
reverse Ig-T 215nm A
reverse sample B
Ellipticity @ 215nm (mdeg)
Temperature (°C)
1
0
-1
-2
-3
-4
0
10
20
30
40
50
60
70
80



FigureII 9. Disulfide bond analysis of the Ig-T of Als5p: Western blot analysis of soluble Als5p$^{1\text{-}431}$ on 4-20% non-SDS gel and probed with anti-V5 antibody. 10µl of 0.38mg/ml protein solution with 10mM DTT (dithiothreotol) added to lane 2, lane 1 with no DTT added, and run at room temperature.

Effect of number of repeat regions on Als5p aggregation: Predicting that the TR region is not just there to increase the length of the protein, but plays a role in adhesion, we investigated the function of the TR region. Non-flocculent *S. cerevisiae* expressing Als5p with 0, 2 & 4 repeats were induced to aggregate in the presence of ligand coated beads. *S. cerevisiae* expressing Als5p without the Ig-T bind poorly to fibronectin coated beads. Aggregates increased as the number of repeats increased (FigureII 10) (Rauceo *et al*. 2006). The TR by itself mediates binding both to ligand and to self, although the binding is not as strong as Ig-T (data not shown).



FigureII 10. Effect of number of Repeats on Als5p Aggregation: *S. cerevisiae* expressing Als5p cells with 0, 2, or 4 repeats were cultured in rich media. The harvested cells were washed and induced to aggregate in the presence of fibronectin coated magnetic beads. Images were acquired under a microscope.

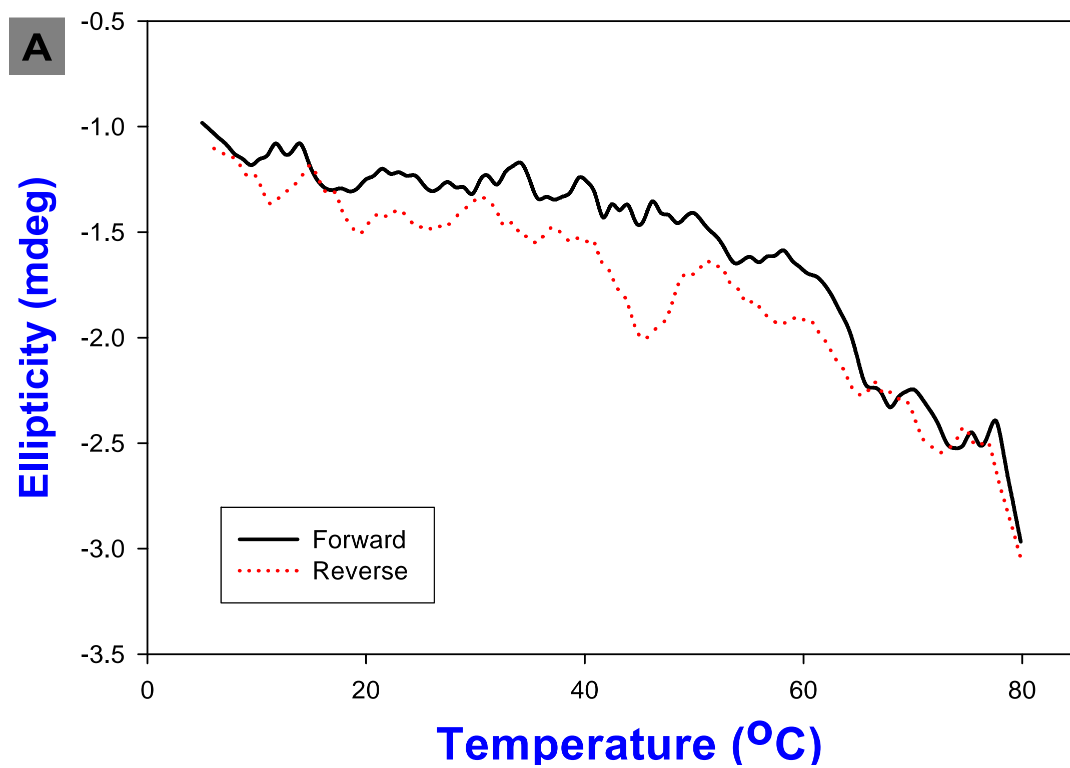
(C) Solution studies of Als5p:

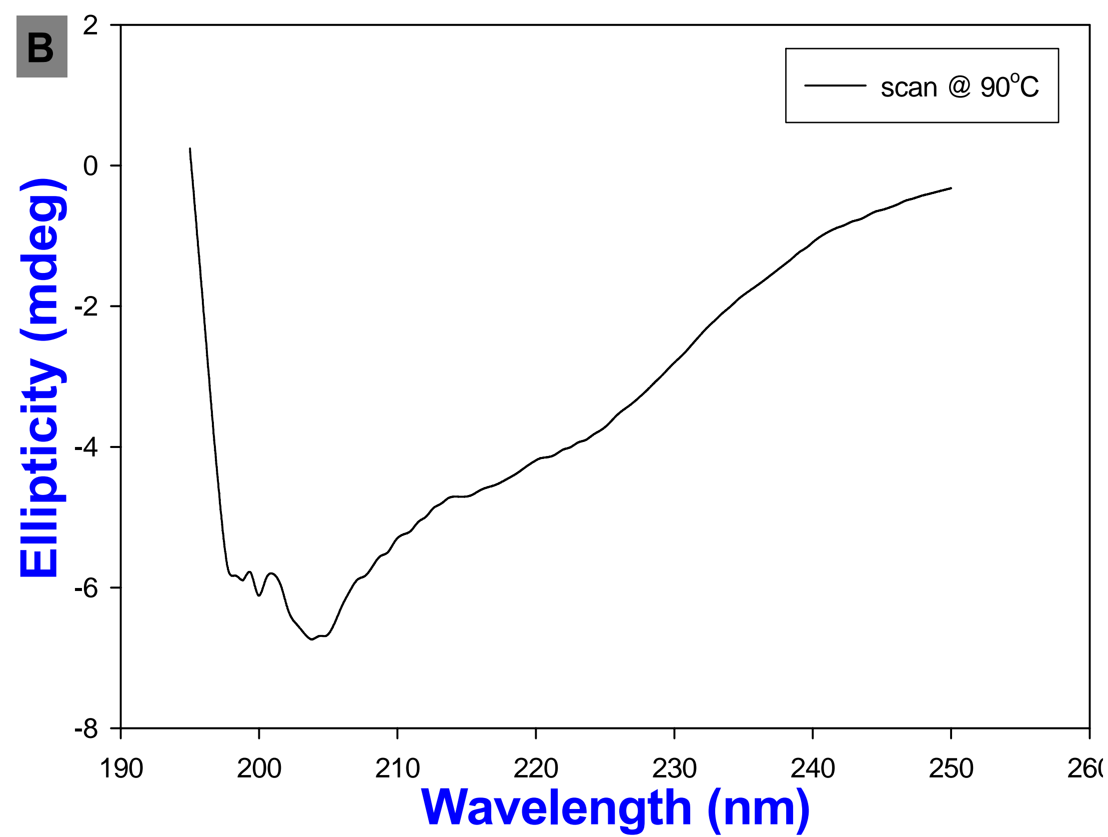
Temperature Stability of Als5p: Cellular aggregation experiments mediated by Als5p show that cells expressing the protein can aggregate cells at temperature between 5°C - 65°C and even aggregate after boiling (FigureII 1A, Rauceo *et al*., 2004). With this knowledge, we set out to study the stability of the protein in the presence of increasing temperatures. The Far UV CD of purified $Alsp^{1-664}$ shows that the purified soluble Als5 protein is able to refold from as high as 80°C (FigureII 11A) and the protein is not completely denatured at 90°C (FigureII 11B). The CD is not that of a random coil and has good signals at 222nm and 215nm. The CD also shows a minimum at about 206nm which does not conform to a completely unfolded structure. These observations imply that there is a significant amount of secondary structure still present and that the protein is not completely denatured.

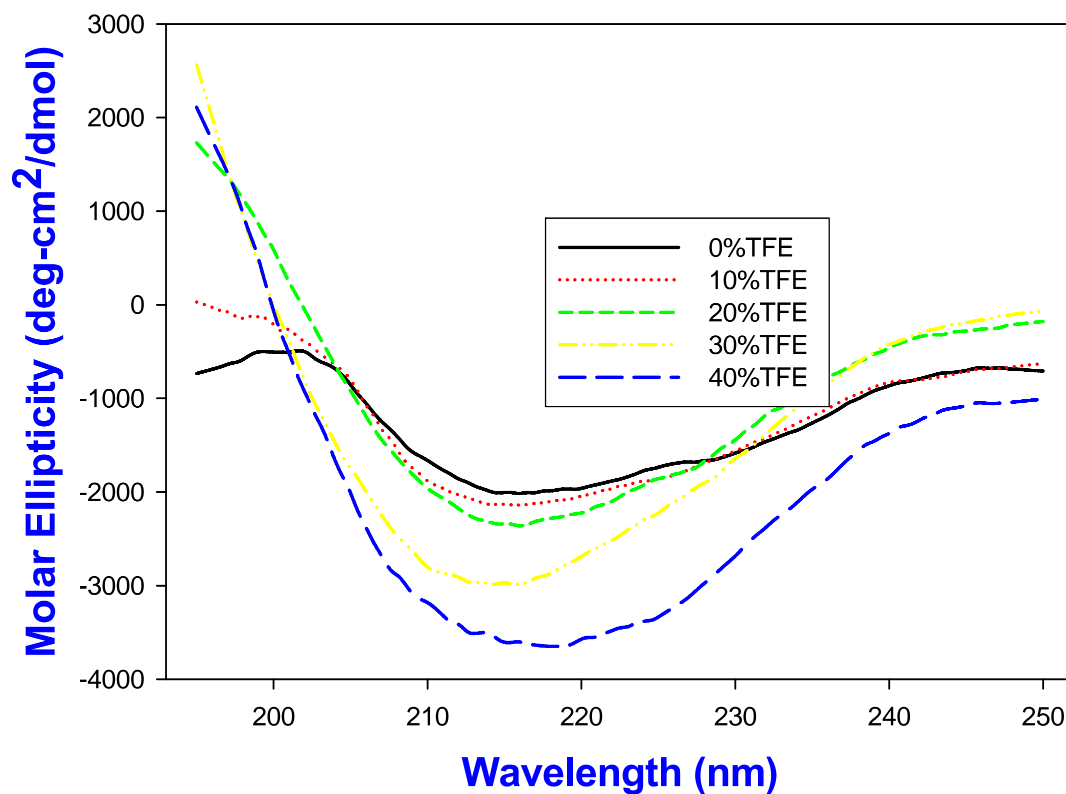
Effect of TFE and NaCl on Als5p conformation: To study the environmental effects of alcohol and salt on the soluble protein conformation, we employed CD spectroscopy. The Far UV spectra were obtained for increasing concentration of TFE from 10% to 40%, titrated against the protein solution (FigureII 12). The curve shows common minima at about 215nm for all the concentrations, and a shoulder around 225nm. The minima intensity increased in general with increasing concentration of TFE. In FigureII 14, sodium chloride was titrated to soluble purified $Als5p^{1-664}$ to concentrations of 50-200mM at 50mM increments and the Far UV spectra was obtained at 25°C within a wavelength of 195nm-250nm. The spectra show that the structure of the protein is not significantly affected by NaCl within this concentration range (FigureII 14). Figure 13 shows the

calculated secondary structural percentages at the various TFE concentrations. The table shows that the percentage of regular helix increases slightly while distorted helix content decreases with increasing TFE concentration, and percentage of distorted beta sheets increases while regular beta sheets decreases. The percentages of turns and unordered structures did not change significantly. A sigmoidal curve fit of the molar ellipticity values at 222nm and 215nm with increasing TFE concentrations show a critical TFE concentration around 20% (FiguresII 13A&B). The data seem to show that the alpha helix secondary structure of the protein is increased through local chain interactions in the presence of TFE and the tertiary interactions of the protein may be perturbed, as shown by variations at 230-250nm.

