

**Investigation into the lipid activation of calpain
and its role in cataract formation**

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ABSTRACT

Age-related cataract is the commonest cause of treatable blindness in the world today. It is an ever-increasing problem with prolonged life-expectancy and a burgeoning elderly population. Although successfully treated by modern, sophisticated cataract surgery, the resources needed to provide a surgical set-up and the prolonged training time required to produce a competent surgeon can challenge the available finances of developed economies and result in unacceptable waiting times while they may simply be unaffordable in developing economies and result in high prevalence of blindness. Surgical treatment, although effective in dramatically reversing blindness, is associated with a small risk of sight-threatening complications. Against this background, it is worth considering an alternative, simpler, medical means of treating cataracts that is easily administered and equally effective.

Calpains are Ca^{2+} -dependent intracellular proteases and several of these enzymes are believed to participate in cataractogenesis. Calpain 2 is the major isoform of calpain involved in human cataractogenesis and its activation appears to be modulated by lipid / membrane interaction. The main aim of this work is to investigate the mechanism of lipid activation of calpains as an essential link towards understanding the molecular and cellular dynamics of cataractogenesis *in vivo*. A clear picture of the enzymatic events in cataractogenesis will form the basis of drug development to cause selective enzyme inhibition.

In chapter 2, atomic absorption spectroscopy shows that the progressive uptake of extra-lenticular Ca^{2+} by porcine lenses correlates with increases in levels of lens opacification with $8.0 \mu\text{moles Ca}^{2+} (\text{gm wet lens weight})^{-1}$ corresponding to cataract occupying approximately 70% of the lens cell volume. This degree of cataract

was reduced by approximately 40%, when a calpain inhibitor, SJA6017 (final concentration 0.8 μM), was included in the extra-lenticular medium, therefore suggesting that the observed porcine opacifications involve the Ca^{2+} -mediated activation of calpains and that cataract could be retarded by the topical administration of calpain inhibitors.

In chapter 3, DWIH (Depth weighted insertion hydrophobicity) analysis shows the small subunit of several calpain 2 isozymes to each possess a segment with the potential to form a lipid / membrane interactive α -helix (DWIH values *circa* 7.0). Extended hydrophobic moment analysis shows these segments to be potential oblique orientated α -helix formers ($\langle \mu_{\text{H}} \rangle$ *circa* 0.5). This potential is confirmed by hydropathy plot and graphical analyses, which show each α -helix to possess a significant N \rightarrow C hydrophobicity gradient. It is suggested that the lipid / membrane activation of calpain 2 may involve oblique angled membrane penetration by an α -helical segment in domain V of the enzyme.

In chapter 4, VP1, a peptide homologue of this domain V segment, is shown to be strongly haemolytic (half-maximal lytic dose = 1.45 mM). FTIR conformational analysis shows VP1 to adopt α -helical structure (20% - 65% of primary structure) in the presence of lipids. These levels are maximal in the presence of anionic lipid (65% of primary structure) and monolayer studies show the peptide to exhibit high levels of anionic lipid monolayer penetration with surface pressure changes (ΔSP) of 5-6 mN m^{-1} at 30 mN m^{-1} , which are reduced by approximately 40% \pm 15% in the presence of 100 mM NaCl. It is suggested that membrane penetration by the domain V α -helix of calpain 2 may proceed *via* electrostatic interactions and snorkelling, involving associations between an arginine residue located in the polar face of this α -helix and anionic membrane lipid.

It has been suggested that lipid / membranes may modulate calpain 2 activity by lowering the enzyme's *in vivo* Ca^{2+} requirements. In chapter 5, colorimetric assay of calpain 2 shows that the enzyme requires 4 mM Ca^{2+} for 100% proteolytic activity, as defined by this assay. In the presence of 1 mM Ca^{2+} , negligible calpain 2 proteolysis is detected but at this level of Ca^{2+} , in the presence of either Dimyristoyl phosphatidylinositol(DMPI), Dimyristoyl phosphatidylserine(DMPS), Dimyristoyl phosphatidylcholine(DMPC) or Dimyristoyl phosphatidylethanolamine(DMPE), calpain 2 shows proteolytic activity which ranges between 37% and 77% of the protein's full enzymatic activity. The large subunit of calpain 2 (LS-calpain 2) is proteolytically active in the absence of the calpain 2 small subunit and it has been suggested that this latter subunit is dispensable for the lipid activation of calpain 2. LS-calpain 2 is assayed under conditions corresponding to those used here for calpain 2 assay and is shown to require the presence of 6 mM Ca^{2+} for 100% enzymatic activity. These Ca^{2+} levels are unaffected by the presence of either: DMPI, DMPS, DMPC or DMPE, and based on these combined results, it is suggested that the lipid activation of calpain 2 requires the presence of the small subunit. In addition, it is shown that when compared to zwitterionic lipid under corresponding conditions, anionic lipid induces an approximate twofold enhancement of calpain 2 proteolytic activity (70% - 77% as compared to 37% - 49%) and a similar enhancement in its average rates of lipid monolayer interaction ($\Delta\text{SP} = 1.5 \times 10^{-3} \text{ mN M}^{-1} \text{ sec}^{-1}$ at 10 mN M^{-1} as compared to $\Delta\text{SP} = 5.0 \times 10^{-4} \text{ mN M}^{-1} \text{ sec}^{-1}$ at 10 mN M^{-1}). It is suggested that calpain 2 may possess an electrostatically driven preference for anionic lipid, which contributes to lowering the enzyme's Ca^{2+} requirement for activation.

In chapter 6, these combined data are discussed in relation to a model for the lipid activation of calpain 2 and proposals for future work are presented.

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ABBREVIATIONS

| | |
|-------------------------|--|
| DMPC | - dimyristoyl phosphatidylcholine |
| DMPI | - dimyristoyl phosphatidyinositol |
| DMPE | - dimyristoyl phosphatidylethanolamine |
| DMPS | - dimyristoyl phosphatidylserine |
| DWIH | - depth weighted insertion hydrophobicity |
| FTIR | - Fourier transform infrared spectroscopy |
| $\langle \mu_H \rangle$ | - the mean hydrophobic moment |
| $\langle H \rangle$ | - the mean hydrophobicity |
| HEPES | - N-[2-Hydroxyethylpiperazine-N']-[2-ethanesulphonic acid] |
| SUV's | - small unilamellar vesicles |
| EDTA | - ethylene diamine tetra-acetic acid |
| EGTA | - ethylene glycol bis (β aminoethylether-N,N,N tetra-acetic acid) |
| Ppm | - parts per million |
| LM | - lens medium |
| IC ₅₀ | - half-minimum inhibitory concentration |
| LD ₅₀ | - half-maximal haemolytic dose |

SINGLE LETTER ABBREVIATION FOR AMINO ACIDS

A = Alanine, C = Cysteine, D = Aspartic acid, E = Glutamic acid, F = Phenylalanine,
G = Glycine, H = Histidine, I = Isoleucine, K = Lysine, L = Leucine,
M = Methionine, N = Asparagine, P = Proline, Q = Glutamine, R = Arginine,
S = Serine, T = Threonine, V = Valine, W = Tryptophan, Y = Tyrosine.

CHAPTER 1

Cataract and calpains

Biswas, S., Harris, F., Dennison, S., Singh, J. and Phoenix, D. A. (2003) The role of calpains in cataract and their potential to act as targets in cataract therapy. *J. Equa.* In press; **Biswas, S., Harris, F., Dennison, S., Singh, J. and Phoenix, D. A. (2003)** Calpains: targets of cataract prevention? *Trends Mol. Med.* In press.

1.1 INTRODUCTION

The ocular lens is a highly specialised non-vascular tissue, which focuses an image onto the retina of the eye, triggering electrical impulses that will then be interpreted by the brain as vision. (Figure 1.1; Harding *et al.*, 1997). In cataract, opacification of all or part of the lens prevents visible light from reaching the retina, reducing optical performance and is most commonly manifested by decreased visual acuity, glare and contrast sensitivity (Truscott, 2003; Johnson *et al.*, 1998).

Cataract accounts for the majority of cases of treatable blindness world-wide and is a major detriment to the quality of life. The disorder can be caused by a variety of factors including: ageing (Harding, 2002) metabolic disturbances, radiation, trauma, drugs (Brown and Bron, 1996a) and congenital disorders (Francis *et al.*, 1999). Aetiologically, the most common types of cataract are those associated with ageing (Harding, 2002), followed by those associated with diabetes (Bron *et al.*, 1998). Age-related cataract involves both changes in the lens cortex, giving rise to cortical cataract, and in the lens nucleus, leading to nuclear cataract (Bron *et al.*, 2000). Below the age of 50, women have a lower incidence of cataract than men, but above that age the incidence is greater. A hormonal influence in cataractogenesis has been suggested and indeed, women undergoing hormone replacement therapy have a significantly reduced incidence of cortical cataract when compared to men (Brown and Bron, 1996b). In human diabetic cataracts, opacities are observed in various regions of the lens, and the posterior, subcapsular or cortical types are common in the early stage. In the cortical type of diabetic cataract, opacities spread along lens fibres from the equator to the centre and the cortex appears to be the lens region predominantly involved in human cataract under long-term diabetic stress (Bron *et al.*, 1998).

Figure 1.1 A schematic cross-section of the human eye

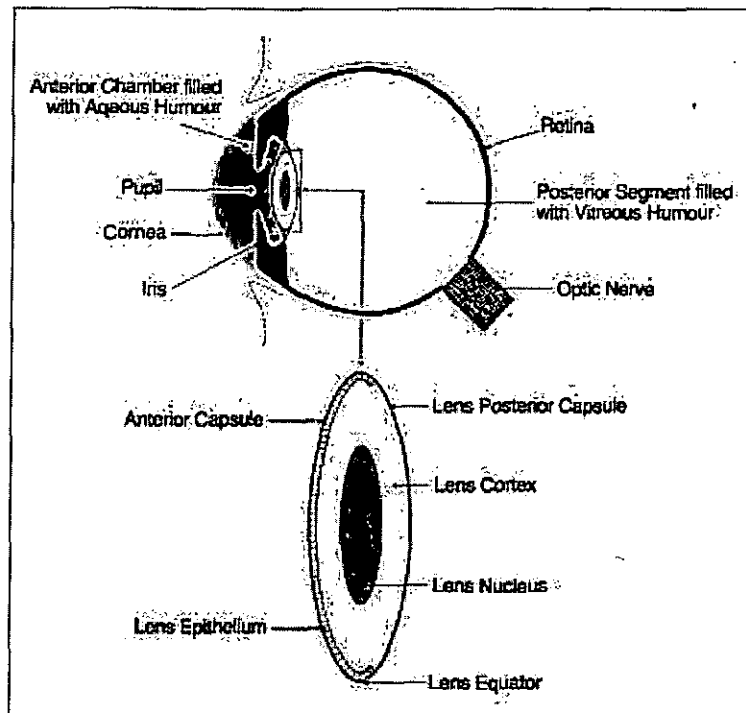


Figure 1.1 was adapted from Biswas et al., (2004) and shows the location of the lens in the human eye. The ocular lens is a highly specialised non-vascular tissue, which focuses an image onto the retina of the eye, triggering electrical impulses that will then be interpreted by the brain as vision. In cataract, opacification of all, or part, of the lens prevents visible light from reaching the retina, which reduces optical performance and is most commonly manifested by decreased visual acuity, glare and contrast sensitivity (Bron, 2000). Also shown in Figure 1.1 is a magnified image of the lens depicting its major anatomical regions each of which on its own or in combination can undergo opacification to form localised cataract or a total 'mature' cataract. Cataracts caused by steroid treatment are localised to the posterior sub-capsular region of the lens. Age-related cataract, which is the most common group of cataracts worldwide, can start either in the cortex, or in the nucleus, although it eventually affects both regions and this is also true of cataracts in diabetic people. The anterior epithelial layers and cortical regions of human cataract have been shown to contain calpain 2, a key enzyme implicated in the genesis of some forms of cataract (Sanderson et al., 2000).

In many cases the precise underlying mechanisms that lead to cataract are not fully understood (Biswas et al., 2003). The lens changes involved in cataractogenesis may be considered to be either specific or general in nature. Specific lens changes are related to the aetiology of the cataract, a major example of which is seen with

diabetics where the effects of sugar-mediated changes to the lens are perhaps, not surprisingly, much higher than in age-matched non-diabetics (Bron *et al.*, 1998). General changes associated with the normal ageing of the lens are also observed in cataract although such changes are generally greater in the case of cataract (Harding, 2002; Bron *et al.*, 2000). These lens changes include: alterations to membrane lipid structure, thereby compromising membrane functions (Tang *et al.*, 2003a; Zheng *et al.*, 1999; Ahuja *et al.*, 1999) changes in the levels of lens enzyme activity, particularly in anti-oxidant enzyme systems (Lou, 2003; Lou, 2000) and the structural modification of a variety of membrane associated proteins (Sanderson *et al.*, 2000; Clark *et al.*, 1999; Matsushima *et al.*, 1997; Andersson *et al.*, 1996). However, in most cases of cataract, the major change associated with reduced optical performance of the lens is the structural modification of the water-soluble crystallins (Figure 1.2).

Crystallins are primarily responsible for lens transparency and their structural modification usually leads to compromised function, aggregation and insolubilisation with elevated levels of these processes resulting in cataract (Dubbelman *et al.*, 2003; Fujii, 2003; Derham and Harding, 2002; Fujii *et al.*, 2001). A number of lens insults are known to induce crystallin modification and lens opacification (Franke *et al.*, 2003; Stitt, 2001; Spector, 2000; Ottonello *et al.*, 2000) but over the last decade it has become increasingly clear that members of the calpain family of proteases may participate in these processes in response to the elevation of internal Ca^{2+} levels that are observed in cataractogenesis (Figure 1.2; Biswas *et al.*, 2003; Biswas *et al.*, 2001). Here, recent research into the roles played by calpain 2, calpain 10 and lens specific isoforms of calpain 3 in cataractogenesis is reviewed with a focus on crystallin proteolysis, the activation of these calpains in the lens and the potential use of calpain inhibitors as anti-cataract agents.