FigureII 11. Effect of High Temperature on Als5p$^{1-664}$: The Far-UV wavelength scans were obtained for Als5p$^{1-664}$ in 20mM sodium phosphate buffer pH 6.2. Spectrum (A) is **t**he forward and reverse scan at 222nm between 5°C and 80°C at 1°C interval with 5mins incubation at each temperature and spectrum (B) is the complete wavelength scan at 90°C. The protein was equilibrated for 20 minutes before acquisition of spectrum.

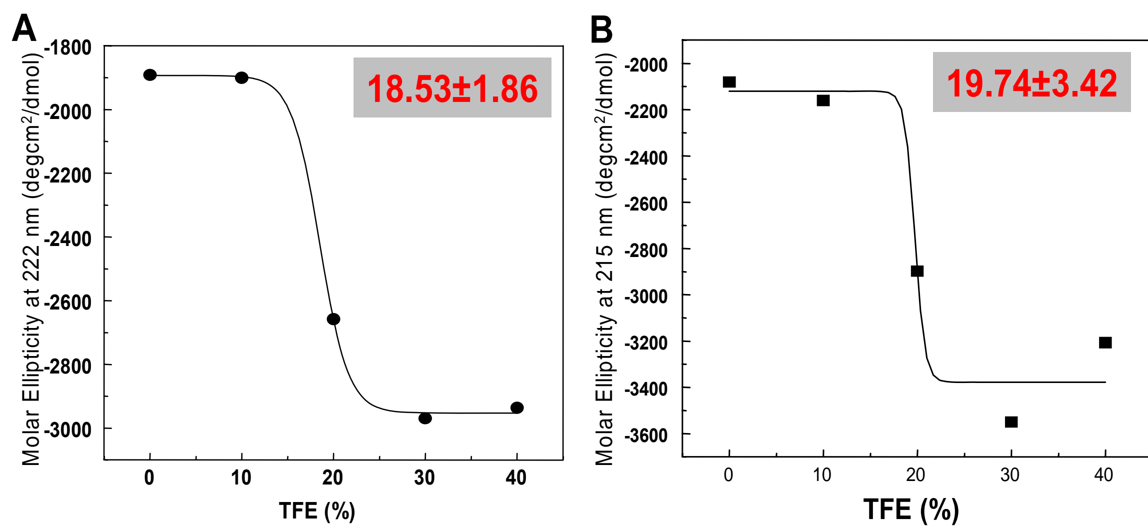

A
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
0
20
40
60
80
Ellipticity (mdeg)
Temperature (OC)
Forward
Reverse


B
2
0
-2
-4
-6
-8
190
200
210
220
230
240
250
Ellipticity (mdeg)
Wavelength (nm)
scan @ 90oC

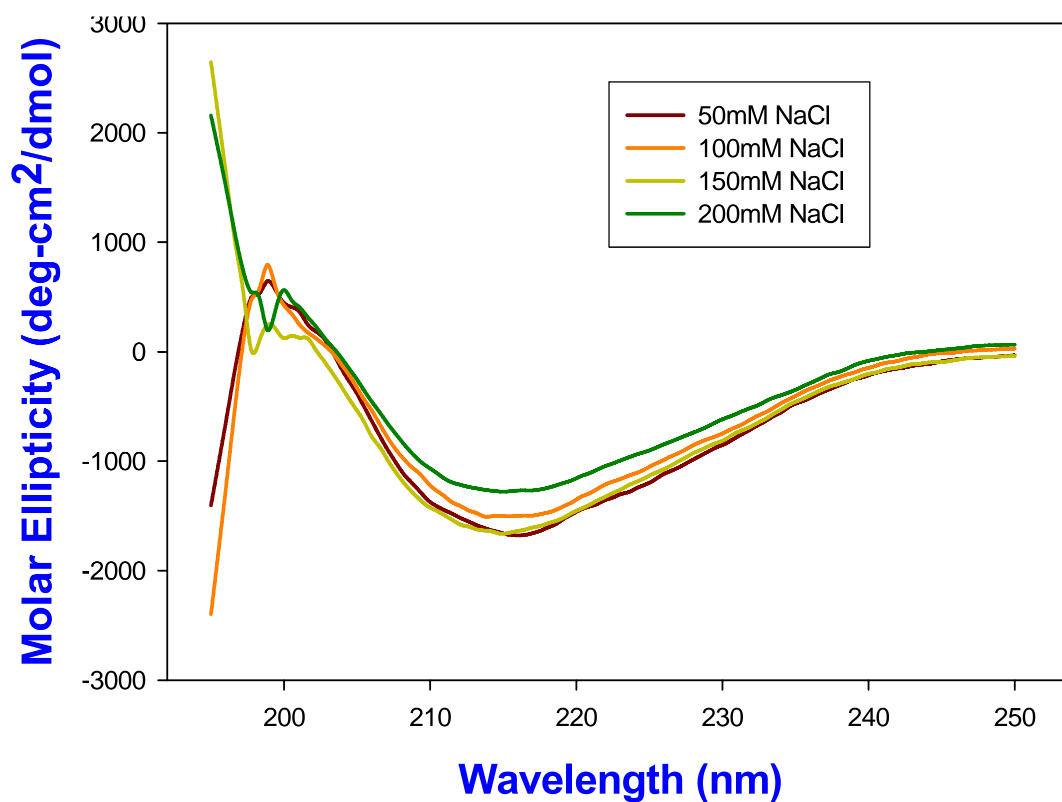


FigureII 12. Effect of TFE on Als5p$^{1-664}$ conformation: The Far UV CD spectra of purified Als5p$^{1-664}$ were acquired at 25°C between 250-195nm wavelengths in the presence of 0, 10, 20, 30 and 40% TFE concentrations titrated against the protein.

FigureII 13. Als5p$^{1-664}$ TFE sigmoidal fit: The molar ellipticity values from the TFE experiment in figureII 12 were plotted against the percentage TFE and the Boltzman sigmoidal fit was calculated for at (A) 222nm and (B) 215nm. The inserted table shows the secondary structural percentages at 0, 10, 20, 30 and 40% TFE concentrations that were estimated with SELCON3, using 56 reference proteins (I-Basis 10). Averages and standard deviations were calculated for the estimated values.



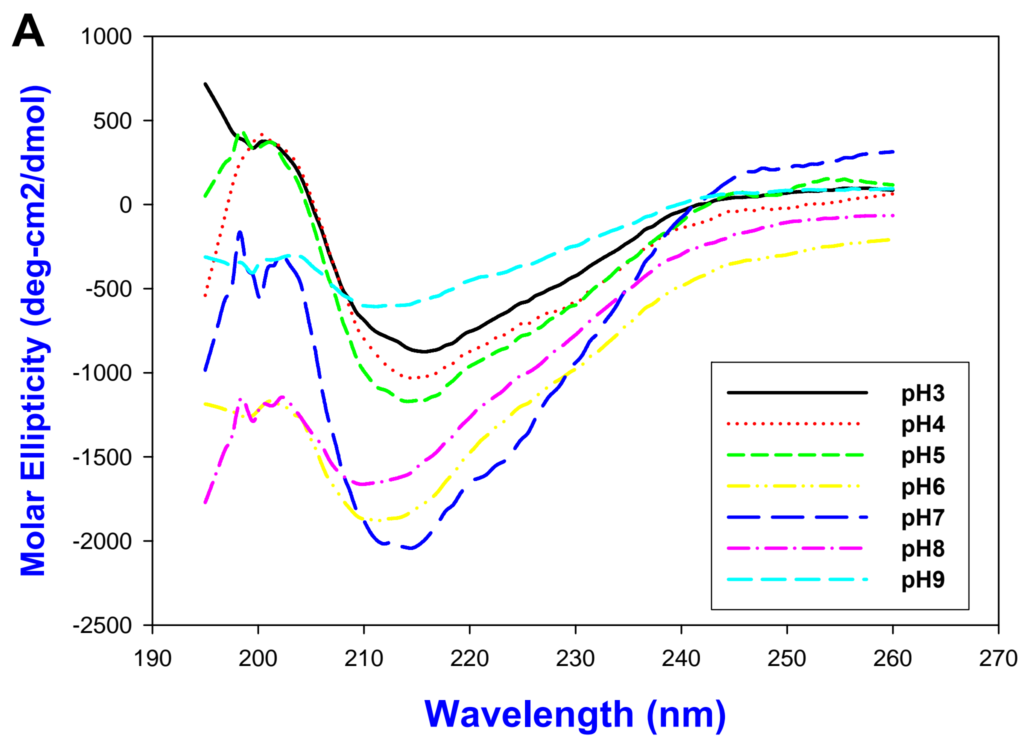
	Alpha Helix		α-helix	Beta sheets		β-sheet	Turns	Unordered
% TFE	r	d	Total	r	d	Total		
0%	0.3±0.1	7.4±1.3	7.7	32.3±2.6	11.7±1.7	44	22.7±1.1	31.1±0.6
10%	0.4±0	7.3±0.9	7.7	30.2±2.5	11.2±1.4	41.4	22.7±0.9	31.6±0.1
20%	0.8±0.1	8.1±0.3	8.9	31.8±1.1	11.4±0.8	43.2	22.0±0.1	31.5±0.7
30%	3.4±0.2	5.8±1.5	8.2	19.1±1.0	18.2±0.2	37.3	22.2±0.4	30.9±1.4
40%	3.5±0	6.7±0	10.2	23.0±0	18.0±0	41	23.7±0.9	30.0±0.4

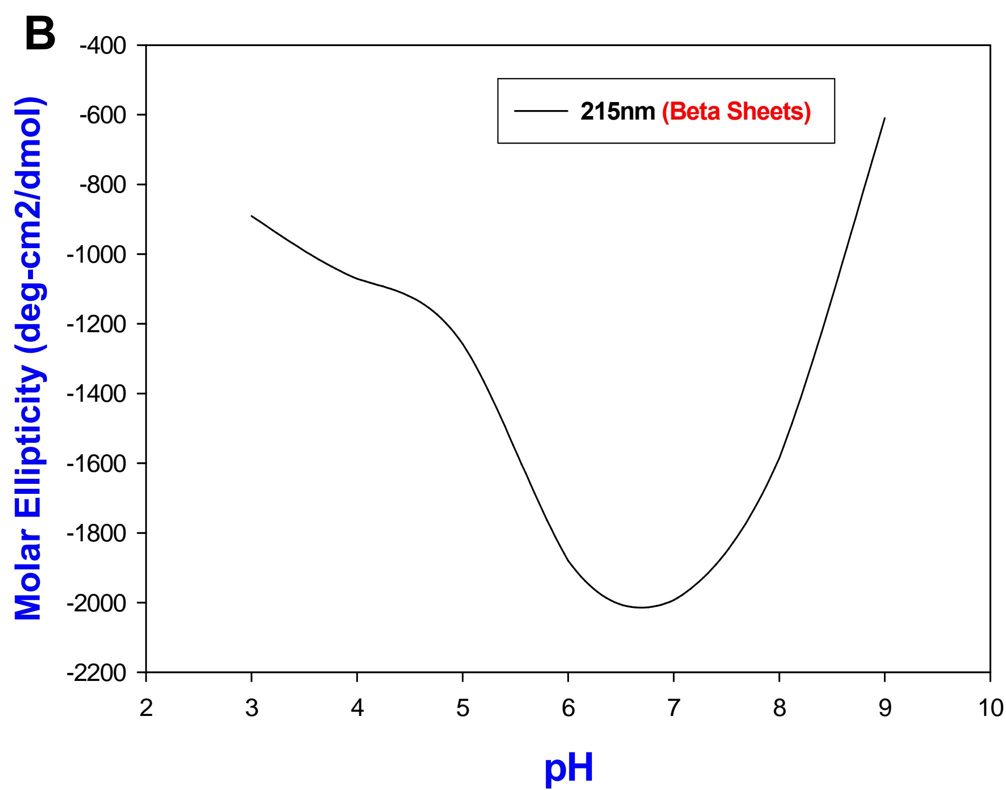


FigureII 14. Effect of NaCl on Als5p$^{1-664}$ conformation: The Far UV CD spectra of purified Als5p$^{1-664}$ were acquired at 25°C between 250-195nm wavelengths in the presence of 50, 100, 150 and 200mM NaCl concentrations titrated against the protein.

Effect of pH on Als5p structure: With the observation that Als5p expressing cells aggregate in a broad and extreme range of pH values, we studied the conformation of the soluble purified protein at different pH values. Far UV CD spectra analysis of soluble Als5p shows that the conformation of the protein is influenced by pH (FigureII 15A). At pH 3 and 9 there is an increase in un-ordered structure, which is an indication of some denaturation. FigureII 15B shows that as pH is gradually increased from 3 to ~7, the beta sheet content increases accordingly, but decreases from ~pH 7 on with increasing pH. The results seem to show that there is more ordered secondary structure formation near the physiological pH of 7. The results also imply that at the extreme pH values of 3 and 9 studied the secondary structure of the protein is weakened (perturbed) but not completely unfolded.

FigureII 15. Far UV CD analysis of Als5p$^{1\text{-}664}$ secondary structure at different pH values: Purified Als5p$^{1\text{-}664}$ (0.175mg/ml) was dialysed against 20mM sodium phosphate buffer with pH 3-9. Wavelength scan of these solutions were obtained between 260nm to 195nm at 25°C (A). An average of three scans were obtained, baseline subtracted and the molar ellipticities were calculated based on the concentration of the solution after dialysis. B is data from A plotted at 215nm to see the effect of pH on the protein conformation.


A
Molar Ellipticity (deg-cm2/dmol)
Wavelength (nm)
pH3
pH4
pH5
pH6
pH7
pH8
pH9


B
Molar Ellipticity (deg-cm2/dmol)
pH
215nm (Beta Sheets)

DISCUSSION

There has been extensive research on Als gene regulation in *Candida albicans*. The current studies investigate the physical and biochemical nature of Als5p protein fragments (Ig-T and Ig-T-TR) and the effect of environmental forces on the conformation of the Als5 protein Ig-T-TR. The yeast has been and continues to be a problem for indwelling devices (Eloy *et al*., 2006).

Als5p fragment purifications: Aggregation mediated by Als5p occurs in a broad pH range (2-10) and temperature (5^oC-65^oC) (Gaur and Klotz, 1997; FigureII 1). In studying the correlation between surface expressed Als5 proteins and the soluble solution proteins, we investigated the biochemical comformational behavior of the protein at different environmental conditions of pH, temperature, salt and alcohol. The major hurdle in all laboratories has been the quantity and aggregation of the purified soluble protein. Due to the difficulty in obtaining a good quantity of soluble Als protein there are few previous biochemical studies of the protein. This work solved the problem of obtaining sufficient quantity of protein and also minimized formation of protein aggregates.

TR influences Als5p conformation and increases binding to substrate: The structure of the substrate binding region has been modeled and this research confirms the structure, as the percentages of the secondary structures are similar (FigureII 4) (Hoyer, 2001; Sheppard *et al*., 2004). These studies further look at the next domain, TR, which most people believe has no function. To attain this goal, both Ig-T-region and Ig-T-TR domains were purified from cultures, and the 36mer peptide (one repeat sequence) was

chemically synthesized. The TR-region alone, although constructed, was not successfully secreted into media (Rauceo et al, 2006). For this reason CD data of the TR-region was obtained as spectral difference in Ig-T and Ig-T-TR CD data. There are repeat regions in the other Als proteins and therefore knowledge gained from these studies on the structure and function of the repeat region can be extrapolated.

Circular Dichroism spectra investigations of the secondary structure of the domains indicate that the Ig-T-TR-region has about the same beta-sheets and alpha helix contents as Ig-T but a little higher unordered structure (FigureII 4, page 52). The high unordered structure may give more flexibility to the protein and more likely cause an increase in aggregate formation as it may become more hydrophobic upon conformational induction. This will help the protein to adapt different structural states and conformations. The presence of an additional shoulder around 227nm in the Ig-T-TR, which may be due to the presence of tyrosine residues, indicates a difference in the tertiary structure of the Ig-T and Ig-T-TR proteins. Thus the presence of the TR causes a change in the conformation of the protein.

Secondary structural percentages from a single repeat of 36 amino acids show a high percentage of unordered structure, 39 ± 1.9% and a low percentage of alpha helix 7.7 ± 0.3%. The high unordered structure predictably will allow more flexibility of the protein to different tertiary states and conformations. Indeed, the melting temperature experiments from FiguresII 6-8 indicate that the TR adds flexibility to the protein, as Ig-T melting spectra shows no major change (FigureII 7) as compared to Ig-T-TR melting spectra that showed multiple conformational changes (FigureII 7). The flexibility of the

protein will allow a change in conformation of the protein to a more hydrophobic protein that further increases the adhesiveness of the protein as proposed by Rauceo et al. (2004).

When Als1p expressing cells are cultured in solution, aggregates are visible, but expression of Als5p, with a lower number of repeats, does not induce such visible aggregates. This and the fact that a truncated version of the protein without the Ig-like region still has residual binding (Rauceo et al., 2006) indicates a possible function for the repeat region in binding, although it was thought to just add length to the protein for presentation of the binding region. The reduction in aggregation in the absence of TR and the increase in aggregation with increase in TR length indicates that the TR aids (supports) binding and this may be due to changes in the tertiary structure of the protein when TR is present. The melting temperature experiments supports the idea that the presence of the TR gives more flexibility and hence more conformational change to the protein as the Ig-T-TR spectra have more conformational change than the Ig-T spectra (FiguresII 6, 7 & 8). The Ig-T is more conformationally restricted because of the presence of disulfide bonds (FigureII 9), and therefore the TR will be the region were the highest conformational shift occurs as the stalk region is more heavily glycosylated than the TR (Hoyer, 2001; Rauceo et al., 2004). The presence of Cys residues, all in the Ig-like region, had been speculated to form disulfide bonds but was not confirmed. This work shows that indeed the Cys residues in the Ig-like region form disulfide bonds and these bonds strengthen the protein structure.

Als5p is resistant to complete denaturation by pH and temperature: CD **s**pectroscopy shows that the protein is resistant to complete denaturation by temperature

and pH. The multiple melting transitions shown by the protein imply that the unfolding of the protein is not well co-ordinated (less co-operative), and that the different domains may be unfolding differently. If there were good co-operativity in the transitions, we would have seen a clear and wide two state transition from say a high beta content to a denatured protein with high unordered structure, with reduced beta sheets (lost secondary structures). Thus the TR, T and Ig-like regions may be all unfolding at different temperatures, and even maybe the Ig-like region may not be unfolding significantly compared to the rest of the protein due to the presence of disulfide bonds in the Ig-like region (FigureII 9).

Effect of TFE and salt on Als5p$^{1\text{-}664}$: A study of the environmental effects of the alcohol TFE and salt NaCl on the structure of the protein, shows that TFE interferes with the bonds involved in regular beta sheet formation but promote the formation of regular alpha helices in the protein. Regular beta sheets are converted to distorted beta sheets and helices with increasing TFE concentrations, from a critical TFE concentration of about 20% (FiguresII 12&13). Turns and unordered secondary structures are not significantly affected by TFE. Overall, the TFE experiment seems to indicate a subtle switch of secondary structure from beta sheets towards alpha helix.

Salt has a marked effect on the stability, structure and function of many globular proteins due to its ability to influence the electrostatic interactions (Kumar et al., 2004). In hexokinase A for example, at 100mM concentration NaCl has a destabilization effect on the protein as it also does on the leucine zipper of GCN4 (Kumar et al., 2004). However, NaCl does not seem to have significant influence on the bonding activities

involve in the protein folding in Als5p (FigureII 14). Thus electrostatic interactions may not be playing a significant role in the folding of the protein.

pH effect on Als5p$^{1\text{-}664}$: The conformation of the protein is affected by pH but the protein is not completely unfolded at extreme pHs of 3 and 9, thus the core of the protein is still folded. It can be deduced that hydrogen bonds may be playing some role in the secondary or tertiary structure of the folding of the protein but not a strong role in the conformational stability of the protein core. Hydrophobic interactions may be playing the most significant role as the protein has a high beta sheet composition and easily aggregates.

Summary: Als5p was successfully purified (overcoming the problem of the protein aggregation) and the TR has been shown to aid the Ig-T region to better bind to substrate (fibronectin) and self (self-self aggregation). The TR has been proposed to also affect the conformation of Als5p as well as add flexibility to the adhesin to better adapt to function in different environments. These investigations show that purified Als5p aggregates at low protein concentrations into multimers which are resistant to dissolution by SDS and GuHCl. It also shows that the protein is very resilient (stable) to temperature as confirmed by temperature experiments that, the Als5p$^{1\text{-}664}$ is not denatured completely (only partially unfolded) at a high temperature of 90°C (close to boiling), and refolds from 80°C. Also the soluble protein is robust at the extreme pHs of 3 and 9 tested. All the above behaviors of the protein are characteristics exhibited by amyloid proteins which have been characterized extensively. Thus Als5p may be amyloid.

CHAPTER III

Amyloidicity of Als5p & Function of the Threonine Conserved Region

ABSTRACT

C. albicans expresses a family of cell wall proteins called agglutinin like sequence proteins (Als) which have been implicated in adhesion, invasion and biofilm formation. The physical and biochemical properties of cell-bound Als5p and purified soluble Als5p$^{1-664}$ resemble characteristics of amyloid proteins (Rauceo et. al, 2004; chapters II & III). Both cell surface and soluble Als5 proteins are highly resistant to extremes of pH and temperature, and readily forms aggregates at low protein concentrations. The aggregated multimers of soluble Als5p are resistant to dissolution by SDS and GuHCl. The computation program TANGO identified a short heptapeptide sequence (IVIVATT) in the Threonine Conserved region of Als5p that has 93% beta aggregation propensity (http://tango.embl.de). A synthetic 13mer peptide (SNGIVIVATTRTV) that includes the heptapeptide sequence aggregated into a gel. This aggregated peptide solution tested positive for amyloid by showing increased absorbance and a red shift when bound to Congo red, increased Thioflavin T fluorescence intensity. The aggregates consisted of fibrils of ~22nm in diameter with Transmission Electron Microscopy. Purified Als5p$^{1-431}$ (Ig-T) and Als5p$^{1-664}$ (Ig-T-TR) aggregated from solution into amyloid-like fibrils and bound Congo Red. Thus the 13mer peptide and soluble fragments of Als5p conform to amyloid (Nilsson, 2004).

INTRODUCTION

Candidiasis which recently was the fourth most common nosocomial agent (Rauceo et al., 2004) is now the third nosocomial agent in the United States of America (Wisplinghoff et al., 2004; Ashraf et al, 2006). The resistance of Candidiasis associated with present anti-fungals even complicates the reduction of this disease. Mortality rates reported are as high as 40-50% and even more (up to 80%) in bone marrow transplant and chemotherapy patients (Barberino et. al., 2006). The persistence and re-occurrence of candidemia frustrates treatment and put patients at risk. Part of the problem of the resistance is due to the formation of biofilms (communities of cellular and non-cellular components) by the yeast which then act as a source of persistent infection and protects the yeast from drugs targeting the yeast. It has been shown that after adherence of *C. albicans* to HEp2 cells, there is an observed up-regulation of genes that encode members of known host recognition adhesins including *ALS5* and *ALS2*, which may be critical to successful colonization and invasion of the host organism (Sandovsky-Losica *et al*., 2006).