Figure 1.2 Representative lens pathways leading to calpain activation

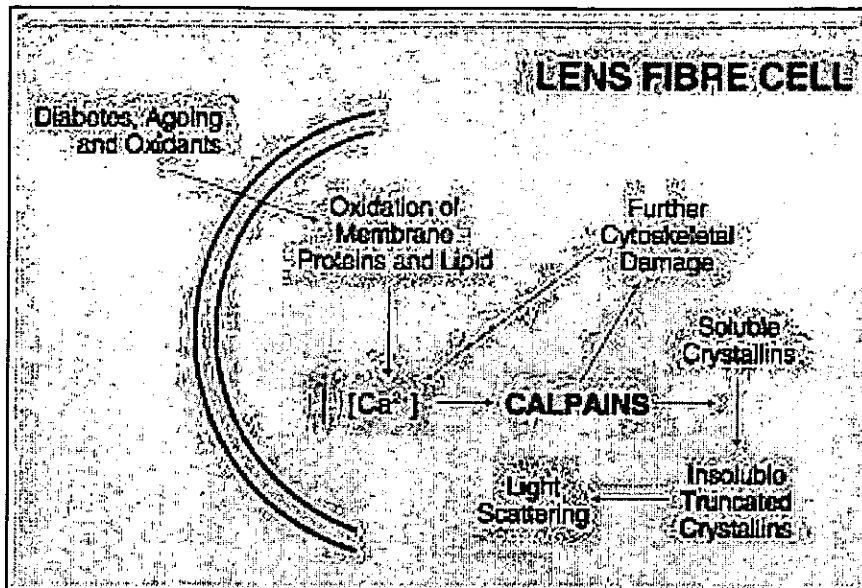


Figure 1.2 was adapted from Biswas et al., (2004) and shows biochemical pathways that can induce light scattering and cataract. The ageing process and lens insults such as oxidation and diabetes, can lead to impaired membrane function, which results in pathologically elevated levels of lenticular Ca^{2+} (Lou, 2003; Stitt, 2001). Under these Ca^{2+} conditions, calpains are over-activated and the resulting deregulated proteolysis of soluble crystallins leads to their insolubilisation and aggregation, thereby compromising their function(s) in maintaining lens transparency and producing the reduced lens performance associated with cataract. Over-activated calpains also proteolyse lens cytoskeletal proteins such as vimentin, which, can further elevate lenticular Ca^{2+} , thus excavating calpain over-activation and cataract formation (Biswas et al., 2001; Sanderson et al., 2000). Calpains have been implicated in a number of cellular processes (Ray et al., 2003; Nixon, 2003; Huang and Wang, 2001) but for clarity, these biochemical pathways are not shown.

1.2 CRYSTALLIN FUNCTION IN THE LENS

Essentially, the bulk of the lens is formed from densely packed elongated fibre cells, which are arranged in concentric shells (Harding, 1997) with a single-celled surface layer of epithelial cells (Bhat, 2001). The transparency of the lens is a function

of the highly ordered arrangement of the macro-molecular components of its cells and the extracellular matrix. The epithelial cell surface layer of the lens and its capsule neither scatter or reflect light, essentially because their combined refractive index is the same as that of the eye's aqueous humour (Paterson and Delamere, 1992). The regular organization of the lens fiber cells in a hexagonal, densely packed cellular arrangement increases the transmission properties of the lens (Francis et al., 1999). Functionally, lens fibre cells are highly specialised. During development, these cells become devoid of nuclei and organelles to reduce light scattering, and are thus, metabolically inactive, being entirely dependent upon the epithelial cells (Bhat, 2001) and a network of gap junctions for metabolic and ionic homeostasis (White and Bruzzone, 2000; Varney and Weiler, 2000). When mature, these cells become specialized for the production of crystallins; which are responsible for the high refractive index of the lens. Birefringence, as shown by the ocular lens, is an optical property arising from a high degree of order in a structure and it has been shown that at high concentrations, crystallins are capable of a high degree of packing regularity (Duncan *et al.*, 1997). It is generally accepted that lens transparency is predominantly the result of such packing by the crystallins with the high levels of short range spatial order produced by these packing arrangements allowing light scatter from individual molecules to cancel each other out (Delaye and Tardieu, 1983).

1.3 CRYSTALLINS IN CATARACT

Crystallins comprise three main classes: α -crystallins β -crystallins and γ -crystallins (Horwitz, 2003; Jaenicke and Slingsby, 2001 Slingsby and Clout, 1999). In addition to their established role in maintaining transparency, it has now been shown

that α -crystallins function as molecular chaperones by suppressing the unfolding of crystallins, other proteins and probably certain lens enzymes or by mediating in the refolding of unfolded proteins (Derham and Harding, 2002; Goenka *et al.*, 2001; Horwitz, 2000).

Functional α -crystallins and β -crystallins are multimeric protein complexes whilst γ -crystallins are monomeric with each of these protein classes primarily possessing β -sheet structures (Horwitz, 2003; Jaenicke and Slingsby, 2001; Slingsby and Clout, 1999). The crystallins can be structurally modified in response to ageing and a number of lens insults (Zhang *et al.*, 2003; Lapko *et al.*, 2003; Davies and Truscott, 2001; Hanson *et al.*, 2000; Zarina *et al.*, 2000; Liang and Ahktar, 2000; Hanson *et al.*, 1998; Lampi *et al.*, 1998). For example, the non-enzymatic glycation of crystallins involves the attachment of carbohydrate molecules to their amino acid residues, modifications which have been shown to inhibit crystalline function and to lead to reduced optical performance in ageing lenses and to contribute to cataract (Pokupec *et al.*, 2003; Lehman and Ortwerth, 2002; Ulrich and Cerami, 2001). However, the major form of crystallin modification resulting from lens insult involves the oxidation of their thiol containing residues. To maintain clarity of the lens, it is generally necessary for the methionine and cysteine amino acid residues of crystallins to be maintained in the reduced state and their oxidation can lead to cross-linking by disulphide bridge formation, thereby facilitating crystallin aggregation and insolubilisation. These effects are prone to occur with crystallins possessing surface-located thiol groups and contribute to the reduced optical performance normally observed with the general ageing process. However, when these effects are associated with lens insult, they generally involve higher levels of residue oxidation, increased unfolding of crystallins and the exposure of buried cysteine groups to oxidation. The

disulfide bonds thereby generated, cause high levels of crystalline aggregation and insolubilisation and generally cataract (Lou, 2003; Lou, 2000; Hanson, 2000; Lund et al., 1996).

In addition to oxidative damage, it has become increasingly clear over the last ten years that pathological structural modifications to lens crystallins also arise from their proteolytic cleavage. It is now believed that Ca^{2+} -mediated insult of the lens may lead to crystallin processing *via* the over-activation of lens proteases, thereby contributing to both age-related changes in the lens and some forms of cataract.

1.4 Ca^{2+} AND CATARACT

Ca^{2+} plays an essential role in many lens processes and the maintenance of Ca^{2+} homeostasis is critical to lens clarity with free Ca^{2+} levels in the lens needing to be kept in the submicromolar range, lower than that of the aqueous humour (Duncan et al., 2002; Duncan et al., 1994). To maintain this homeostasis, the inward passive diffusion of Ca^{2+} *via* lens membrane channels (Cooper et al., 1986) is countered by the use of a variety of Ca^{2+} -adenosine triphosphatases (Ca^{2+} -ATPases) and $\text{Ca}^{2+}/\text{Na}^+$ exchangers, variously located in the plasma membrane, sarcoplasmic membrane and endoplasmic reticular membrane (figure 1.3; Liu et al., 2002; Bian et al., 2000; Churchill and Louis, 1999; Liu et al., 1999; Duncan et al., 1994; Ye and Zadunasky, 1992).

The mechanisms that regulate Ca^{2+} homeostasis in lens fibre cells are not well understood. Deep lens fibers do not possess organelles and therefore have lost the means to synthesise ATP to a significant extent, although low concentrations of ATP

may be produced by glycolysis (Bassnett, 2002). Earlier studies have shown that there is no significant Ca^{2+} -ATPase activity in the lens nucleus (Borchman et al. 1988).

Figure 1.3 Mechanisms involved in the regulation of lens cell internal Ca^{2+}

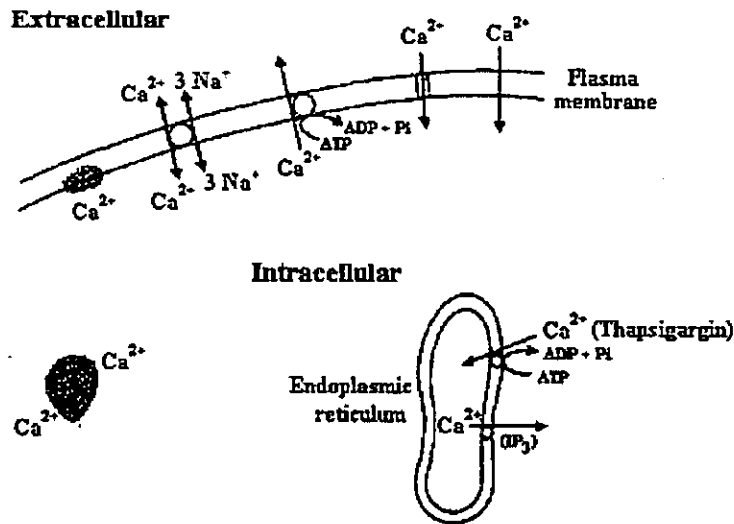


Figure 1.3 was adapted from Duncan et al., (1994) and shows mechanisms involved in Ca^{2+} -homeostasis in a representative lens cell. These mechanisms include passive influx through different channel types and efflux *via* at least two active transport mechanisms. Most Ca^{2+} efflux in the resting cell is performed by Ca^{2+} -ATPases with contributions from $\text{Ca}^{2+}/\text{Na}^{+}$ exchangers, depending upon cellular conditions. Calcium storage can also occur through sequestration in the cytosol and membrane, represented above by shaded areas, and through incorporation into dynamic cytoplasmic stores within the endoplasmic reticulum.

Borchman et al. 1989). However, it has been recently suggested that gap junctions may play a role in the Ca^{2+} homeostasis of lens fibre cells (Baruch et al., 2001). Gap junctions are formed by hexameric structures of connexin molecules that interact with their counterparts in neighbouring cells to form membrane aqueous pores (Evans et al., 2002). These pore systems appear to mediate the transport of Ca^{2+} (Paemeleire et al., 2000) and $\alpha 3$ connexin is a gap junction protein expressed in lens fibre cells (Vaney and Weiler, 2000). Knockout mice for $\alpha 3$ connexin (-/-) have been generated (Gong et al., 1998) and when lenses from $\alpha 3$ (-/-) mice were recently compared to

those of wild type mice, an age-dependent rise in the total Ca^{2+} of the lens nuclear regions of the genetically modified mice was observed. These elevations in nuclear Ca^{2+} were due to both increased Ca^{2+} influx and reduced Ca^{2+} efflux and correlated spatially and temporally, with nuclear cataract formation (Baruch et al., 2001).

Recent studies have suggested that the maintenance of Ca^{2+} homeostasis in lens epithelial cells may involve gap junctions (Churchill et al., 2001) but currently, the primary mechanism involved in this maintenance appears to be Ca^{2+} -ATPase activity (Duncan et al., 1994). During normal ageing processes, the increased entry of Ca^{2+} into the clear lenses is usually compensated for by increased activity of Ca^{2+} -ATPases (Zeng et al., 1995; Duncan et al., 1993). However, age related changes that are detrimental to the lens can decrease the efficiency of these compensatory mechanisms and in cataract, their efficiency is greatly reduced – showing approximately 50% of their normal cellular activity (Paterson et al., 1997). Ca^{2+} -ATPase are particularly sensitive to oxidation and it has been suggested that progressive oxidative damage to Ca^{2+} -ATPases leads to decreased activity of these proteins, resulting in the elevated Ca^{2+} levels associated with cataract and the reduced optical performance of aged lenses (Ahuja et al., 1999). Ca^{2+} -ATPases are also sensitive to membrane lipid order (Zeng et al., 1999; Delamere et al., 1991), which changes during cataractogenesis (Paterson et al., 1997; Borchman et al., 1993) and ageing (Zeng et al., 1999), and several studies have suggested that decreased Ca^{2+} -ATPase activity may be related to structural changes in membrane lipid (Paterson et al., 1997; Borchman et al., 1993). Most recently, studies on human lenses showed that that lens lipids have a high capacity to bind Ca^{2+} and that decreases in this capacity lead to elevated Ca^{2+} levels and increased light scattering from lipids and especially proteins, possibly indicating a further mechanism that may lead to elevated Ca^{2+}

levels in the lens (Tang et al., 2003b). Moreover, studies involving Ca^{2+} -sensitive microelectrodes to probe localised cortical opacities, and surrounding clear regions, have shown that Ca^{2+} is elevated only in the disrupted areas (Duncan, 1997).

Elevated Ca^{2+} levels are known to be associated with a number of processes that are detrimental to the lens (Rhodes et al., 2003; Tang et al., 2003a; de Valle, 2002; Biswas et al., 2001; Hightower and Misiak, 1998) including the overactivation of Ca^{2+} -dependent enzymes. Transglutaminase for example has been shown to process a number of enzymes associated with cataractogenesis (Shridas et al., 2001; Sanderson et al., 2000; Clement et al., 1998; Lorand *et al.*, 1998) but the major enzymes implicated in this eye disorder are the calpains.

1.5 CALPAINS AND CATARACT

Calpains are a growing superfamily of Ca^{2+} -dependent structurally related intracellular cysteine proteases (Goll et al., 2003; Perrin and Huttenlocher, 2002; Sorimachi and Suzuki, 2001; Wang, 2000). Calpain 2 (Shearer et al., 2000; Azuma et al., 1997), calpain 10 (Ma et al., 2001) and the calpain 3 splice variants, *Lp82* (Ma et al., 1998a; Ma et al., 1998b) and *Lp85* (Ma et al., 2000a) are the major calpains known to be expressed in the lens (Figure 1.4). Numerous studies have shown that calpain inhibitors can retard and inhibit cataractogenesis (Biswas et al., 2003; Nakamura et al., 2002; Quian and Shichi, 2000; Wang and Yuen, 1994) whilst recent studies have suggested that multiple calpains may be involved in cataractogenesis (Azuma et al., 2001; Takeuchi et al., 2001; Tomohiro et al., 1997). There is accumulating evidence that in response to elevated levels of Ca^{2+} , the proteolysis of crystallins by calpain 10, *Lp82*, *Lp85* and calpain 2 may contribute to cataractogenesis (Huang and Wang, 2001; Shearer et al., 2000; Azuma et al., 1997).