Amyloid fibril formation is associated with biofilm formation in *Escherichia coli* (Cherny *et al*, 2005). Curli, an extracellular matrix amyloid protein produced by many Enterobacteriaceae has been implicated in pathogenic processes of *Escherichia coli* and *Salmonella* spp. The Curli fibers mediate adhesion to surfaces, cell aggregation, and biofilm formation according to Barhart and Chapman, 2006. Biofilms such as thrush on the tongue caused by *C. albicans* are known to be very difficult to get rid of and cause increase resistance to drugs (Nobile *et al*., 2006). Als1p and Als3p have been implicated in biofilm formation (Nobile and Mitchell, 2006).

Cellular aggregation studies of Als5p expressing cells shows that the protein mediates ordered aggregation, with the protein becoming more hydrophobic upon ligand binding, and that the protein may have amyloid characteristics, because it binds Congo Red strongly (Rauceo *et al*., 2004). Also biochemical studies of Als5p reveal a protein that easily aggregates in solution, has high beta sheet content, and forms aggregates which are resistant to SDS and GuHCl dissolution (Rauceo et al, 2006; chapter II). All these properties of the protein are similar to properties of amyloids.

Amyloid proteins cause in vivo aggregative protein disorders such as Alzheimer's disease, Huntingtons disease, prion disease and type II diabetes. Amyloids are very adhering to each other, stable, and bind Congo Red (Cherny *et al*, 2005; Barhart and Chapman, 2006; Stathospulos *et al.*, 2004). Various tests have been proposed and used for the classification of a peptide or protein as an amyloid. One of the latest revisions to the classification and test for amyloidicity was by Nilsson, (2004) which includes formation of gel, binding to Congo Red dye, binding to Thioflavin T, and the formation of fibrils. We therefore tested directly for amyloid formation by Als5p of *C. albicans* and a short heptapeptide sequence in the Threonine rich (T) region predicted by the computational algorithmic program Tango to have 93% propensity to form β-aggregates.

MATERIALS & METHOD

Materials: Thioflavin T and Congo Red were purchased from Sigma. Sodium chloride, potassium phosphate and sodium phosphate were purchased from Fisher Scientific. The 13mer peptide (SNGIVIVATTRTV) was purchased from Arizona State University Peptide facility.

Peptide Aggregation: Briefly 2mg of 13mer peptide was dissolved in 1ml distilled water and stirred at 5°C for 2 days, then left unstirred for 3days at 5°C (Juszczak, 2004).

Unaggregated peptide: 2mg/ml peptide solution was dissolved in 1ml distilled water and left unstirred at 5°C.

Thioflavin T Amyloid Assay: A 0.8mg/ml ThT phosphate buffer stock solution was made (10mM phosphate, 150mM NaCl, pH 7.0) and filtered through a 0.2µm syringe filter. The solution was kept in the dark. Two 50 fold serial dilutions of ThT stock solution were made and the absorbance determined at 440nm. The fluorescence intensity of a 1ml working solution was measured using a Fluorolog (JOBIN YVON-SPEX) fluorescence spectrometer at an excitation of 440nm and emission 482nm (Nilsson, 2004).

Congo red Spectroscopic Amyloid Assay: A 0.7mg/ml Congo red stock solution in buffer (5mM potassium phosphate, 150mM NaCl, pH 7.4) was filtered through a 0.2µm syringe filter prior to use, and spectra were obtained between 400nm and 700nm with an

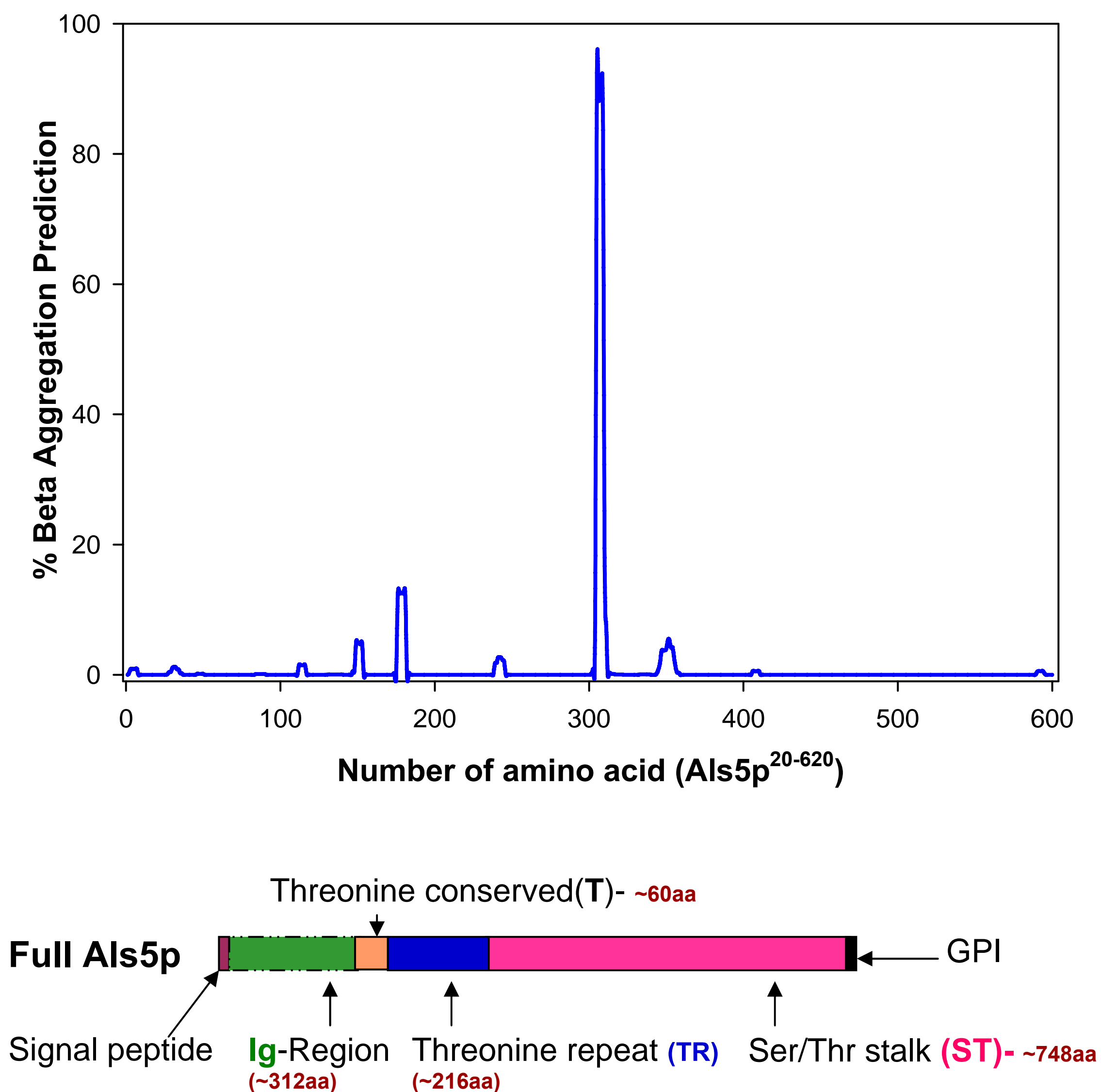
AVIV UV-vis spectrophotometer. 10μl of non-aggregated peptide/protein sample was added, and incubated at room temperature for 30mins. The contents of the cuvette were well mixed and the spectrum recorded between 400nm and 700nm. The procedure was repeated for aggregated peptide/protein sample (Nilsson, 2004).

Transmission Electron Microscopy (TEM): A small amount of sonicated aggregated protein/peptide sample was placed on a carbon coated 400 mesh nickel grid and excess solution was dried using filter paper. The non-dried sample was then stained with a small amount of 2% uranyl acetate and excess liquid removed. The grid with sample was air dried at room temperature. The images were examined using Hitachi H-7000 Electron Microscope between 4,000x to 70,000x magnifications.

RESULTS

TANGO Prediction: Als5p cellular aggregation experiments (Rauceo et al., 2004) and biochemical studies of soluble Als5p (chapters II, Rauceo et al., 2006) implied that the Als5p protein may be amyloid. Therefore the sequence of Als5 protein was evaluated with the TANGO program for beta aggregation prediction (Fernandez-Escamilla et al., 2004). In FigureIII 1, a short hepta-peptide (IVIVATT) from the Threonine Conserved region of the protein showed a 93% prediction of beta aggregation propensity. The rest of the protein did not show high propensity for beta aggregation (FigureIII 1). Based on these findings, a synthetic 13mer peptide (SNGIVIVATTRTV) corresponding to the hepta-peptide with tripeptide flanking sequences included was purchased.

Spectral Amyloid Dye Test for 13mer: To test if the peptide would aggregate into an amyloid, we had to make aggregates first from the peptide solution. To this end a 2mg/ml peptide solution was stirred, leading to the formation of a gel after two days (Juszczak, 2004). The presence of gel is a positive sign of presence of amyloid fibrils (Nilsson 2004). The gel solution was tested for its binding characteristics to Congo red (CR) and Thioflavin T dyes. Binding to CR is a traditional method for the test of presence of amyloid (Eisert *et al*., 2006). Congo red is used in post-mortem to identify the presence of amyloid because it binds and stains tissue sections that contain amyloids (Nilsson, 2004). Amyloids bind CR with increased absorbance at about 540nm in spectral studies.



FigureIII 1. Tango Beta Aggregation Prediction: Prediction of Beta aggregation propensity of Als5p$^{20-321}$ (Ig-like region), Als5p$^{321-381}$ (T-region) and Als5p$^{381-597}$ (TR-region) with the program Tango at 25°C, and pH 7. The rest of the protein sequence has negligible beta aggregation potential.

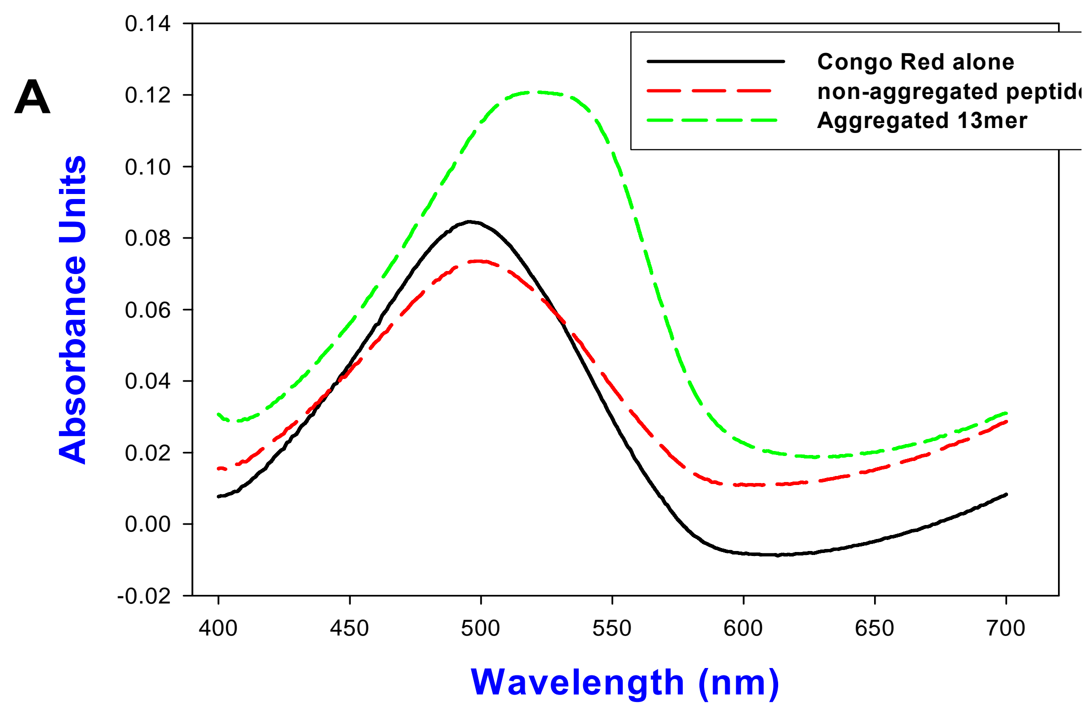
The aggregated 13mer peptide (SNGIVIVATTRTV) bound Congo red with the peak red shifted to about 541nm (FigureIII 2A). The non-aggregated (unstirred) peptide solution serving as a control, did not affect the spectrum of Congo Red.

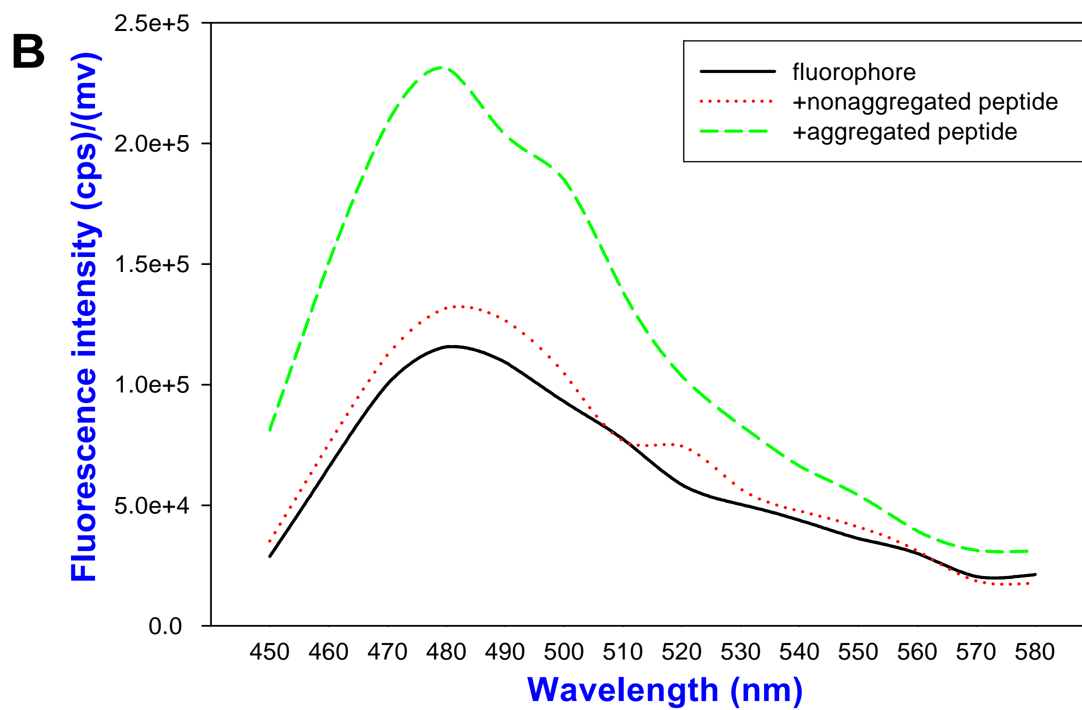
Thioflavin T (Th T) dye, like Congo red, is used to test for the presence of amyloids. When bound to amyloids, Th T fluoresces with a higher intensity. In this study, Thioflavin T had higher fluorescence intensity at about 480nm in the presence of aggregated 13mer peptide than the non-aggregated peptide (FigureIII 2B). Thus the aggregated peptide displayed amyloid characteristics, while the non-aggregated peptide did not display any amyloid characteristics.

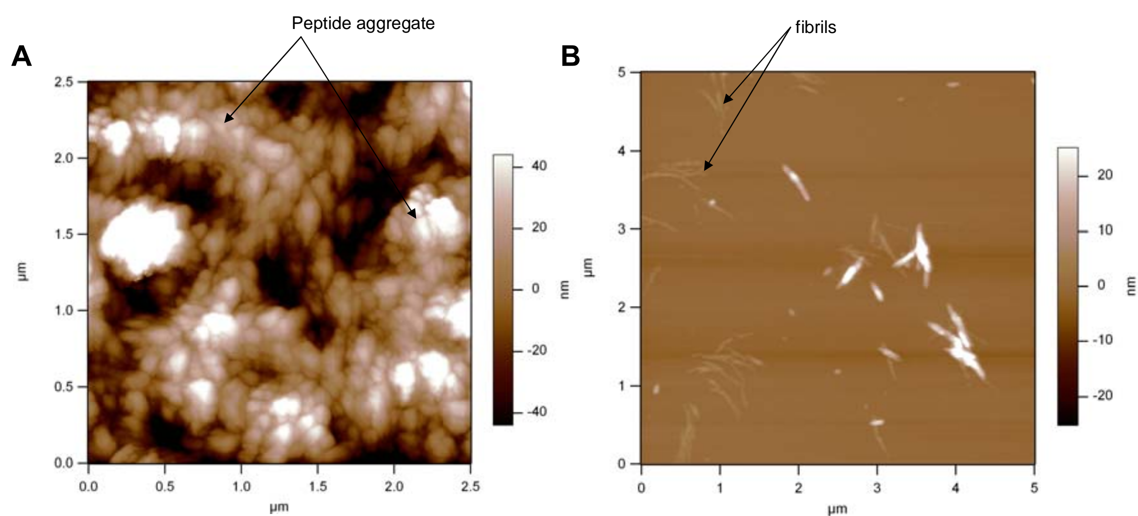
Transmission Electron Microscopy (TEM): The positive results of the peptide aggregates (SNGIVIVATTRTV) in both Congo red and Th T spectral tests prompted the need for a final confirmation of the presence of amyloid fibrils. Atomic force microscopy images showed that the sonicated aggregated peptide formed amyloid-like fibrils (FigureIII 3). Also the aggregated 13mer peptide was negatively stained with 2% uranyl acetate solution and observed under high Transmission Electron Microscopy (FigureIII 4). The aggregated peptide formed a mesh work of fibrils with a mean width of 22.2nm (FigureIII 4). These results further confirmed that the aggregated 13mer peptide had indeed formed amyloid fibrils. The unsonicated peptide only showed presence of aggregates (globules of aggregates) and no fibrils.

FigureIII 2. Congo Red & Thioflavin T Spectra Test Of 13mer peptide.

(A) The absorbance spectrum between 400nm-700nm was obtained for a 0.7mg/ml Congo red solution (5mM potassium phosphate pH7) using a 1cm path length cuvette. 10µl of non-aggregated or aggregated 2mg/ml samples were added, and spectra obtained between 400-700nm. (B) The fluorescence emission of ThT fluorophore was measured between 450nm to 580nm at an excitation of 440nm. The Raman spectra were subtracted. 10µl of non-aggregated peptide solution (2mg/ml), and aggregated peptide (2mg/ml) were added and fluorescence intensity measured.

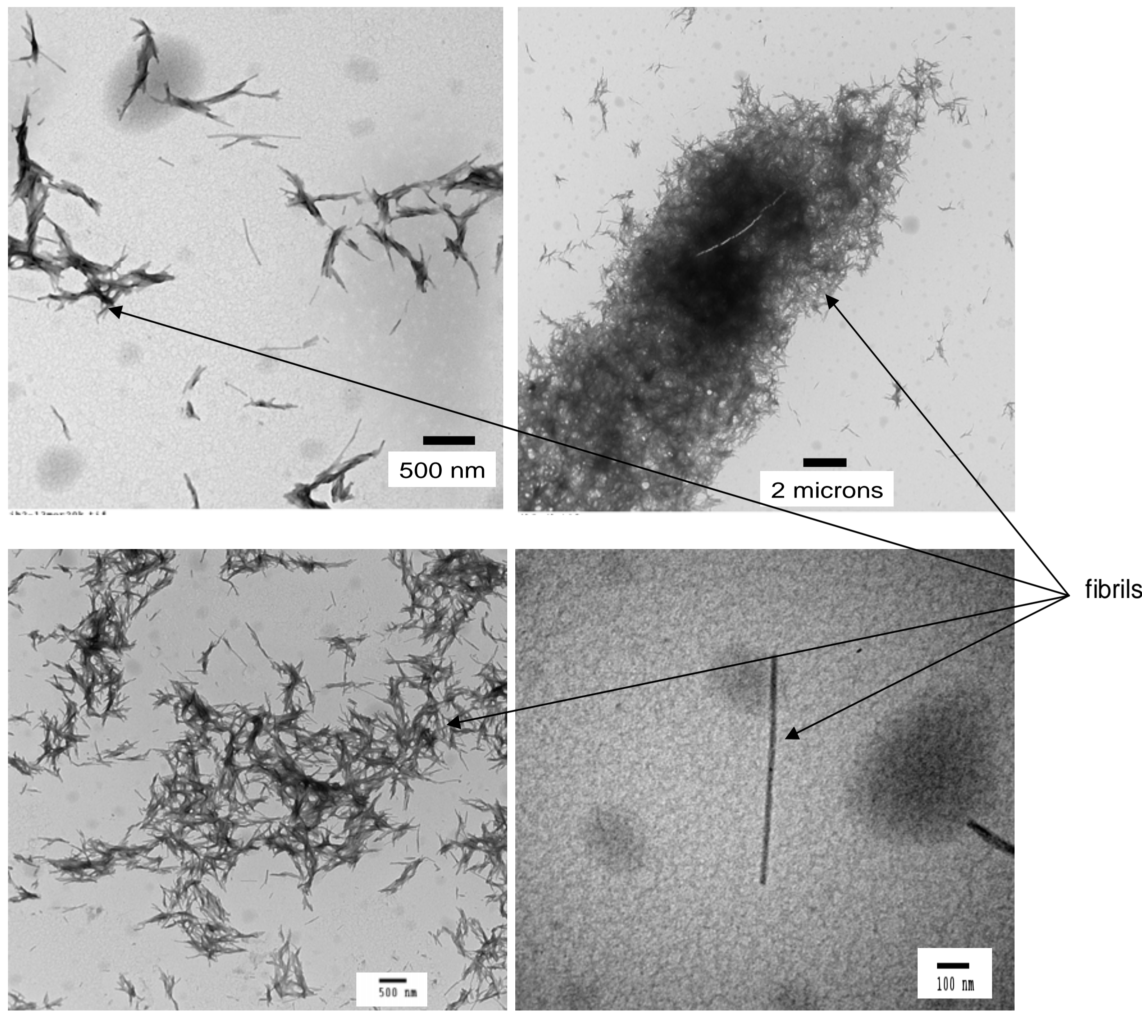

A
Absorbance Units
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
400
450
500
550
600
650
700
Wavelength (nm)
Congo Red alone
non-aggregated peptid
Aggregated 13mer


B
Fluorescence intensity (cps)/(mv)
2.5e+5
2.0e+5
1.5e+5
1.0e+5
5.0e+4
0.0
450
460
470
480
490
500
510
520
530
540
550
560
570
580
Wavelength (nm)
fluorophore
+nonaggregated peptide
+aggregated peptide



FigureIII 3. Atomic Force Microscopy images of aggregated 13mer:

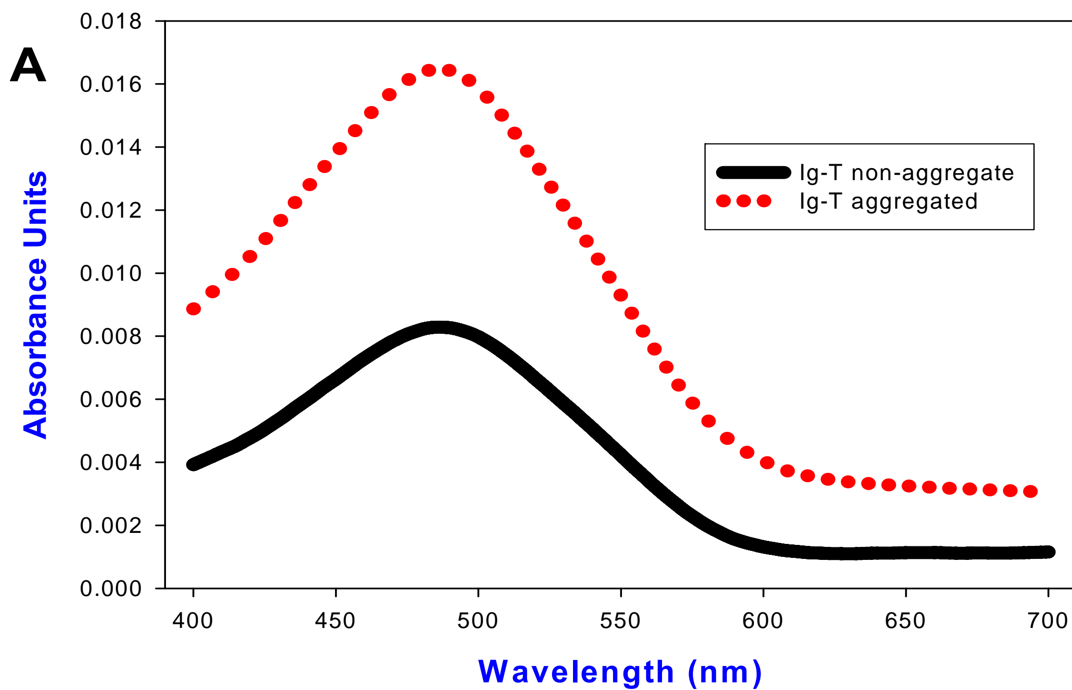
10µl of unsonicated aggregate of 13mer (A) and sonicated aggregate of 13mer (B) were dried on mica at room temperature and images were obtained at room temperature. Images were provided by Dr. Hiroshi Matsui at Hunter College, Dept. of Chemistry.

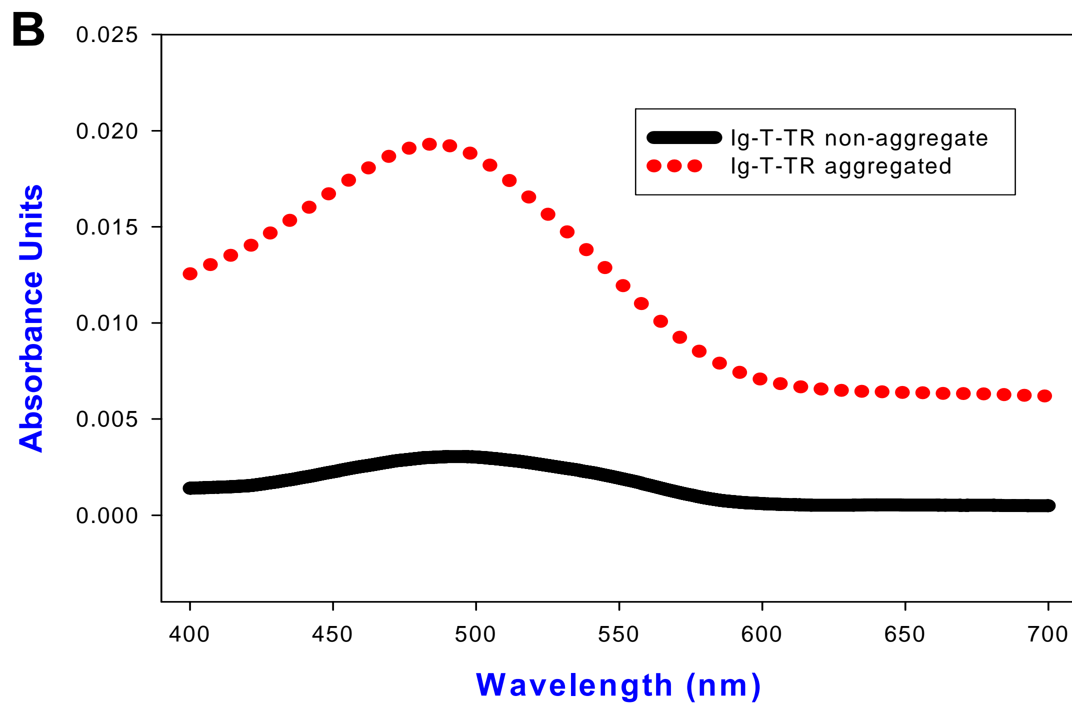


FigureIII 4. Transmission Electron Microscopy of 13mer Peptide Aggregates. Aliquots of aggregates of 13mer peptide were air dried on carbon coated nickel grids, with 400 mesh size. The partially dried samples were stained with 2% uranyl acetate. Images of stained aggregate were obtained at magnification between 4,000x to 200,000x. Unstirred solutions did not form aggregates nor fibrils (not shown).

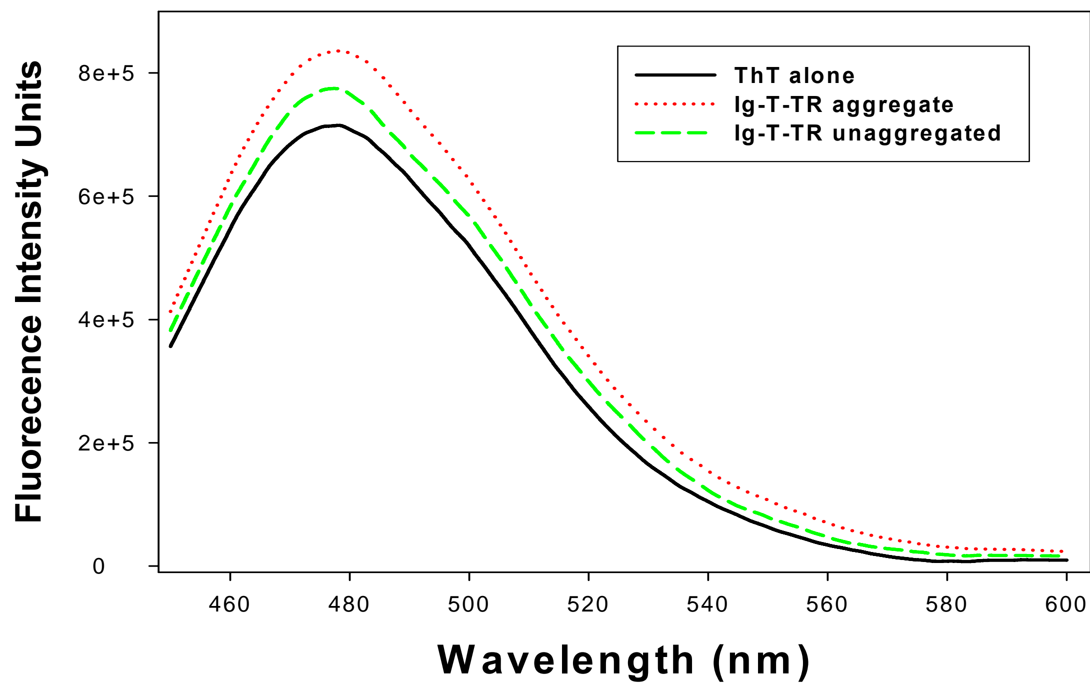
Als5p amyloid formation: Once the peptide fibrils were seen, we went back to look at the aggregated Als5 proteins that form during the purification. Aggregated preparations of Als5p$^{1-431}$ (FigureIII 5A) and Als5p$^{1-664}$ (FigureIII 5B) bound Congo red with increased absorbance compared to non-aggregated proteins. Aggregated Als5p$^{1-431}$ and Als5p$^{1-664}$ also enhanced the fluorescence of ThT (FigureIII 6). Both Als5p$^{1-431}$, and Als5p$^{1-664}$ aggregates were stained with 2% uranyl acetate and TEM confirmed the presence of fibrils (FiguresIII 8 & 9). The unsonicated proteins show globules rather than fibrils (FigureIII 7) like the unsonicated aggregated peptide solution (Figure III 3). The aggregated Als5p$^{1-431}$ formed two types of fibrillar structures with diameters ranging from 26.3nm-166.7nm (Figure III 8). Aggregated Als5p$^{1-664}$ formed more diverse of fibrillar structures, with diameters ranging from 78nm-260nm (FigureIII 9). The aggregates from the peptide are straight, un-branched and thin compared to the aggregated proteins that are thick, branched, of uneven thickness and globules along the length of the fibrils. These results conclusively confirm that the 13mer peptide, and both Als5p$^{1-431}$ and Als5p$^{1-664}$ become amyloid upon aggregation.

FigureIII 5. Congo Red Spectral Test for Als5p$^{1-431}$ & Als5p$^{1-664}$. Purified Als5p$^{1-431}$ (Ig-T) & Als5p$^{1-664}$ (Ig-T-TR) non-aggregated and aggregated proteins were tested for their binding to 0.7mg/ml Congo red solution at 25°C, and wavelength 400nm-700nm (A) and (B) respectively. 10µl of non-aggregated and aggregated samples were added to the Congo red solution and spectra obtained between 400-700nm. The baselines have been subtracted.

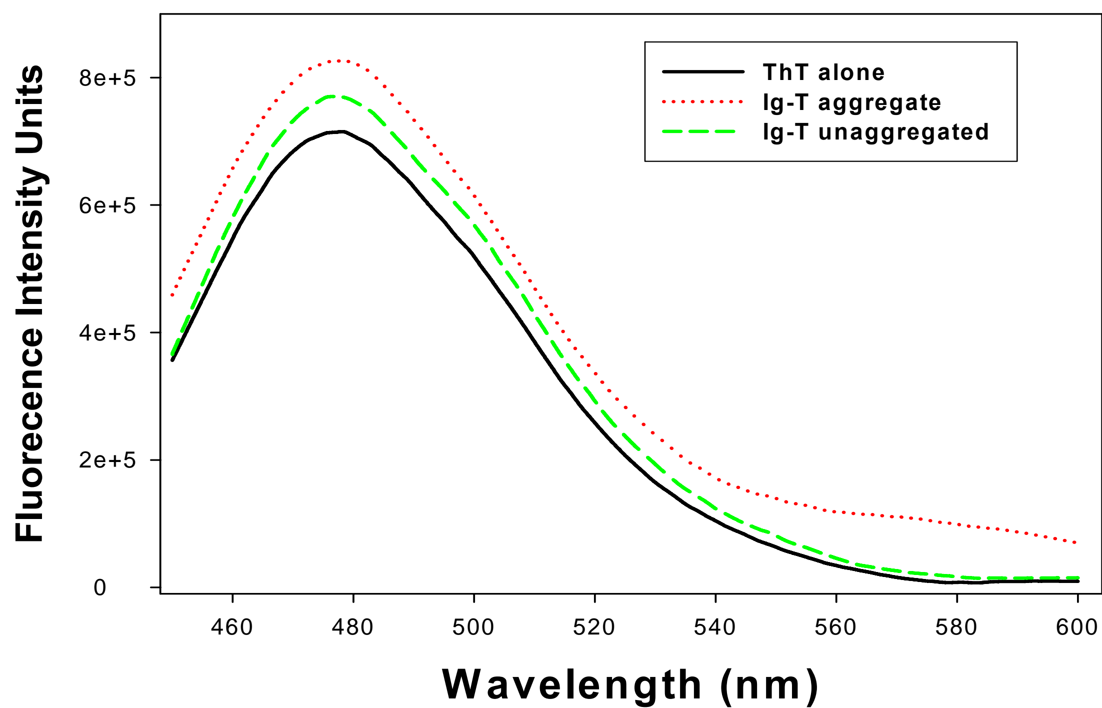

A
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Absorbance Units
Ig-T non-aggregate
Ig-T aggregated
400
450
500
550
600
650
700
Wavelength (nm)