Figure 1.4 The structural organisation of calpains expressed in the lens

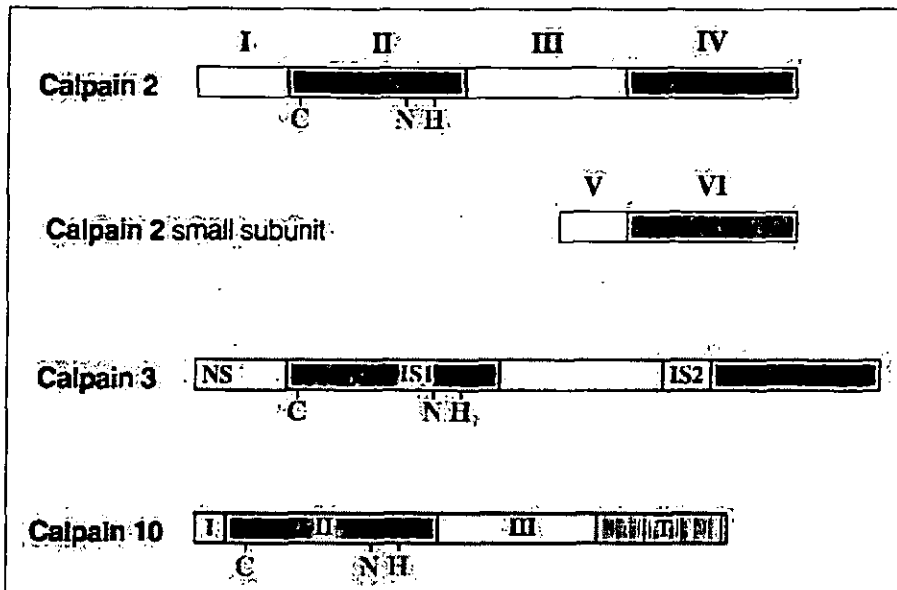


Figure 1.4 was adapted from Biswas et al., (2004) and shows the structural organisation of lens calpains implicated in cataractogenesis. Calpain 2 (and calpain 1) is dimeric, possessing a large subunit comprising domains I-IV and a smaller subunit comprising domains V-VI. Calpain 10 and calpain 3 are monomeric, possessing the structural organisation of the calpain 2, larger subunit, except that calpain 3 possesses a novel N-terminus (NS) and the unique inserts, IS1 and IS2, in domains II and IV respectively. *Lp82* (and *Lp85*) is a lens specific variant of calpain 3, lacking IS1 and IS2, and possessing a modified N-terminal region (AX1). Also represented above, in domain IV of calpain 2 and calpain 3 are five EF-hand motifs, which appear to be primarily responsible for the Ca^{2+} binding ability of these enzymes and other calpains. However, calpain 10 lacks domain IV and these EF-hands, instead possessing a divergent T domain (Goll et al., 2003; Ma et al., 2001; Ma et al., 2000). Annotated in domain II of the above calpains is the triad of amino acid residues that forms the active site of all calpains, C, H and N. Essentially, the thiol group of this cysteine residue directly participates in catalytic cleavage of the substrate peptide bond. The histidine residue, which is in close spatial proximity is also essential for the cysteine protease activity of calpains whilst other nearby residues provide groups for general acid-base catalysis (Reverter et al., 2001). A three-dimensional representation of residues forming the active site of calpain 2 is included within Figure 1.5.

1.5.1 Calpain 10

Polymorphisms in both calpain 10 and its parent gene, *CAPN10*, have been associated with human diabetes mellitus (Harris et al., 2003; Horikawa et al., 2000). Recently, Sakamoto-Mizutani *et al.*, (2002) studied calpain 10 in the lenses of Wistar Bonn/Kobori (WBN/Kob) rats, which spontaneously exhibit diabetic cataract (Nakama et al., 1985). These lenses were fractionated and immunoblot analysis showed calpain 10 to be predominantly present in the water-insoluble fraction of the lens cortex (Sakamoto-Mizutani *et al.*, 2002). Male WBN/Kob rats develop cortical cataract at 1 year of age and co-incident with the onset of this cataract, lenses from these rats showed large rises in intracellular Ca^{2+} , which correlated with the over-activation of calpain 10. Based on these results, it was suggested that calpain 10 may be involved in the development of diabetic cataractogenesis in male WBN/Kob rats (Sakamoto-Mizutani *et al.*, 2002). Consistent with these results, the highest concentrations of calpain 10 were found in the water-insoluble fraction of lens tissues derived from a rat selenite cataract model, with over-activation of the enzyme detected in the lens cortex and epithelial cells, and coincident with the onset of cataractogenesis (Ma *et al.*, 2001). However, whilst selenite-induced cataract is known to involve the Ca^{2+} induced over-activation of calpains, these cataracts are specifically located in the lens nucleus (Shearer et al., 1997) as opposed to the cortical location identified for calpain 10. In order to explain these results, it was observed that domain III of calpain 10 possesses a putative nuclear localisation signal and that the enzyme localises to the nucleus of α TN4-1 mouse cells, suggesting a role for calpain 10 in signal transduction (Ma *et al.*, 2001). Moreover, elevated Ca^{2+} levels were found to enhance this nuclear localisation of calpain 10, and based on these combined results, it was suggested that the normal function of the enzyme may be in

epithelial cell differentiation but that elevated intracellular Ca^{2+} may lead to the increased uptake of calpain 10 into the nuclei of lens epithelial cells and a contribution to selenite cataract formation. Some support for this suggestion could come from the observation that the highest levels of calpain 10 expression in the lens of non-diabetic mice were in regions of the lens associated with the differentiation of epithelial cells (Reed *et al.*, 2003).

Clearly, the results described above imply that calpain 10 may be over-activated in response to the elevated Ca^{2+} levels associated with lens cataract (Tang *et al.*, 2003). Nonetheless, a requirement for Ca^{2+} by calpain 10 has not yet been definitively demonstrated (Reed *et al.*, 2003) and the enzyme lacks domain IV of the typical calpains, which contains the EF-hand motifs generally believed to be responsible for calpain Ca^{2+} -binding activities (figure 1.4; Reverter *et al.*, 2002; Reverter *et al.*, 2001a; Reverter *et al.*, 2001b). However, it has been reported that TRA-3, a homologue of calpain 5 from *Caenorhabditis elegans*, which lacks EF-hands, shows Ca^{2+} dependent proteolytic activity, (Sokol and Kuwabara, 2000). Moreover, domain III of TRA-3 is homologous to domain III of calpain 2, which has recently been shown to possess Ca^{2+} -binding activity (Tompa *et al.*, 2001). Viewed in total, these results are suggestive of a role for calpain 10 in cataractogenesis. Further investigation of this putative role would be particularly merited when it is considered that the lens opacification exhibited by WBN/Kob rats is generally accepted as a good mimic of human diabetic cataract (Sakai, 1991).

1.5.2 Calpain 3 splice variants: *Lp85* and *Lp82*

CAPN3 encodes calpain 3 and has been implicated in several genetic disorders (Huang and Wang, 2001), including muscular dystrophy (Jia *et al.*, 2001; Gordon *et*

al., 2001; Richards et al., 1999) and diabetes mellitus (Harris et al., 2003; Horikawa et al., 2000). Multiple variants of *CAPN3* have been described in rodents (Herasse et al., 1999) and the best characterised of these at the protein level are the lens specific *Lp82* and *Lp85* (Ma et al., 2000a).

Lp82 has been localised to the nuclear and cortical regions of lens from new born mice (Ma et al., 2000b) and has been shown to co-localise with lens crystallins in the elongating fibres, and central fibres, of embryonic and post-natal mouse lenses (Reed *et al.*, 2003). However, these latter studies that found in mature mouse lenses, levels of *Lp82* were greatly reduced (Reed *et al.*, 2003). It is known that the normal post-translational proteolytic processing of crystallins is completed prior to adulthood in mice (Ueda et al., 2002a) and that this processing proceeds at a slower rate in the cortical fibres of adult murine lenses (David et al., 1994). Based on these observations, it was suggested that the normal function of *Lp82* may be in the post-translational processing of crystallins and that in adult mouse lenses, only low levels of the enzyme may be required in order to maintain crystalline architecture (Reed et al., 2003).

Previous studies on *Lp82* have shown that in maturing mouse lenses, mouse cataracts (Ma *et al.*, 1999) and mouse lenses with congenital nuclear cataract, the presence of elevated Ca^{2+} levels leads to increased crystalline proteolysis by the enzyme (Baruch *et al.*, 2001). Most recently, exogenous Ca^{2+} was used to activate endogenous calpains in total soluble protein extracts from mouse lenses (Azuma et al., 2002). These studies demonstrated that *Lp82* was able to proteolyse αA -crystallin, a subunit of the α -crystallin multimeric protein complex (Horwitz, 2003) and additionally, that this proteolysis produced C-terminally truncated forms of αA -crystallin with increased susceptibility to insolubilisation when compared to αA -

crystallin proteolysis by other calpains, suggesting enhanced potential for *Lp82* to induce cataracts (Azuma *et al.*, 2003). Based on these and other studies (Baruch *et al.*, 2001) it has been proposed that *Lp82* was the major calpain involved in mouse cataract formation (Azuma *et al.*, 2003)

Lp82 from rat lenses was found to be 99% homologous to its mouse counterpart (Ma *et al.*, 1999), to be localised to the nuclear regions of the lens (Ma *et al.*, 2000a) and able to proteolyse crystallins (Ueda *et al.*, 2002b). In these latter studies, total rat lens extracts of soluble proteins were taken and endogenous calpain activity inhibited. It was then shown that exogenous *Lp82* was able to proteolyse α A-crystallins, producing insoluble C-terminally truncated forms of the protein, the levels of which were enhanced by the addition of Ca^{2+} . Moreover, these studies found that *Lp82* produced a specific cleavage fragment, αA_{1-168} , which has also been identified in the *Lp82*-mediated proteolysis of mouse α A-crystallins (Azuma *et al.*, 2003). This fragment lacks the final five C-terminal amino acid residues of α A-crystallin and the C-terminal region of this protein, $\alpha\text{A}_{158-173}$, is essential for its chaperone activity with truncation of the $\alpha\text{A}_{158-173}$ region leading to lens crystallin aggregation, insolubilisation and cataract formation (Sharma *et al.*, 1996; Takemoto, 1994). The studies of Ueda *et al.*, (2002b) further showed that in rat lenses, endogenous levels of insoluble C-terminally truncated α A-crystallins were significantly enhanced under the elevated Ca^{2+} conditions of selenite-induced cataract when compared to age-matched lenses from healthy non-diabetic rats. It was found that the highest proportions of these C-terminally truncated α A-crystallins could be associated with *Lp82* proteolysis and the *Lp82* specific crystallin fragment αA_{1-168} whilst a similar selective insolubilisation of truncated crystallins was reported by other authors (Ueda *et al.*, 2002a). Based on these combined results, it was suggested that *Lp82* may be the

major calpain involved in selenite cataract formation in rats (Ueda *et al.*, 2002b). However, these results contrast to those of other authors (Inomata *et al.*, 2002) who studied *Lp82*-mediated crystallin proteolysis in Shumiya cataract rats (SCRs), which also demonstrate spontaneous nuclear cataract. These studies demonstrated that *Lp82* activity occurred in both normal and cataractous lenses with no specific localisation to lens regions associated with cataract formation in SCRs. Taken with the results of other studies (Inomata *et al.*, 2000), it was concluded by Inomata *et al.*, (2002) that *Lp82*-mediated proteolysis made no direct contribution to cataractogenesis in SCRs. Following on from this work, the effect of streptozotocin-induced diabetes (Perry, 1987) on the *Lp82*-mediated proteolysis of rat lens crystallins in the absence of cataract was investigated (Thampi *et al.*, 2002). These diabetic rats were killed prior to the onset of cataract and C-terminal cleavage patterns of crystallins in water-soluble fraction of their lenses analysed using electrospray ionisation mass spectrometry. It was found that levels of C-terminally truncated α A-crystallins and α B-crystallins were significantly higher in diabetic lenses than age-matched non-diabetic lenses. Moreover, the level of the *Lp82* specific crystalline fragment α A₁₋₁₆₈ was similar in both lens groups, whereas C-terminally truncated α A-crystallin fragments specific to other calpains were strongly represented in diabetic lenses but not those of non-diabetics. Based on these results, it was suggested that *Lp82* was not the major calpain activated when the rats were under diabetic stress (Thampi *et al.*, 2002). This suggestion was strongly supported by the results of studies on male WBN/Kob rats. *Lp82* was detected in both the nucleus and cortex of lenses from these rats, but the proteolytic activity of the enzyme disappeared after three months, nine months before the spontaneous appearance of the hereditary diabetes exhibited by these rats. However, other calpains appeared to be specifically activated with the

onset of cataract when under diabetic stress (Sakamoto-Mizutani *et al.*, 2002).

There is therefore some debate as to whether *Lp82* is the major murine calpain involved in lens cell crystallin processing and cataractogenesis. Adding to the debate, the *Lp82* specific crystalline fragment, αA_{1-168} , has previously been identified in various other mammalian lenses (De Jong *et al.*, 1981) and most recently, in bovine lenses, which led to the proposal that the enzyme may be the major calpain in these lenses (Ueda *et al.*, 2001). Whilst both *Lp82* and *Lp85* are believed to be absent in human lenses (Fougerousse *et al.*, 2000), studies on these enzymes may help to elucidate the roles played by calpains in human cataractogenesis. Moreover, it is interesting to note that crystallin fragments corresponding to specific *Lp82* cleavage products from αA -crystallin (Lund *et al.*, 1996; Takemoto *et al.*, 1995) and β -crystallin (Colvis *et al.*, 2000) have been detected in human lenses whilst those from γ -crystallins have been identified in some human cataracts (Baruch *et al.*, 2001). Nonetheless, several studies have clearly shown that *Lp82* does not appear to play a major role in murine diabetic cataractogenesis (Thampi *et al.*, 2002; Sakamoto-Mizutani *et al.*, 2002) and based on the current evidence, this calpain would not appear to be involved in human cataractogenesis.