B
0.025
0.020
0.015
0.010
0.005
0.000
Absorbance Units
Ig-T-TR non-aggregate
Ig-T-TR aggregated
400
450
500
550
600
650
700
Wavelength (nm)

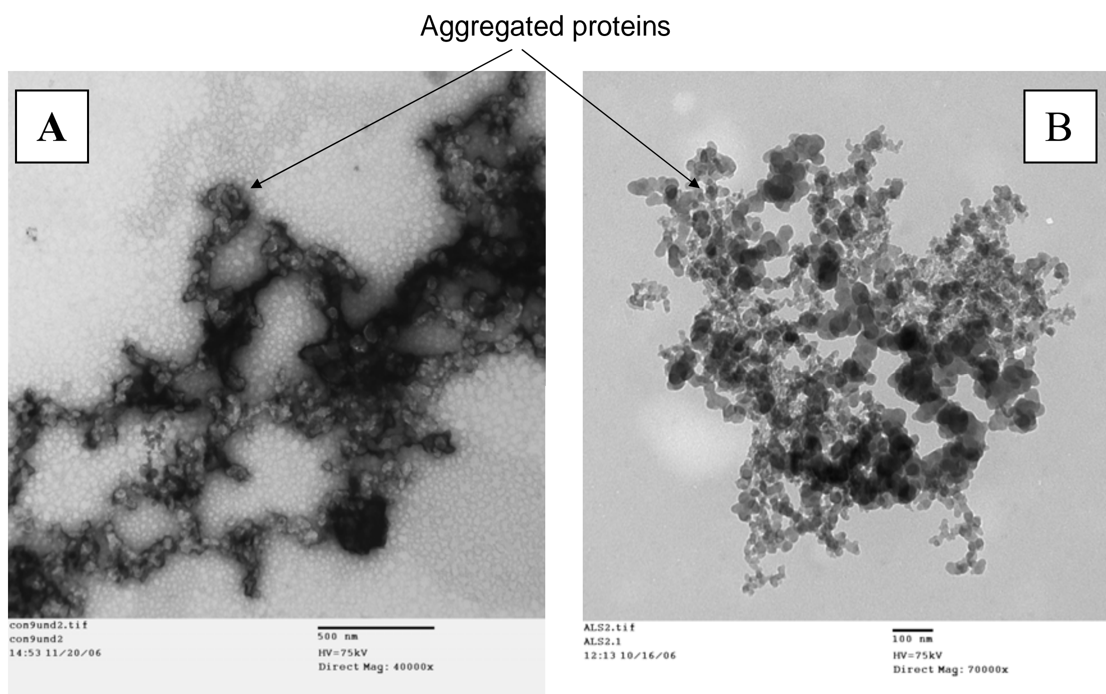
FigureIII 6. Thioflavin T Fluorescence Spectra of Als5p$^{1-431}$ & Als5p$^{1-664}$: Purified Als5p$^{1-431}$ (Ig-T) & Als5p$^{1-664}$ (Ig-T-TR) non-aggregated and aggregated proteins were tested for their characteristic ThT dye binding. The fluorescence intensity of ThT fluorophore was measured between 450nm to 580nm at an excitation of 440nm. 10µl of non-aggregated protein, or aggregated protein solutions were added and fluorescence intensity measured.



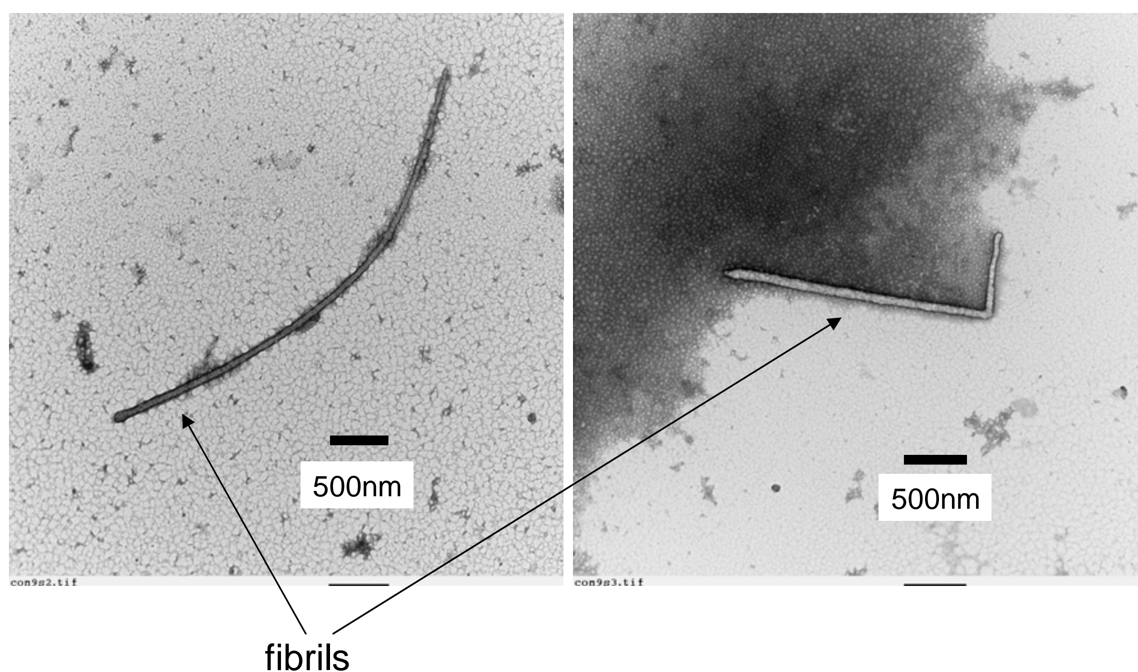
Fluorecence Intensity Units
Wavelength (nm)
ThT alone
Ig-T-TR aggregate
Ig-T-TR unaggregated
0
2e+5
4e+5
6e+5
8e+5
460
480
500
520
540
560
580
600



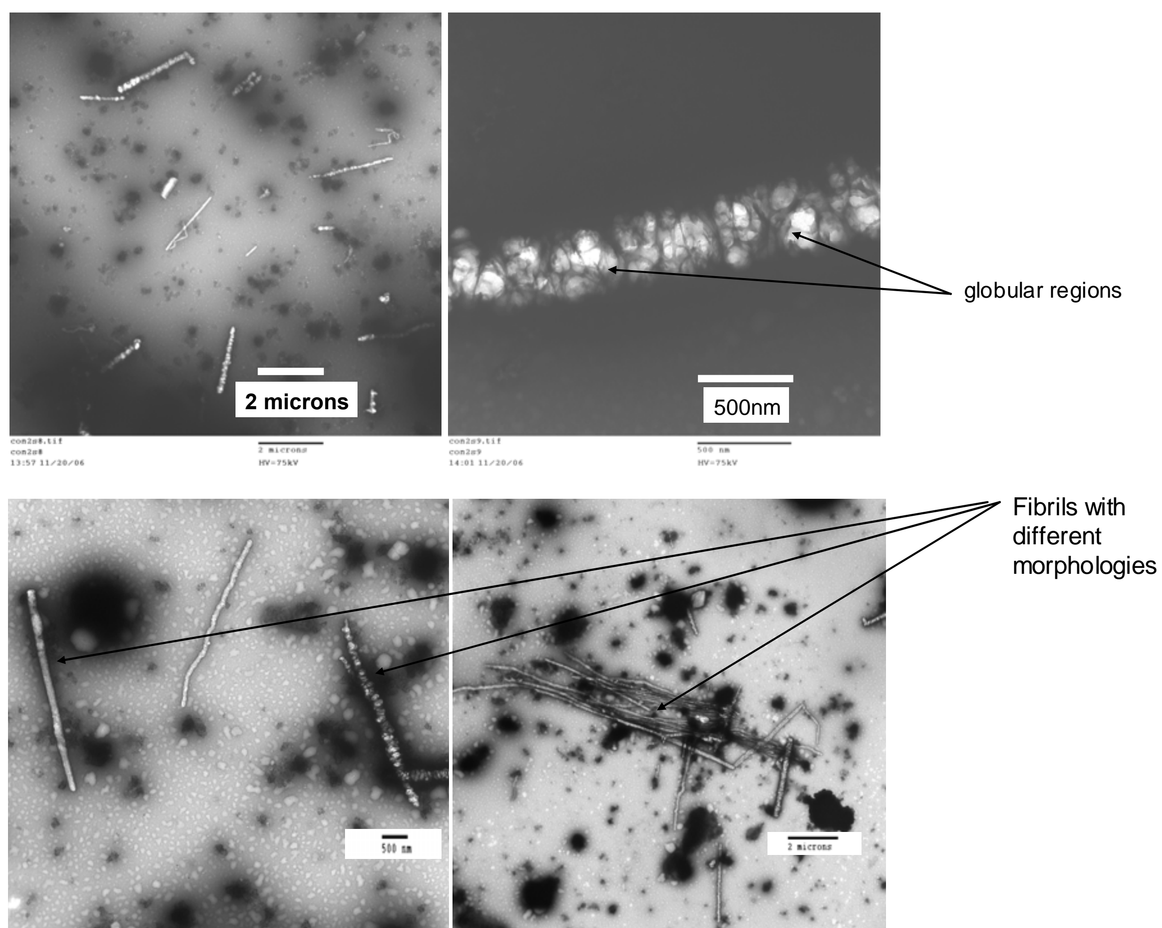
Fluorecence Intensity Units
Wavelength (nm)
ThT alone
Ig-T aggregate
Ig-T unaggregated
0
2e+5
4e+5
6e+5
8e+5
460
480
500
520
540
560
580
600



FigureIII 7. Transmission Electron Microscopy of unsonicated Aggregated Ig-T & Ig-T-TR. Aliquots of unsonicated aggregated Ig-T (A) & Ig-T-TR (B) were air dried on carbon coated nickel grids, with 400 mesh size. The partially dried samples were stained with 2% uranyl acetate. Images of stained aggregate were obtained at high magnification between 4,000x to 200,000x.

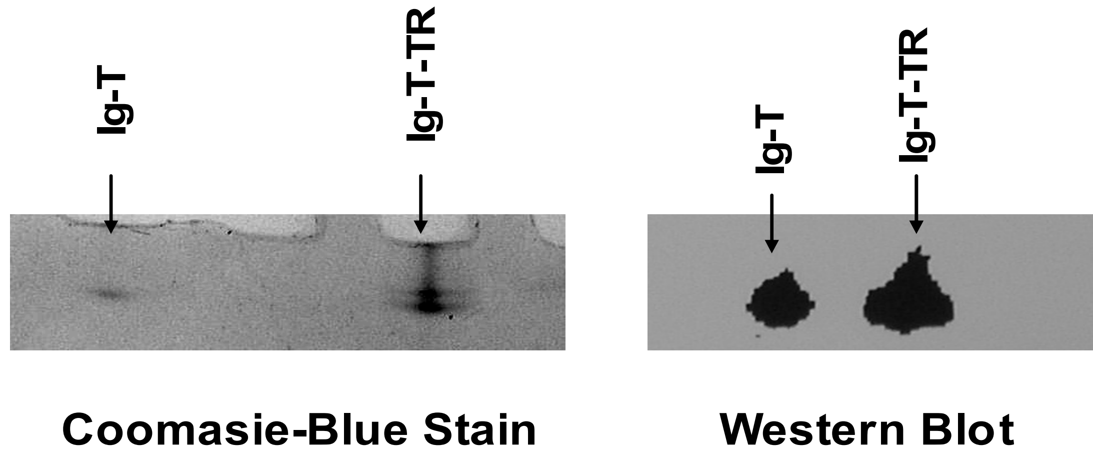


FigureIII 8. Transmission Electron Microscopy of sonicated Aggregated Ig-T: Aliquots of sonicated aggregated Ig-T were air dried on carbon coated nickel grids, with 400 mesh size. The partially dried samples were stained with 2% uranyl acetate. Images of stained aggregate were obtained at high magnification between 4,000x to 200,000x.



FigureIII 9. Transmission Electron Microscopy of sonicated Aggregated Ig-T-TR: Aliquots of sonicated aggregated Ig-T-TR were air dried on carbon coated nickel grids, with 400 mesh size. The partially dried samples were stained with 2% uranyl acetate. Images of stained aggregate were obtained at high magnification between 4,000x to 200,000x.

Electrophoretic Gels of aggregated Proteins. To find out if the aggregated proteins are indeed Als5p, the aggregates were washed and suspended in phosphate buffer, sonicated, and run on parallel 4-20% native gels. One gel was stained with Coomasie blue (FigureIII 10), and the other gel was used for a western blot probed with antibody (Anti-V5-Ab) specific to the proteins (FigureIII 10). The results show that both proteins were stained by the Coomasie blue dye and both aggregates were bound by the antibody. This confirms that the aggregates contain proteins and specifically Als5p.



FigureIII 10. Electrophoretic analysis of Als5p aggregates: Purified aggregates of the Als5p were washed with sodium phosphate buffer, re-suspended in the phosphate buffer, and sonicated. 10µL of the samples were run on two different 4-20% native gels. One gel was stained with coomasie blue (left), while the protein was transferred onto a nitrocellulose and probed with Anti-V5-Ab for the Western blot experiment (right).

DISCUSSION & CONCLUSION

In previous studies we have shown that Als5p mediated aggregation of cells in the presence of a polypeptide ligand. The aggregates had increased cell surface hydrophobicity due to a conformational shift in the structure of the protein (Rauceo *et al*., 2004). The aggregated cells also bound Congo Red. We have also shown previously that Als5p$^{1-431}$ and Als5p$^{1-664}$ have high beta sheet content, a characteristic property of amyloids (Rauceo *et al*., 2006). Further biochemical and physical studies of soluble Als5 protein show that the protein forms SDS-resistant aggregates. These are characteristic properties of amyloid proteins, and thus further pointed to our prediction that the protein may be amyloid (chapter II).

In light of the many ambiguities associated with classifying various proteins and peptides as amyloid or not, Nilsson, 2004, proposed a revised criteria for identification of amyloid fibrils. From this revision a protein/peptide needs to score a total of 4 points in order to be classified as an amyloid. Among the criteria are: gel formation (1pt), Congo Red binding via spectroscopic assay or microscopic assay (2pts), ThT or ThS binding (2pts), beta-sheet structure, and fibrillar morphology.

Spectral studies: The aggregated 13mer peptide, Als5p$^{1-431}$ and Als5p$^{1-664}$ all bound Congo Red and enhanced its absorbance as compared to their non-aggregated forms, indicating that the aggregated peptide and proteins are amyloid.

ThT fluorescence upon amyloid binding also verified that the aggregated peptide was indeed, amyloid. The aggregated peptides enhanced fluorescence more than the non-aggregated peptide.

Fibril Morphology: Cassone et al. 1973 and 1978 used electron microscopy to visualize the cell wall structure of *Candida albicans* and found layers of differing electron densities and fibrils on the cell surface. In 1986, Tokunaga et al. used rapid-freezing techniques to show that these fibrils are brush-like and evenly spaced. It has been predicted that a subset of the mannoproteins on the yeast cell surface formed the fibrillar structures that extended into the surrounding environment (Masuoka & Hazen, 1997; Masuoka, 2004). Masuoka (2004) also reports that the detailed structure of the fibrils is not clear but predicts the possibility of the protein component being globular and the fibril being entirely glycan or alternatively being linear or tightly helical protein core decorated with glycan. Whether the fibrils are covalently attached or trapped within the glucan matrix is not known, reports Masuoka, 2004.

Amyloid fibrils observed, according to Nilsson (2004), could be amorphous, fibrillar, wound around each other and even spherical. Multiple distinct fibrillar morphological in amyloid fibrils are common and aggregates of the Als proteins show similar features (Petkova *et al*., 2005). For example, the β-amyloid peptide ($A\beta_{1-40}$) of Alzheimers disease exhibits different morphological fibrillar structures with morphology sensitive to pH, temperature, buffer composition and peptide concentration (Petkova *et al*., 2005).

Our work sheds light on some of the unanswered questions mentioned by Masuoka (2004). Electron Microscopy images of the Als5p fibrils show the presence of globular regions. Also, I speculate that the protein has four different subunits and not three subunits as has been previously postulated (Hoyer, 2001), the additional unit is the

T-region, which is necessary for fibril formation. This speculation is based on the fact that the 13mer peptide that formed the amyloid-like fibrils is located in the T-region.

Other Als proteins may form amyloids: We predict that other Als protein adhesins like Als1p and Als3p that have similar T-region like Als5p, will form amyloids. Both Als1p and Als3p have been implicated in biofilm formation (Nobile & Mitchell, 2006) and therefore we predict Als5p will mediate biofilm formation as the curli amyloid protein of *E. coli* does (Wang et al., 2006). Amyloid proteins are very hydrophobic and have high affinity for self-self interactions (stick to each other) which will therefore foster cell-cell aggregation in yeasts that expresses such amyloid proteins. The increased cell-cell aggregation helps with cell community formation and hence, biofilm formation. Nobile & Mitchell (2006) believe that initiation of biofilm formation may occur as soon as a cell comes in contact with a surface, based on the Murillo et al. 2005 findings that by 30min after *C. albicans* contacts a polystyrene surface, a gene expression program is initiated that is distinct from that of planktonic cells grown under similar conditions. Genes that encode Als5p and Als2p are known to be upregulated following adherence of *C. albicans* to HEp2 cells (Sandovsky-Losica *et al*., 2006). Thus Als5p and Als2p predictably may be important for biofilm formation, at least during the initial stages.

Importance of Als5p being amyloid: Als5p formation of fibrils may be important as fibrils on yeast surfaces have been found to mediate adhesion to endothelium of renal peritubular and glomerular capillaries (Masuoka, 2004).

The amyloid nature of Als5 protein will likely increase the hydrophobicity of the cell surface and adhesiveness, and therefore predictably make Als5p a better adhesin. Being a better adhesin, the Als5p will predictably aid the yeast in the colonization of host, biofilm formation, and host invasion for pathogenesis. Indeed, the hydrophobicity of *C. albicans* cell surface has been linked to an increase in adhesion and resistance to phagocytosis (Masuoka and Hazen, 2004). Of the same importance is the finding from this work that the T-region is an amyloidogenic region and hence this work has assigned a function to the T-region of Als5p.

Tandem Repeat (TR) increases amyloid aggregate formation: I speculate that the TR also plays a significant role in the fibril formation. There are increases in both aggregates and fibrils formed in Als5p in the presence of the TR compared to in its absence, as well as the difference in morphology of the fibrils when TR is present. This speculation is also based on the findings from other studies that the animal prion protein, yeast Sup35 prion, and bacteria Curli proteins (which is not evolutionarily related to the yeast Sup35 prion and bacteria Curli proteins, but contain short repeat peptides like the yeast Sup35 and Curli proteins) all have their repeats playing important roles in amyloid fibril formation (Cherny et al., 2005; Gophna et al, 2001). These repeats alone have been shown to form fibrils in curli proteins and also in mammals. The prion disease related proteins, mostly inherited, have been also shown to have extra peptide repeats and that the octa-repeat deletions of these peptides slow down disease (Cherny et al., 2005). Thus predictably, the repeat region (TR) of Als5p may be further supporting the amyloid property of the T-region.

In Als5p, the initial monomeric interactions of the proteins could be facilitated by hydrogen bonds between the amide chains and the conformational flexibility of the backbone would assist in amyloid formation assembly. These associations may then be further stabilized by hydrophobic interactions and speculatively, by disulfide bonds too.

Conclusion: This amyloid formation property of Als5p is novel and is an important and useful evolutional survival property for the yeast as it differs from the well known disease association of amyloid formation by proteins and peptides and may assist the yeast to form cell communities (like biofilms) that are resistant to elimination from the host.

CHAPTER IV

Summary, Conclusion & Future work

Summary: The Als5p domains are scantily purified from *S. cerevisiae* and the purified proteins easily aggregate, but they were successfully produced in quantities that allowed these studies to be conducted. The problem with aggregation was drastically reduced and therefore protein loss was reduced (chapter II).

The Threonine Repeat (TR) region previously had no function assigned. We now know that the TR supports the substrate binding Ig-region to better bind ligand and also increases cell to cell aggregation in the yeast. CD spectroscopy indicates that the addition of the TR to the Ig-T does not change the average secondary structure of the protein very much, but induces a change in the tertiary structure and flexibility of the protein. Structural studies of one synthetic repeat sequence (36mer) showed that the addition of TFE induced a conformation shift in the peptide from an otherwise low helical content to a significantly higher alpha helix structure and at the same time reduced the percentage of beta sheets significantly. The results from the TFE experiment on the 36mer peptide further seems to supported the prediction that the shift in conformation in the Als5p upon binding to a substrate to a more hydrophobic protein may predominantly occur at the TR region (Chapter II).

The biochemical nature of soluble Als5p$^{1-664}$ was investigated by employing Far UV CD spectroscopy (chapter II). We knew that Als5p mediates aggregation within a wide pH (2-10) and temperature range (5°C to 65°C). Data from the investigation shows that the soluble protein aggregates into multimers which are resistant to dissolution by SDS and GuHCl. The soluble non-aggregated protein is stable at 90°C, refolds from 80°C, and has multiple temperature transitions, most of which are non-cooperative in the unfolding of the protein. The conformation of the protein is also affected by pH, with the

protein not being completely unfolded (denatured) at the extreme pH values of 3 and 9 tested in this experiment, thus indicating that there is a very stable core of protein. Sodium chloride did not have any significant effect on the conformation of the protein above the physiological concentration of 150mM however the presence of TFE significantly altered the percentages of the secondary structural elements of the protein. TFE weakened the intermolecular forces in the regular beta sheets but apparently strengthened and increased the forces that held regular alpha helices together, with most of the critical conformational changes occurring at about 20% TFE.

In chapter III, predicting from the biochemical studies that Als5p may be amyloid, the beta aggregative propensity of sequences of the protein were investigated with the program TANGO. Results showed that a short peptide in the Threonine conserved region had about 93% propensity to form an amyloid. A synthetic peptide aggregated to form a gel, tested positive for Congo Red and Thioflavin T fluorescence amyloid tests, and more importantly formed amyloid fibrils in Transmission Electron Microscopy images. This information led to the testing of the purified protein aggregates Ig-T and Ig-T-TR for their amyloidicity. Both aggregated proteins tested positive for the Congo red amyloid test and formed amyloid fibrils as observed from Transmission Electron Microscopy images.

Conclusion: The results from this investigation expanded knowledge about the Als5p. We now know that the protein easily aggregates into multimers (different aggregate sizes), and is very stable to pH and temperature. Unfolding is mostly non-cooperative and occurs predominantly at the repeat region, and that Als5p is an amyloid protein with the

amyloidogenic region being the T-region. Thus this work has shown for the first time two regions of Als proteins that play very important roles in the adhesive function of the protein, and a property of Als5p that is novel and extremely important. The biochemical behavior of the protein is now known and these studies can be extended to the rest of the Als proteins which share similar sequence structures. Drugs can be targeted towards the aggregated colonies with the approach of an amyloid protein being involved in the aggregate formation.

Future work: Mutation experiments of the 13mer (SNG***IV***IVATTRTV) shows that the sequence of the peptide is important for fibril formation (data not shown). Mutation of the sequences in the T-region of the protein will further fortify the function of the T-region. Also the biofilm formation property of Als5p will be investigated.

CHAPTER V

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