At present, research on *Lp85* in relation to *Lp82* appears to be limited. Immunolocalisation studies have shown low levels of *Lp85* to be present in the lens fibre cells of developing and post natal mice but that these levels were greatly decreased in mature mouse lenses, consistent with calpain autolysis and over-activation (Reed *et al.*, 2003). It has also been shown that the proteolytic activity of *Lp85* is Ca^{2+} -dependent (Ma *et al.*, 2000a) but presently no role for the enzyme in cataractogenesis has yet been established. Nonetheless, the intimate association of this calpain with lens processes suggests that this possibility should not be excluded.

Structural studies on *Lp82* and *Lp85* have shown that in relation to calpain 3, these enzymes lack the unique insert regions, IS1 and IS2 (figure 1.4), and possess a new N-terminal region (AX1) encoded by an alternative exon (Herasse *et al.*, 1999). In addition, *Lp85* possesses a further alternative splice, which leads to the addition of a short segment to domain IV, the calpain Ca²⁺-binding region (Ma *et al.*, 2000a; Ma *et al.*, 1998a). These structural changes appear to give *Lp82* greater stability than wild type calpain 3 (Ma *et al.*, 2000b) and studies on calpain 3 splice variants have suggested that the novel AX1 N-terminus of *Lp82* and *Lp85* may play an important role in the ocular tissues of rodents (Nakajima *et al.*, 2001). Interestingly, these same studies isolated *CAPN3* isoforms encoding the AX1 region from human ocular tissue (Nakajima *et al.*, 2001). However, the functional significance of the structural differences shown between *Lp82* and *Lp85*, and calpain 3, and whether these differences influence the *Lp82* and *Lp85* activation mechanisms, are currently unknown.

1.5.3 Calpain 2

Encoded by *CAPN2*, calpain 2 is the best characterised member of the calpain superfamily (Goll *et al.*, 2003). The enzyme is highly conserved across mammalian species (Daman *et al.*, 2000) and has been implicated in a range of pathological conditions (Huang and Wang, 2001), ranging from spinal cord injury (Ray *et al.*, 2003) to Alzheimers disease (DiRosa *et al.*, 2002). However, over the last two decades, mounting evidence from numerous studies has suggested that the enzyme is involved in cataractogenesis. Earlier investigations established the ability of calpain 2 to proteolyse α -crystallins and β -crystallins and that calpain inhibitors could prevent

the progression of rodent cataract, which had been induced by the activation of calpain 2 in response to elevated Ca^{2+} levels. Based on these (Shearer, 2000; Azuma et al., 1997; Andersson et al., 1996, Shearer and Davis, 1991) and more recent studies (Goll et al., 2003; Inomata et al., 2000), it is now generally accepted that calpain 2 plays a major role in rodent lens opacification. Most recently, calpain 2 has been shown to similarly induce cataractogenesis, and to cleave crystallins, in the lenses of a variety of other mammalian species including: mice and guinea-pigs (Ueda et al., 2002a; Ueda et al., 2002b; Fukiage et al., 1997), monkeys and rabbits (Nakajima et al., 2001) and calves (Ueda et al., 2001). Nonetheless, a role for calpain 2 in human cataractogenesis has not been definitively demonstrated, although the available evidence is strongly suggestive. The presence of calpain 2 in human lenses is generally accepted whilst Andersson et al., (1994) showed that the enzyme was the major calpain in the epithelial cells of human lenses exhibiting age-related cortical cataract. Human lenses with cortical cataract are known to have increased levels of intracellular Ca^{2+} (Duncan and Bushell, 1975) whilst treating these lenses with extracellular Ca^{2+} leads to discrete cortical cataracts (Hightower and Farnum, 1985).

The activity of calpain 2 has previously been demonstrated in the cortex of human lenses (David et al., 1989) but it was only recently, that the Ca^{2+} -induced activation of calpain 2 in this type of human cataract was demonstrated under physiologically relevant conditions (Sanderson et al., 2000). It was shown that when Ca^{2+} overload was induced in cultured human lenses using ionomycin and physiologically relevant extracellular Ca^{2+} concentrations, cortical opacification resulted, accompanied by a significant loss of crystallins from the soluble fraction of the lens. These effects were strongly inhibited when extracellular Ca^{2+} was chelated with EGTA. Moreover, analysis of the insoluble fraction from human lenses showed that elevated Ca^{2+} levels

had led to high levels of vimentin proteolysis. Vimentin is a cytoskeletal protein substrate of calpain 2 in the lens, and other tissues, and vimentin proteolysis is taken as a biomarker of calpain 2 activation (Goll et al., 2003). Previous studies on animal lenses have shown that elevated Ca^{2+} levels lead to almost total degradation of vimentin but that this effect can be strongly reduced by calpain inhibitors (Sanderson et al., 1996; Marcantonio et al., 1991). Taken overall, these results clearly imply that calpain 2 plays a role in at least cortical human cataractogenesis.

Several studies have strongly suggested that calpain 2 is the major calpain activated in murine diabetic cataractogenesis. Sakamoto-Mizutani *et al.*, (2002) showed that in spontaneously diabetic male WBN/Kob rats, the onset of cataractogenesis was accompanied by increased levels of proteolytic activity due to the specific activation of calpain 2. Moreover, Thampi *et al.*, (2002) showed that there were high levels of the α A-crystalline cleavage fragment, α -A₁₋₁₆₂, which is specific to calpain 2, in the diabetic lenses of streptozotocin-treated rats but that the fragment was absent from the lenses of age-matched non-diabetic rats. In studies on human lenses, these latter authors showed that levels of C-terminally truncated α A-crystallins were significantly higher in diabetic cataracts than in age-matched non-diabetic cataracts but were not able to ascribe the crystalline cleavage sites to calpains. In addition, other studies have shown that α A-crystallin chaperone activity is strongly decreased in human diabetic cataracts with these levels of activity less than in age-matched senile human cataracts (Cherian-Shaw et al., 1999; Cherian and Abraham, 1995; Thampi and Zarina, 2002). At present there appears to be no clear demonstration of calpain 2 activity in diabetic lenses. However, it is known that calpain activity in normal human lenses is low, *circa* 3% of that in rat lenses (David et al., 1989) and most recently it has been shown that overall levels of Ca^{2+} found in the

lenses of diabetic cataract are statistically no different to those of normal lenses, implying that the elevated levels of Ca^{2+} known to be associated with diabetic cataract may be highly localised to specific lens regions (Tang et al., 2003).

1.5.3.1 The activation of calpain 2

Despite many years of intensive study, full details of the Ca^{2+} -mediated activation of calpain 2 have not been elucidated (Goll et al., 2003; Perrin and Huttenlocher, 2002). Some progress was made in this direction when the recently solved crystal structures of Ca^{2+} free, rat calpain 2 (Hosfield et al., 1999) and human calpain 2 (Strobl et al, 2000) suggested a structural basis for the Ca^{2+} dependence of the enzyme. These studies confirmed the dimeric nature of the enzyme, which possesses an 80 kDa large subunit with the four domain organisation (I, II, III and IV) of typical calpains, and a 30 kDa smaller regulatory subunit comprising domains VI and V (figure 1.4; Sorimachi and Suzuki, 2001). Moreover, the high-resolution data provided by the studies of Strobl et al, (2000) and Hosfield et al., (1999) revealed that domain II of calpain 2, which houses the active site of the enzyme, is divided into two sub-domains, IIa and IIb. Between them, these sub-domains contain the amino acid residues necessary to form this site (Figure 1.5) but in the absence of Ca^{2+} , structural constraints hold these subdomains apart, thus maintaining the active site in a disassembled state. The N-terminal segment of domain I appears to anchor domain IIa to domain VI in the regulatory subunit whilst Domain IIb makes a number of contacts with domain III, which is centrally placed within the molecule, making contact with each other domain of the enzyme. Effectively circularising the inactive catalytic subunit, domain III is connected *via* a short linker to Domain IV, which, in turn

associates with domain IV of the regulatory subunit (Strobl *et al.*, 2000; Hosfield *et al.*, 1999). This association of domains IV and VI has previously been shown to proceed *via* predominantly hydrophobic interactions and facilitates the heterodimerisation of the calpain subunits (Blanchard *et al.*, 1997; Lin *et al.*, 1997). Based on these results, a number of authors have proposed mechanisms for core events involved in the Ca^{2+} -mediated activation of calpain 2 (Figure 1.5; Reverter *et al.*, 2001a; Reverter *et al.*, 2001b; Strobl *et al.*, 2000; Hosfield *et al.*, 1999). Essentially, these mechanisms propose that Ca^{2+} binds to domains IV and VI, which each contain Ca^{2+} binding sites consisting of a series of linked α -helices forming potential EF-hand motifs (Dutt *et al.*, 2000; Kretsinger, 1997). This binding induces conformational changes in domains IV and VI, which leads to release of the domain I N-terminal anchor and initiates a series of structural events, including conformational changes, resulting in the realignment of domains IIa and IIb and the assembly of the calpain 2 papain-like cysteine protease domain (figure 1.6; Reverter *et al.*, 2002; Reverter *et al.*, 2001a; Reverter *et al.*, 2001b; Strobl *et al.*, 2000; Hosfield *et al.*, 1999). Additionally, whilst generally supporting this proposed mechanism, several studies have indicated the possibility that Ca^{2+} binding to domain II and domain III may also play a role in the activation of calpain 2 (Hata *et al.*, 2001; Tompa *et al.*, 2001; Moldoveanu *et al.*, 2001; Hosfield *et al.*, 2001).

Whilst the above results provide insight into the internal molecular events involved in the activation of calpain 2, an important need with regards to external environmental events associated with activation of the enzyme is clear elucidation of the threshold levels of Ca^{2+} required for this activation. Under normal lens cell condition, where Ca^{2+} levels are in the nanomolar range, calpain 2 exists as an inactive proenzyme (Azuma *et al.*, 1997) and several models have been proposed for

the Ca^{2+} -mediated trigger used by the enzyme for activation. It has been postulated that calpain 2 activation may involve a “calpain cascade” in which

Figure 1.5 The Ca^{2+} dependence of calpain 2 activation

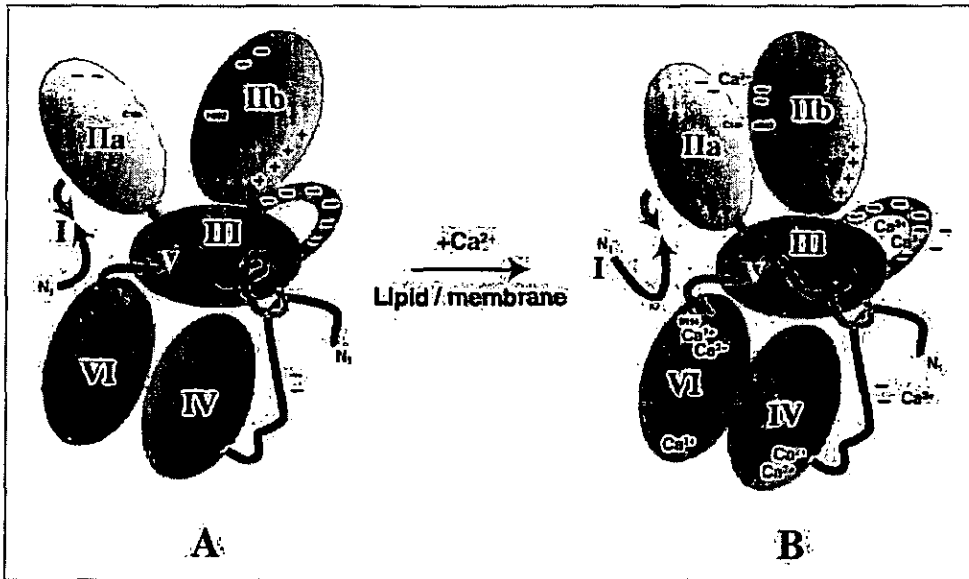


Figure 1.5 was adapted from Biswas et al., (2004) and is a representation of core events, which form the basis of the calpain 2 dependence on Ca^{2+} for activation. Figure 1.5A shows calpain 2 with subdomains IIa and IIb distal and thus, the enzyme’s active site in a disassembled state. Figure 1.5B shows Ca^{2+} binding to EF hands in domain VI, which disrupts a salt bridge between lysine 7 of domain I of the catalytic subunit and asparagine 154 of domain VI of the smaller subunit. Disruption of this salt bridge facilitates the liberation of the N-terminal region of domain I, thereby initiating a series of structural movements that bring domain IIb into close proximity with domain IIa and assembly of the calpain 2 active site, represented in figure 1.5B by cysteine 105 and histidine 262. These processes may be assisted by Ca^{2+} binding to acidic residues on both sides of the active site and lipid / membrane mediated Ca^{2+} binding to an acidic loop in domain III. Also associated with the lipid / membrane mediated activation of calpain 2 is Ca^{2+} binding to domain IV (figure 1.5B), which works in concert with the above Ca^{2+} binding events to promote other features of the enzyme’s activation process, including autolysis and subunit dissociation (figure 1.6, Azuma et al., 1997). Additionally, figure 1.5B shows that domain V of calpain 2 (in white) has a surface location, which would give this region access to lipid bodies / the membrane, consistent with the recent proposal that a membrane interactive α -helix within domain V may play a role in calpain 2 activation (Brandenberg et al., 2002).

calpain 1, which is activated in the presence of $\mu\text{molar Ca}^{2+}$, proteolyzes calpain 2, thereby reducing its Ca^{2+} requirement to a level that would permit the latter calpain to be active at physiological levels of Ca^{2+} (Tompa et al., 1996). However, more recent studies showed that calpain 1 caused no detectable proteolytic degradation of calpain 2 and did not change the Ca^{2+} concentration required for the proteolytic activity of calpain 2. It was concluded that calpain 1 was unlikely to act as an activator of calpain 2 in living cells (Thompson et al., 2000). Croall and Demartino, (1991), proposed the autolytic model of calpain 2 activation (Figure 1.6). According to this model, increases in intracellular Ca^{2+} trigger the autolytic cleavage of N-terminal segments from domain I and domain V of calpain 2. These structural modifications then lead to conformational change in the calpain molecule and separation of the truncated subunits, accompanied by activation of the enzyme (Johnson and Guttman, 1997; Azuma et al., 1997). However, there are several difficulties associated with this model. The autolysis of domain V of calpain 2 involves the cleavage of the 86 N-terminal residues from the enzyme's smaller subunit. This cleavage does not affect the enzyme's Ca^{2+} requirement and its significance is not well understood (Elce et al., 1997a). In contrast, it is well established that cleavage after residue 9 of domain I of the enzyme's larger subunit, and later possibly after residue 19, greatly reduce the enzyme's Ca^{2+} requirement for activation (Goll et al., 1992). However, a number of studies have shown that autolysis of the domain I N-terminal segment is neither strictly necessary for activity (Molinari *et al.*, 1994) nor sufficient for activation (Elce *et al.*, 1997b). Furthermore, how the autolysis model of calpain 2 activation would be triggered under normal *in vivo* conditions is unclear. It is well established that, *in vitro*, calpain 2 requires levels of Ca^{2+} in the mM range for activation (Perrin and

Huttenlocher, 2002) and clearly, these levels of Ca^{2+} do not occur in normal cellular metabolism, which are generally $\leq 1 \mu\text{molar}$ (Goll *et al.*, 1992). One possible explanation has been proposed by Azuma *et al.*, (1997) who suggested that transient, regionally specific elevations in Ca^{2+} may activate calpain 2. Nonetheless, taken overall, these results suggested that in addition to autolysis, some other factor(s) are involved in the *in vivo* activation of calpain 2. As described above, membrane-associated forms of calpain 2 have been detected in the lens and it has been shown that lipid can lower the Ca^{2+} requirement for activation of the enzyme to near physiological levels (Shearer, 2000; Azuma *et al.*, 1997). Based on these observations, Mellgren (1987) proposed the membrane activation model for calpain 2. The original model proposed by the latter author has since been developed to account for more recent data and is shown in its present form in Figure 1.6. The model shown in Figure 1.6 indicates that elevated intracellular Ca^{2+} triggers the translocation of calpain 2 from the cytosol to the membrane. There, at physiological levels of Ca^{2+} and the presence of membrane effectors such as specific phospholipids, the dissociation of the calpain 2 subunits is promoted, accompanied by activation of the enzyme. Once so activated on the membrane, calpain may diffuse into the cytosol or may be confined to acting on membrane-associated proteins (Molinari and Carafoli, 1997, Kawasaki and Kawashima, 1996). Whilst a generally accepted model, there is some debate with regards to events involved in the membrane activation model of calpain 2. Some authors have disputed the involvement of subunit dissociation in the activation of the enzyme (Dutt *et al.*, 1998; Elce *et al.*, 1997a; Zheng and Mellgren, 1996). Other studies would support the view that the subunit dissociation of calpain 2 is accompanied by autolysis, producing the N-terminally truncated large subunit of the enzyme as the active species (Dainese *et al.*, 2002; Nakagawa *et al.*, 2001; Kitagaki *et*

al., 2000). Yet other studies would suggest that subunit dissociation occurs without autolysis and that the intact lone large subunit is the enzymatically active form of calpain 2 (Cottin et al., 2001; Pal et al., 2001; Bessiere et al., 1999; Yoshizawa et al., 1995). At present, whilst subunit dissociation is generally accepted as a feature of

Figure 1.6 Activation pathways of calpain 2

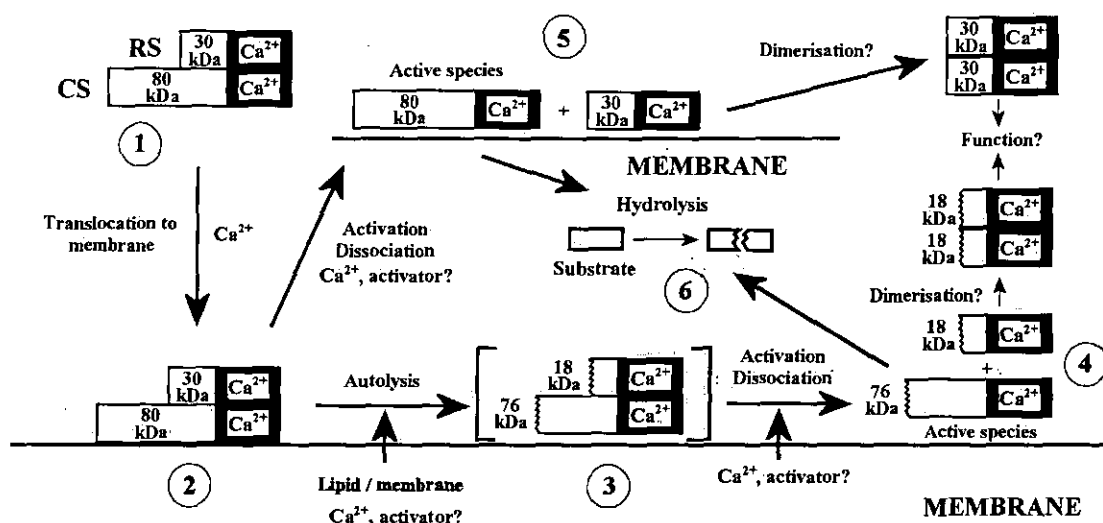


Figure 1.6 shows a schematic representation of the membrane mediated activation of calpain 2 and was adapted from Suzuki and Sorimachi (1998). In the presence of elevated intracellular Ca²⁺, dimeric calpain 2 (1) is translocated to the membrane (2). Then, in the presence of lipid / Ca²⁺ / activators(?) subunit dissociation occurs, either directly and leading to the formation of the activated 80 kDa form of calpain 2 (5), or preceded by autolysis and leading to the activated 76 kDa form of the enzyme (4). It has been suggested that an end product of both forms of activation may be the dimerisation of liberated calpain 2 small subunits.

calpain 2 activation, no proposed mechanism of activation for the enzyme has been definitively rejected. Indeed, it has been suggested that calpain 2 may employ more than one activation mechanism with choice depending upon cellular conditions (Figure 1.6; Sorimach and Suzuki, 1998; Azuma et al., 1997). A number of recent studies have progressed understanding of the membrane activation model for calpain 2. Domain III of the enzyme was found to contain an EF hand motif and was shown to

bind lipid in a Ca^{2+} dependent manner (Tompa et al., 2001). Moreover, this domain was shown to form an anti-parallel β -sheet sandwich, which is structurally related to C2 domains (Strobl *et al.*, 2000; Hosfield *et al.*, 1999) and believed to modulate enzyme activity *via* Ca^{2+} regulated lipid binding (Rizo and Sudhof, 1998). It has been proposed that a similar mechanism may allow domain III to modulate the activation of calpain 2 *via* electrostatic interactions with domain II (Reverter et al., 2002; Reverter *et al.*, 2001a; Reverter *et al.*, 2001b). Domain V of calpain 2 was shown to possess a C-terminal segment with the sequence $\text{G}_{17}\text{TAMRILGG}$, which appeared to be required for the lipid activation of calpain 2 (Crawford et al., 1990). More recent investigations, using peptides homologous to various regions of domain V, demonstrated that although the presence of the TAMRIL sequence was required for these lipid interactions, the presence of a polyglycine sequence was also necessary for such interaction (Arthur and Crawford, 1996). It was concluded by these latter authors that their data supported the general membrane activation model proposed for calpain 2 (Figure 1.6; Suzuki and Sorimachi, 1998) but no further investigations into the lipid / membrane interactions of these domain V segments appears to have been performed.

1.6 Calpain inhibitors and cataract

In view of the major role played by calpains in cataractogenesis, a number of authors have suggested that calpain inhibitors may have potential as anti-cataract agents (Biswas et al., 2001; Donkor, 2000; Wang and Yuen, 1994). However, to perform this function, these inhibitors must possess a number of characteristics, including high specificity for lens calpains, water solubility and membrane permeability. The only known compound with absolute specificity for calpains is their

endogenous inhibitor, calpastatin (Todd et al., 2003) but due to its high molecular mass, and thus membrane impermeability, this inhibitor is of little therapeutic use (Goll et al., 2003). In response, calpain inhibitors have been derived from naturally occurring sources and produced by both semi-synthetic and fully synthetic routes (Hernandez and Rousch, 2003; Ray et al., 2003; Donkor, 2000; Wells and Bihovsky, 1998; Chatterjee, 1998). Amongst the best characterised of first generation calpain inhibitors are the epoxysuccinyl peptides, which include E64 and its analogues (Figure 1.7; Wang and Yuen, 1994; Azuma et al., 1992). These inhibitors possess electrophilic centres within their structure that form an irreversible covalent bond with the thiol group of the cysteine residue in the calpain active site, thereby blocking calpain proteolytic action (Wang, 1990, Parkes et al., 1985). E64 was found to show some ability to reduce the proteolysis of β -crystallins and retard cataractogenesis in rodent models (Lampi et al., 1992; Azuma et al., 1992) but showed poor selectivity for calpain, inhibiting other cysteine proteases, and suffered from poor membrane permeability due to the presence of charged carboxylate and guanidinium groups within its structure (Wang and Yuen, 1994). The esterification of the E64 carboxyl group and the modification of the compound's guanidinobutane group to an alkyl group produced E64d, which showed improved membrane permeability. Moreover, *in vivo*, E64d is readily hydrolysed by cellular esterases to an active form, which has been shown to prevent induced-cataract in lens culture models (Ray et al., 2003; Azuma et al., 1992). A further well characterised class of first generation calpain inhibitors are the peptide aldehydes, which bind calpains in a reversible Ca^{2+} -dependent manner and include leupeptin, calpain inhibitor I, calpain inhibitor II calpeptin (Iqbal et al., 1997) and MDL28170 (Wang and Yuen, 1994). Electrophilic centres within these inhibitors, usually a functional group such as an aldehyde or a

ketone, which contains a carbonyl moiety, react with the thiol group of the active site cysteine of calpains. These reactions form covalent hemithioacetal or hemithioacetal adducts, which effectively allows these peptide aldehydes to function as competitive inhibitors of calpains (Wang, 1990). The peptide aldehydes showed improved membrane permeability and calpain selectivity over E64 and its analogues and demonstrated some success in retarding calpain-mediated cataractogenesis (Azuma et al., 1992). However, low water solubility and toxicity to lenses largely prohibited their use as pharmacological tools and anti-cataract agents (Biswas et al., 2001; Sanderson, 1996; Lampi et al., 1992).

As potential therapeutic agents in a variety of disorders, the problems of poor membrane permeability and calpain selectivity have led to the development of a large number of novel compounds directed against calpains (Ray et al., 2003; Di Rosa et al., 2002; Wang and Yuen, 1994) and indeed cysteine proteases in general (Hernandez and Rousch, 2003). These efforts have produced a chemically diverse range of novel compounds, including: reversible calpain inhibitors such as peptidyl aldehydes, α -ketocarboxyls and α -ketoamides, and irreversible calpain inhibitors such as fluoromethylketones, vinyl sulphones, diazomethylketones, acyloxymethylketones, sulphoniummethylketones, haloacylhydrazines and epoxysuccinyl peptides (Hernandez and Rousch, 2003; Ray et al., 2003; Donkor, 2000; Wells and Bihovsky, 1998). As recent examples: Novel compounds, based on short peptides with heterocyclic components bearing α,β -unsaturated carbonyl functionalities have been shown to be potent calpain inhibitors (Mann et al., 2002). Several authors have employed a peptidyl α -hydroxamate scaffold for the design of reversible calpain inhibitors and identified a hydroxamate with an N-alkoxy substituent that enhanced inhibitory potency by strengthening the hydrogen bonding between the hydroxamate

NH moiety and residues in the active site of calpain (Josef et al., 2001; Donkor et al., 2001). Peptidyl methyl ketone inhibitors have been designed using 1-hydroxybenzotriazole and other peptide-coupling reagents to produce a number of compounds, which are potent irreversible inhibitors of the enzyme (Tripathy et al., 2000). Calpastatin has been used as a structural template and it was found that the fusion of an eleven poly-arginine peptide to the inhibitor yielded a peptide with enhanced membrane permeability but no reduction in its ability to inhibit calpains when compared to naturally occurring calpastatin (Wu et al., 2003). Peptidomimetics have been introduced into the design of reversible calpain inhibitors. The substitution of peptide constituent amino acid residues with piperidine carboxamides or benzothiazine led to the identification of novel ketoamide and aldehyde inhibitors of calpains with high selectivity over other cysteine proteases (Lubisch et al., 2000; Wells et al., 2001). A number of non-peptide calpain inhibitors have been developed and recently, α -mercapto acrylate derivatives have been synthesised, which interact with the Ca^{2+} -binding domains III, IV and VI rather than the active site of calpain (Lin et al., 1997; Wang et al., 1996). One such derivative, PD150606, was reported to be highly membrane permeable and highly selective for the inhibition of calpains relative to other cysteine proteases (Wang et al., 1996), although more recently, the compound has been shown to inhibit motor neuron death *via* inhibition of kainate-induced Ca^{2+} influx and not *via* calpain inhibition (Van den Bosch et al., 2002).

In general, the testing of these novel calpain inhibitors as anti-cataract agents is in the infancy stage. Nonetheless, the lessons being learnt, coupled to the recently gained insights into calpain activation mechanisms (Reverter *et al.*, 2001a; Reverter *et al.*, 2001b; Strobl *et al.*, 2000; Hosfield *et al.*, 1999), will allow structure based

inhibitor design (Hernandez and Rousch, 2003) and the production of highly specific lens calpain inhibitors with physiochemical properties tailored for lens entry.

1.7 CONCLUSIONS

Here, an overview of current research into the role of calpains in cataractogenesis has been described and the body of evidence strongly suggests that these enzymes participate in the eye disorder. In murine lenses, and possibly those of other animals, calpain 2 and *Lp82* are the major candidates for these roles with the possibility of contributions from calpain 10 and *Lp85*. Calpain 2 appears to be the major calpain involved in animal models of diabetic cataractogenesis. *Lp82* and *Lp85* have not been detected in human lenses and it is tempting to extrapolate from animal models and suggest that calpain 2 may play a major role in at least some forms of human cataractogenesis.

As yet no definitive mechanism(s) for the activation of calpain 2 under physiological conditions appears to have been presented although the involvement of lipid / membranes in these mechanisms seems probable and it is worthy of note that high levels of Ca^{2+} -induced calpain 2 activity have been demonstrated in the membrane fraction of human lenses (Sanderson *et al.*, 2000). In response to physiologically relevant elevations of lens Ca^{2+} , there appears to be the possibility of multiple mechanisms of calpain 2 activation, leading to different enzymatically active forms of the enzyme. It would seem that the mechanism of calpain 2 activation used depends at least in part, upon the cellular location of the enzyme. It may be that in the

lens, physiological functions are performed by different isoforms of calpain 2, generated according to the location of the enzyme's activation. However, under pathological conditions such as cataract where intracellular levels of Ca^{2+} are in the mM range and above (Tang et al., 2001), autolysed forms of calpain 2 have been consistently detected (Goll et al., 2003; Shearer, 2000; Azuma et al., 1997). These observations clearly suggest that pathological levels of Ca^{2+} are able to directly induce the activation of calpain 2 *via* autolytic mechanisms. Nonetheless, it has been observed that pathological levels of lens Ca^{2+} are also likely to directly induce the conformational changes associated with the production of non-autolysed forms of activated calpain 2 (Shearer, 2000; Azuma et al., 1997). It may therefore be that in cataract, multiple mechanisms of calpain 2 activation lead to the unregulated proteolysis of crystallins and lens opacification.

1.8 Aims of the study and Scope of the work

The aim of this study was to develop an *in vitro* model of cataract in order to study the effect of calpain inhibitors in retarding this process. Here, the ability of Ca^{2+} to induce cataractogenesis in porcine lenses is investigated for the first time, extending the range of *in vitro* lens sources available for such studies. Atomic adsorption spectroscopy is used to investigate the relationship between levels of porcine lens Ca^{2+} and degree of opacification within these lenses whilst calpain inhibitors are used to confirm the participation of calpains in this opacification (chapter 2).

These investigations imply a role for calpain 2 in porcine cataract and the remaining work presented in this thesis attempts to extend the understanding of the

role played by lipid / membranes in the lens activation of this enzyme corroborating the hypothesis that membrane phospholipids play a significant role in the activation of calpain 2 *in vivo*. Hydrophobic moment plot based methodologies, hydropathy analysis and the DWIH measure of membrane interactive potential are used to identify segments within the primary structure of domain V of calpain 2 for the potential to form lipid interactive secondary structure (chapter 3).

A peptide homologue of the domain V segments thus identified is then tested experimentally for the ability to penetrate membranes, using haemolytic analysis and monolayer studies. Additionally, the ability of the peptide to adopt lipid interactive secondary structure is investigated in conjunction with German collaborators using FTIR conformational analyses (chapter 4).

A role for domain V of calpain 2 in the membrane mediated activation of the enzyme is then investigated. The large subunit of calpain 2 (LS-calpain 2) is proteolytically active in the absence of the calpain 2 small subunit. LS-calpain 2 and calpain 2 are assayed for proteolytic activity in the presence of various phospholipids in order to see if these lipids can reduce the threshold Ca^{2+} requirement of these enzymes, which is clearly demonstrated for calpain 2 but not LS-calpain 2. In addition, the lipid preferences of calpain 2 are investigated using monolayer studies (chapter 5).

In combination, the results of these studies are used to construct a putative model for the lipid / membrane activation of calpain 2 and in the discussion of the potential of calpain inhibitors to act as anti-cataract agents (chapter 6).

CHAPTER 2

The *in vitro* ability of the peptide aldehyde, SJA6017, to inhibit calpain-mediated porcine opacification

Biswas, S., Harris, F., Singh, J. and Phoenix, D. A. (2003) The *in vitro* ability of the peptide aldehyde, SJA6017, to inhibit calpain-mediated porcine cataractogenesis. *Cell. Mol. Biochem.* In press

2.1 ABSTRACT

Calpain inhibitors show the potential to support non-surgical alternatives in treating cataract. Here, the recently developed calpain inhibitor, SJA6017, and calpain inhibitor II are tested *in vitro* for their ability to inhibit opacification in porcine lenses. These lenses were incubated in increasing levels of extra-lenticular Ca^{2+} (5 mM – 30 mM). Atomic absorption spectroscopy was used to determine the total Ca^{2+} content of these lenses and a correlation between lens Ca^{2+} uptake and levels of lens opacification was found with a lens total Ca^{2+} content of the order of $5.0 \mu\text{moles Ca}^{2+} (\text{gm wet lens weight})^{-1}$ corresponding to the onset of opacification. A total lens Ca^{2+} content of the order of $8.0 \mu\text{moles Ca}^{2+} (\text{gm wet lens weight})^{-1}$ corresponded to opacity occupying approximately 70% of the lens cell volume. This degree of opacification was reduced by approximately 40%, when SJA6017 (final concentration $0.8 \mu\text{M}$) was included in the extra-lenticular medium, suggesting that the Ca^{2+} -mediated activation of calpains may be involved in the observed opacification. Supporting this suggestion atomic absorption spectroscopy showed that the effect of SJA6017 (final concentration $0.8 \mu\text{M}$) on lens opacification was not due to the compound restricting porcine lens Ca^{2+} uptake. Calpain inhibitor II showed no ability to retard porcine opacification and the results of this study are discussed in relation to the development of SJA6017 as an anti-cataract agent.

2.2 INTRODUCTION

With the realisation that calpains are involved in a spectrum of human disorders, investigation into the potential of calpain inhibitors to act as therapeutic agents has led to the production of a plethora of agents (section 1.6). One of the most potent of these agents is the recently characterised compound, SJA6017 (figure 2.1; Nakamura et al., 2003; Fukiage et al., 1997).

Figure 2.1 The chemical structure of SJA6017

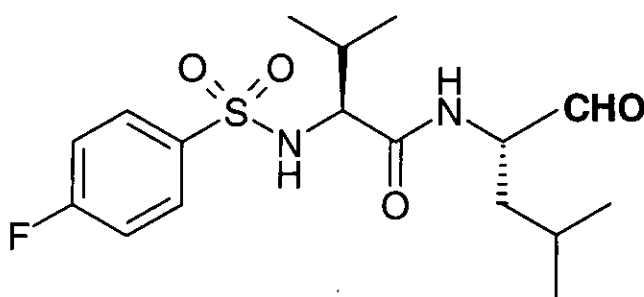


Figure 2.1 was adapted from Nakamura et al., (2003) and shows the chemical structure of the recently characterised calpain inhibitor, SJA6017 (N-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal), which is essentially a peptide aldehyde. The functional aldehyde of the compound (in bold) forms a hemithioacetal with the active SH on the cysteine residue of calpains, thereby reversibly inhibiting the proteolytic activity of the enzyme (section 1.6; Inoue et al., 2003; Fukiage et al., 1997).

The chemical structure of SJA6017 is shown in figure 2.1 and it can be seen that the inhibitor is a peptide aldehyde. Essentially, SJA6017 was synthesised by treating L-valine with 4-fluorobenzenesulfonyl chloride to give N-(4-fluorophenylsulfonyl)-L-valine. This product was then coupled with L-leucinol and the resulting compound oxidised, using a sulphur trioxide-pyridine complex, to give N-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal (Fukiage et al., 1997). SJA6017 has been

shown to be a potent inhibitor of calpain 2 (Nakamura et al., 1999; Fukiage et al.1997) and *Lp82* (Nakamura et al., 1999). In relation to other calpain inhibitors, SJA6017 shows high specificity for calpains, exhibiting only limited efficacy against other cysteine proteases and relatively low cytotoxicity (Inoue et al., 2003). Based on these therapeutically desirable characteristics, SJA6017 has been studied as a potential agent for the treatment of a variety of human disorders including: brain injury (Ray et al., 2002; Kupina et al., 2001), Alzheimers disease (DiRosa et al., 2002), retinal cell damage (Tamada et al., 2002) and angiogenesis (Tamada et al., 2000). Additionally, SJA6017 is one of the few recently developed calpain inhibitors to be tested as an anti-cataract agent. Studies on rats showed that systemic SJA6017 was taken up by the lens and inhibited *in vivo* selenite cataract formation (Tamada et al., 2001) whilst studies on cultured lenses from rats (Mathur et al., 2000; Fukiage et al., 1997), guinea pigs and rabbits (Fukiage et al., 1998) have found the inhibitor to possess high efficacy in preventing Ca^{2+} -induced models of cataract. There appears to have been no investigation into the effects of SJA6017 on calpain-mediated cataractogenesis in other mammalian lenses and in terms of physical dimensions, porcine lenses are close to human lenses. In the present study, the *in vitro* ability of SJA6017, and the well-characterised calpain inhibitor, calpain inhibitor II, to inhibit Ca^{2+} -induced opacification in porcine lenses was investigated. To ensure that this ability was not related to reduced lens cell uptake of Ca^{2+} , the total Ca^{2+} content of opacified lenses, which had been treated with either SJA6017 or calpain inhibitor II, and their corresponding controls, were determined using atomic absorption spectroscopy. The results of these studies showed that neither calpain inhibitor II nor SJA6017 were able to significantly influence porcine lens cell uptake of Ca^{2+} and that SJA6017 alone retarded opacification in these lenses.

2.3 MATERIALS AND METHODS

2.3.1 Materials

The calpain inhibitor, SJA6017, was donated by Senju Pharmaceuticals (Japan). Calpain inhibitor II and Analar calcium chloride were purchased from Sigma (UK). All other reagents were of Analar grade with $\text{Ca}^{2+} < 0.01$ ppm and purchased from Sigma (UK) except for concentrated nitric acid (70%, w/v) and lanthanum chloride, which were purchased from Fischer (UK). Aliquots (1 litre) of lens medium (LM) were prepared: A solution containing: NaCl, 137 mM; KCl, 12.7 mM; NaHCO_3 , 12mM; NaH_2PO_4 , 36 mM; Tris, 10 mM, pH 7.5 was autoclaved. The cooled solution was supplemented with: penicillin (final concentration 100 units ml^{-1}), streptomycin (final concentration 100 units ml^{-1}) and sterile glucose solution to give a final glucose concentration of 5.5 mM. The tonicity of the resulting mixture was adjusted to give a value of 300 mOsm Kg^{-1} according to Varma et al., (1986).

2.3.2 Lens photography

Lenses were photographed with their epithelial side up using an SLR camera (Nikon 55 with micro-lens attachment) against a black grid with homogeneous retro-illumination from a light table. When viewed through a transparent lens, the black grid should be visible without any discontinuity of the lines. However, since the porcine lens has a somewhat biconvex shape (as most other lenses), clear visibility of a plane grid was only obtained through the relatively central area of the lens. Some parts of the grid will therefore appear distorted or blurred in the periphery of even a

clear lens. In addition, some optical irregularity is always seen in a natural lens, and being a biological tissue, a lens is never totally transparent. Opacification was determined qualitatively by assessing the loss of visibility of the grids, either as an overall diffuse blurring, segmental discontinuity of the lines or a total loss of visibility when viewed through the central circular area of the lens, as well as the appearance of white patches within the lens substance. The results are presented as original photographs in figures 2.3, 2.4, 2.5 and 2.7.

2.3.3 Isolation of the porcine whole lens system and preliminary analyses

Paired porcine eyes were obtained from a local abattoir (Preston Meats, UK) and washed with sterile saline (0.25 mM) containing 1% (w/v) povidone-iodine. Under aseptic conditions, the lenses of these eyes were removed by gentle dissection from the posterior approach to avoid anterior epithelial damage (Holleschau et al., 1986). Lenses were visually examined and those obviously damaged, or clearly showing pre-existing cataracts were rejected (Figure 2.3A), including the matching lens in the pair. The selected pairs of matched lenses were then cleaned of adherent vitreous material, iris pigment and lens zonules, and immediately weighed. Each lens was placed on its posterior side and photographed as described above.

Preliminary experiments were performed to optimise experimental conditions such that the Ca^{2+} -mediated induction of porcine lens opacification reached desired levels without accompanying lens degradation. Accordingly, a series of matched lens pairs were taken and one lens from each pair immersed in 2 ml of sterile LM. The remaining lenses from the matched lens pairs were similarly placed in LM except that in this case, the medium contained sterile calcium chloride with concentrations that

varied between 5 mM and 30 mM. These samples were then incubated at either: 25°C or 30°C for either: 12, 15, 18 or 24 hr, lens integrity visually assessed and optimal conditions for lens incubation determined.

2.3.4 The effect of calpain inhibitors on porcine lens opacification

A series of matched porcine lens pairs were taken and one lens from each matched lens pair was incubated in sterile LM containing CaCl₂ with a concentration of either: 7.5 mM, 10 mM, 15 mM, 20 mM, or 30 mM. The remaining lens of each matched lens pair was placed in a corresponding medium except that either calpain inhibitor II or SJA6017 had been added. In all cases, the final concentration of each inhibitor corresponded to its IC₅₀ value for calpain 2, which in the case of calpain inhibitor II was 500 nM (Maki et al., 1989) and in the case of SJA6017 was 0.8 μM (Fukiage et al., 1997). Each of these samples was then incubated at 25°C for 18 hours. After incubation, the lenses were extracted, excess medium gently removed using absorbent tissue, and the lenses photographed as described above. Lenses were then placed in sterile containers and frozen at -80°C until further use.

2.3.5 Atomic absorption spectroscopy.

Lenses were allowed to come to room temperature and any visible moisture gently removed using absorbent tissue. The lenses were then weighed, individually placed in a microwave bomb containing 10 ml of concentrated nitric acid (70% w/v) and microwave digested at 180°C for 20 min (CEM Mars X microwave).

The resulting samples were then evaporated to dryness in PTFE beakers using a heating plate and re-dissolved in 25 ml of nitric acid (5% w/v), which also contained lanthanum chloride (0.2 gm l^{-1}).

Figure 2.2 ATI Unicam Flame Atomic Absorption Spectrophotometer

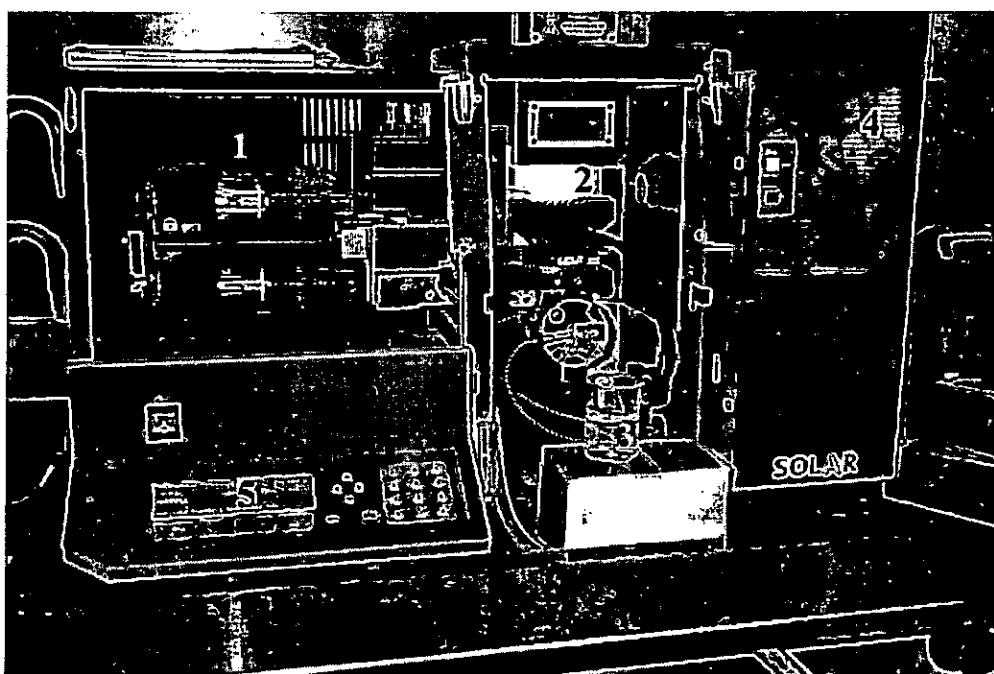


Figure 2.2 shows an interior view of a typical flame atomic absorption spectrophotometer, including: hollow cathode lamps (1), high temperature acetylene flame atomising calcium from sample (2), beaker containing test sample in solution (3), unit housing photo-multiplier diode (4) and console displaying spectrophotometric readings (5).

The Ca^{2+} content of the lens samples was then measured using atomic absorption spectroscopy (ATI Unicam Model 929, Figure 2.2). A set of Ca^{2+} calibration standards were prepared, analysed in triplicate and used to produce a calibration curve for Ca^{2+} across the range 0 ppm to 10 ppm (Figure 2.5). Total lens Ca^{2+} content was determined in triplicate and expressed as $\mu\text{moles Ca}^{2+}$ per gm wet lens weight.

2.3.6 Statistical analyses of porcine lens Ca²⁺ uptake data

Mean Ca²⁺ uptake by porcine lenses was plotted as a function of the extralenticular Ca²⁺ concentration with error bars based on the standard deviation (SD). These plots were analysed to make comparisons between porcine lenses Ca²⁺ uptake in the presence and absence of: 1) calpain inhibitor II, and 2) SJA6017, using a Regression in Groups model (Draper and Smith, 1998):

$$y = \beta_0 + \beta_1x + \beta_2I + \beta_3I \cdot x + \varepsilon$$

where β_0 , β_1 , β_2 , and β_3 are the regression coefficients with β_0 the intercept for x , and β_1 , β_2 , and β_3 the coefficients for x , I and the interaction term $I \cdot x$ respectively; x represents porcine lens Ca²⁺ uptake, I is the dummy variable denoting group membership ($I = 0$ for Ca²⁺ uptake without inhibitor and $I = 1$ for Ca²⁺ uptake with inhibitor) and ε is the error term. The Least Squares estimators for the regression coefficients, β_2 and β_3 , were analysed to establish statistically whether the curves representing porcine lenses Ca²⁺ uptake in the presence and absence of: 1) calpain inhibitor II, and 2) SJA6017, can be considered to be coincident. The results of these analyses are presented in Table 2.1.

In this and Chapters 3, 4 and 5, the results are presented as original photographs, traces and as mean \pm the standard deviation (SD). All data were analysed using MINITAB 13.1 statistical software. A 'p' value of <0.05 was taken as significant.

2.4 RESULTS

Here, the *in vitro* ability of Ca²⁺ to induce opacification in porcine lenses has been investigated. Preliminary experiments showed that when porcine lenses were incubated in LM supplemented with 30 mM Ca²⁺ for 12 hours, substantial lens

degradation occurred (Figure 2.3B and 2.3C). However, similar incubations for a duration of 18 hours at 25°C led to total opacification of the incubated lenses whilst preserving lens integrity (Figure 2.4B'). All future lens incubations were conducted under these conditions.

Control lenses, which had been incubated in LM alone showed no evidence of opacification (Figure 2.4A). However, when incubated in LM, which was supplemented with increasing levels of Ca^{2+} (5.0 mM to 30 mM), lenses showed clear evidence of progressive opacification. LM supplemented with 10 mM Ca^{2+} marked the onset of significant opacity in these lenses whilst a 15 mM Ca^{2+} supplement, led to high levels of opacification, which occupied approximately 70% of lens volume (Figures 2.5C' and 2.5D'). Lenses incubated in LM, which had been supplemented with 30 mM Ca^{2+} showed dense opacification, characteristic of advanced cataract formation.(Figure 2.4B).

Figure 2.3 Examples of porcine lenses found unsuitable for investigation

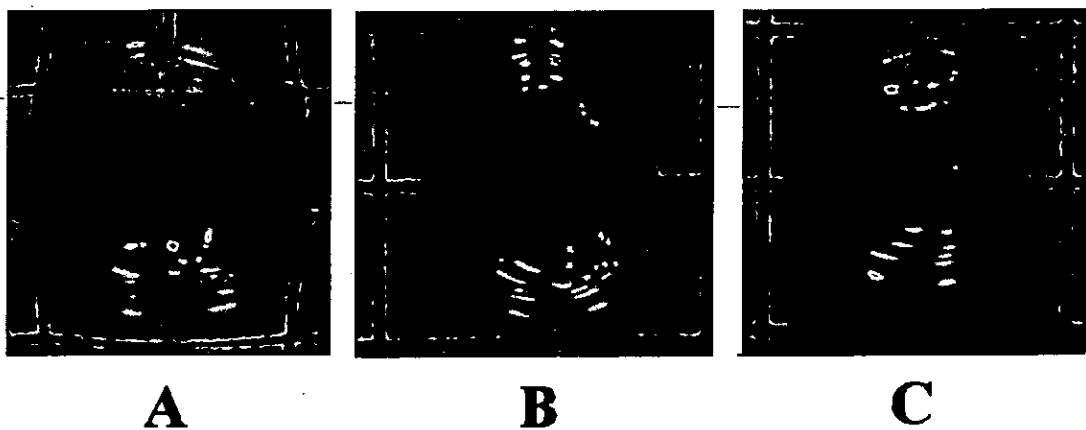


Figure 2.3A shows a porcine lens exhibiting a pre-existing sectoral opacity. Such lenses were not considered for further investigation as clearly, pre-existing opacities will confound the extent of neo-cataract formation. Figures 2.3B and 2.3C show porcine lenses that have been incubated in LM, supplemented with 30 mM Ca^{2+} , for 12 hours and the early stages of disintegration can be seen. For these lenses, disintegration of the lens capsule in a small area causes the slow release of intra-lenticular contents. As a result, the lens then loses its bi-convex spheroidal shape and normal consistency, ultimately becoming an opacified, irregular mass. All photography was performed as described in section 2.3.2.

Atomic absorption spectroscopy was used to determine the total Ca^{2+} content of lenses that had been incubated in LM supplemented with increasing levels of Ca^{2+} . Mean lens Ca^{2+} uptake was plotted as a function of the extra-lenticular Ca^{2+} concentration and it was found that a strong linear correlation (Table 2.1A and 2.1C) existed between porcine lens total Ca^{2+} content and levels of Ca^{2+} in the extra-lenticular LM medium across the range 5 mM to 30 mM (Figures 2.6A and 2.6B). The onset of opacification (10 mM extra-lenticular Ca^{2+}) corresponded to a lens total Ca^{2+} content of 5.8 $\mu\text{moles Ca}^{2+}$ (per gm wet lens weight). Increases in lens total Ca^{2+} content correlated with progressively greater levels of lens opacification (Figure 2.4 and 2.5), clearly showing a link between levels of porcine lens uptake of Ca^{2+} and degree of lens opacity.

The previous experiments were repeated except that either calpain inhibitor II (final concentration 500 nM) or SJA6017 (final concentration 0.8 μM) were included in the extra-lenticular medium. In each case, the Ca^{2+} uptake of lenses was plotted as a function of the extra-lenticular Ca^{2+} concentration and it can be seen from Figures 2.6A and 2.6B that curves representing lens Ca^{2+} uptake in the presence of inhibitors appear to be coincident with curves representing lens Ca^{2+} uptake in the absence of inhibitors.

This was confirmed by analysis of these curves using a fitted Regression in Groups model (Draper and Smith, 1998), which showed that all p-values for the β_2 and β_3 coefficients are not significant at the 5% level (Tables 2.1C and 2.1D). Analysis of the corresponding residuals confirmed that the distributional assumptions of Normality and homoscedasticity were met. These results indicate that neither calpain inhibitor II nor SJA6017 showed any significant effect on levels of lens total

Ca²⁺ content across the range 5 mM to 30 mM (Figure 2.6A and 2.6B), showing that neither inhibited porcine lens uptake of Ca²⁺.

Figure 2.4 Porcine lenses exhibiting advanced opacification

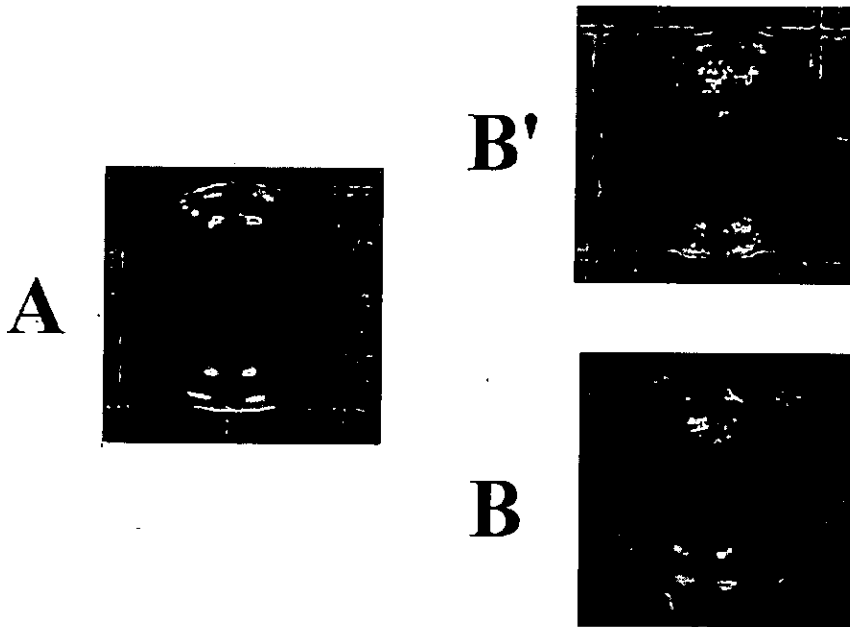


Figure 2.4A shows a lens that has been incubated in LM alone and the background grid can be clearly seen, indicating that under these conditions, opacification was not induced. Figure 2.4B' shows a lens that had been incubated in LM, supplemented with 30 mM Ca²⁺ and the lens is densely opaque with the background grid completely obscured, typical of advanced cataract. A similar result is seen in figure 2.4B where the lens was incubated in LM, supplemented with 30 mM Ca²⁺ and SJA6017 (final concentration 0.8 μ M), indicating that under conditions of high Ca²⁺, these levels of the calpain inhibitor have no detectable effect on levels of lens opacification. All photography was performed as described in section 2.3.2.

Figure 2.5 The retardation of porcine lens opacification by SJA6017

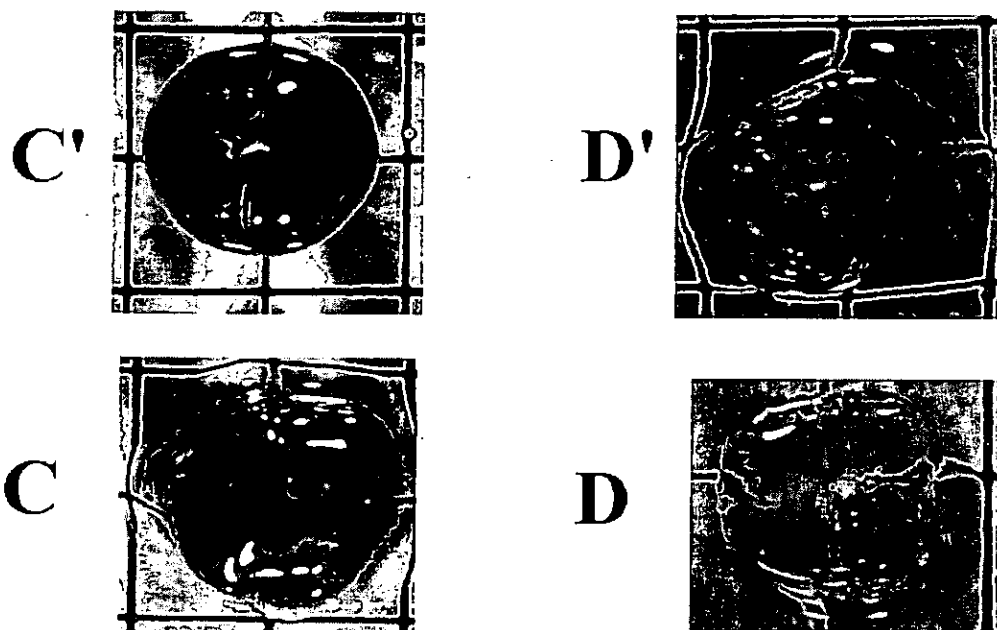


Figure 2.5C' and 2.5D' show porcine lenses that have been incubated in LM, supplemented with 10 mM and 15 mM Ca^{2+} , respectively. In Figure 2.5C', the onset of opacification is evidenced by a developing translucency, which creates a significant blurring of the background grid lines. This effect has strongly progressed in Figure 2.5D' where the background grid lines are largely obscured and the lens shows the development of several patches of localised white opaque areas, which encompasses *circa* 70% of the lens volume.

Figures 2.5C and 2.5D show porcine lenses that had been incubated in media corresponding those of 2.5C' and 2.5D' except that in both cases, SJA6017 had been included in the medium (final concentration 0.8 μM). In contrast to figure 2.5C', figure 2.5C shows that, although some optical distortion is present, the background grid lines can be more clearly seen through the lens than in Figure 2.5C. In Figure 2.5D, whilst opacification has clearly started in the lens with a diffuse obscuration of the black grid lines, this opacity occupies only *circa* 30% of the lens volume, representing a reduction of *circa* 40% of the opacity observed in Figure 2.5D'.

These results clearly show that SAJ6017 is able to retard porcine opacification under our experimental conditions. To highlight areas of opacification, higher levels of illumination were used when photographing these lenses, which were optimised in each case. All photography was performed as described in section 2.3.2.

Figure 2.6 Relationship between lens uptake of Ca^{2+} and extra-lenticular Ca^{2+} levels

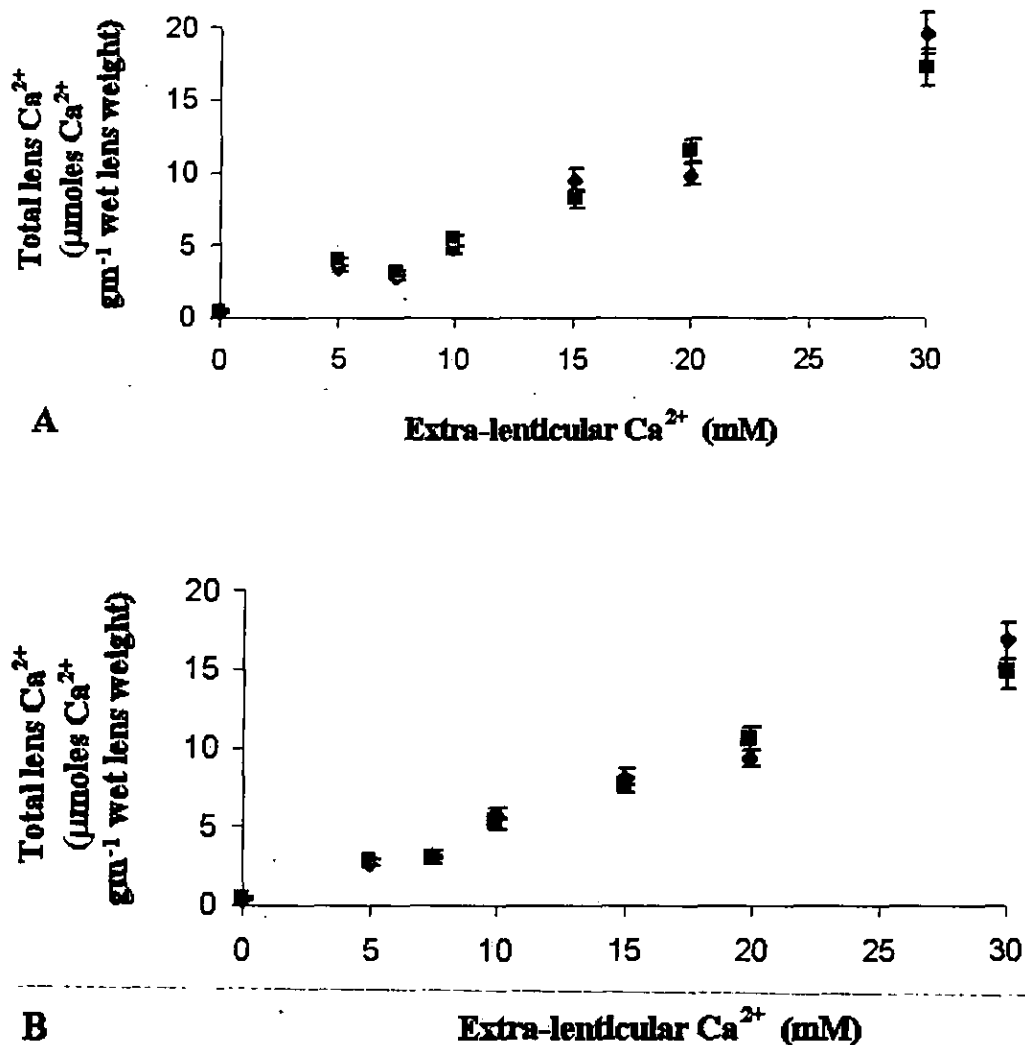


Figure 2.6A shows Ca^{2+} uptake in porcine lenses, which were incubated in LM containing increasing levels of Ca^{2+} , either in the absence (◆) or presence (■) of the calpain inhibitor SJA6017 (final concentration 0.8 μM). Figure 2.6B shows Ca^{2+} uptake in porcine lenses that were treated identically except that incubations were conducted in the absence (◆) or the presence (■) of calpain inhibitor II (final concentration 500 nM). It can be seen that in the absence of these inhibitors, there is a linear correlation between Ca^{2+} uptake levels and the concentration of Ca^{2+} in the extra-lenticular medium. It can also be seen that in both cases, the presence of calpain inhibitor in the extra-lenticular medium led to no significant change in this linear correlation, suggesting that neither inhibitor had any significant effect upon Ca^{2+} uptake by porcine lenses. Experimental procedures were as described in section 2.3.5 and each data point represents the mean of three experiments with error bars \pm SD.

Table 2.1 Statistical analyses of porcine lens Ca²⁺ uptake data

| | Correlation Coefficient | P-value |
|-------------------------------------|--------------------------------|----------------|
| Calpain Inhibitor II present | 0.964 | <0.0005 |
| Calpain Inhibitor II absent | 0.985 | <0.0005 |

Table 2.1A shows the Pearson Product-Moment correlation coefficient for Ca²⁺ uptake by porcine lenses with and without calpain inhibitor II present, against extra-lenticular Ca²⁺ concentrations with P-values for test of significance.

| Regression Coefficient | Least Squares Estimate | P-Value |
|-------------------------------|-------------------------------|----------------|
| β_0 | -0.0445 | 0.908 |
| β_1 | 0.56882 | <0.0005 |
| β_2 | 0.3301 | 0.547 |
| β_3 | -0.04641 | 0.190 |

Table 2.1B shows Regression Coefficients for Regression in Groups fit of Ca²⁺ uptake by porcine lenses with and without calpain inhibitor II present, against extra-lenticular Ca²⁺ concentrations (Adjusted Coefficient of Determination=0.96; Anderson-Darling statistic for Normality of residuals, AD=0.391, P=0.365).

| | Correlation Coefficient | P-value |
|------------------------|--------------------------------|----------------|
| SJA6017 present | 0.983 | <0.0005 |
| SJA6017 absent | 0.975 | <0.0005 |

Table 2.1C shows the Pearson Product-Moment correlation coefficient for Ca²⁺ uptake by porcine lenses with and without SJA6017 present, against extra-lenticular Ca²⁺ concentrations with P-values for test of significance.

| Regression Coefficient | Least Squares Estimate | P-Value |
|-------------------------------|-------------------------------|----------------|
| β_0 | -0.0245 | 0.948 |
| β_1 | 0.53853 | <0.0005 |
| β_2 | 0.1485 | 0.780 |
| β_3 | -0.06559 | 0.590 |

Table 2.1D shows Regression Coefficients for Regression in Groups fit of Ca²⁺ uptake by porcine lenses with and without SJA6017 present, against extra-lenticular Ca²⁺ concentrations (Adjusted Coefficient of Determination=0.96; Anderson-Darling statistic for Normality of residuals, AD=0.391, P=0.365).

The effect of calpain inhibitor II and SJA6017 on the Ca^{2+} -mediated opacification of porcine lenses was tested. These lenses were incubated in LM that had been supplemented with increasing levels of Ca^{2+} (5.0 mM to 30 mM) and either calpain inhibitor II (final concentration 500 nM) or SJA6017 (final concentration 0.8 μM). At extra-lenticular Ca^{2+} concentrations of 10 mM and 15 mM, the presence of SJA6017 (Figures 2.5C and 2.5D) led to reductions in levels of lens opacity when compared to those in lenses that had been incubated in corresponding media but lacking the inhibitor (Figures 2.5C' and 2.5D'). At 15 mM Ca^{2+} , this decrease in lens opacity was of the order of 40% of lens cell volume, showing that SJA6017 was able to retard the onset of porcine opacification (Figure 2.5D). At extra-lenticular Ca^{2+} concentrations of 30 mM, the levels of SJA6017 used in these experiments showed no significant effect on the advanced state of opacification observed in porcine lenses (Figures 2.4B and 2.4B'). In contrast to SJA6017, calpain inhibitor II showed no significant ability to retard Ca^{2+} -induced porcine lens opacification under the experimental conditions used here (Figure 2.7).

2.5 DISCUSSION

In lenses which had been incubated in LM alone, lens total Ca^{2+} content was found to be 0.38 $\mu\text{moles Ca}^{2+} (\text{gm wet lens weight})^{-1}$. Comparable values for total Ca^{2+} in the normal lenses of other mammalian species were reported by Shi et al., (1994), which varied between 0.11 and 0.6 $\mu\text{moles Ca}^{2+} (\text{gm wet lens weight})^{-1}$. As LM was increasingly supplemented with Ca^{2+} , a strong, almost linear response was shown by levels of porcine lens total Ca^{2+} content, implying progressively increased Ca^{2+} uptake by these lenses (Figure 2.6). Moreover, this Ca^{2+} uptake correlated with

Figure 2.7 The effect of calpain inhibitor II on porcine lens opacification

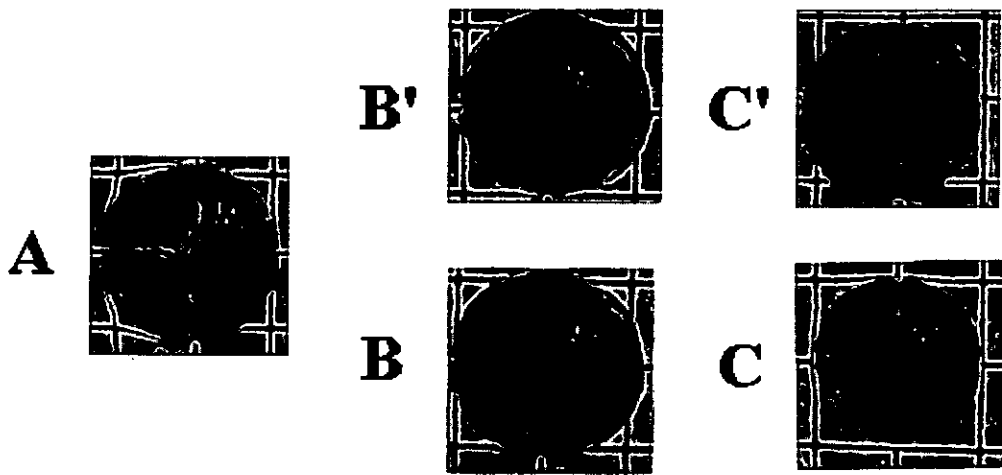


Figure 2.7A shows a porcine lens that has been incubated in LM alone and the background grid can be clearly seen, indicating that under these conditions, opacification was not induced. Figure 2.7B' shows a porcine lens that had been incubated in LM, which was supplemented with 15 mM Ca^{2+} . Similarly to figure 2.7C', the background grid lines are largely obscured with the lens showing the development of opacification, which encompasses *circa* 70% of the lens volume. Figure 2.7B shows a porcine lens that had been incubated in a medium corresponding to that of 2.7B' except that calpain inhibitor II (final concentration 500 nM) had been included in the medium. The levels of opacity shown by this lens shows no appreciable difference to that shown in figure 2.7B' indicating that calpain inhibitor II was unable to retard opacification under these conditions. The lenses shown in figures 2.7C' and 2.7C were incubated in media that corresponded to those of figures 2.7B' and 2.7B, respectively, except that 30 mM Ca^{2+} was included in these media. Again, it can be seen that the presence of calpain inhibitor II led to no apparent reductions in lens opacity, which under these Ca^{2+} conditions gives rise to lenses exhibiting dense opacity, typical of advanced cataract. All photography was performed as described in section 2.3.2.

increasing levels of lens opacification (Figures 2.4 and 2.5) and in combination, these results suggest that the activation of porcine lens calpains could be involved in this opacification. To investigate this suggestion further, the previous experiments were repeated except that either SJA6017 or calpain inhibitor II were included in the extralenticular medium. It can be seen from Figure 2.7 that calpain inhibitor II showed no apparent ability to retard porcine opacification. However, previous studies have shown this compound able to retard the opacification associated with cataractogenesis

(Tamada et al., 2001; Mathur et al., 2000; Fukiage et al., 1998; Fukiage et al., 1997) and a possible explanation for the results found here is that calpain inhibitor II shows low permeability to porcine lens cell membranes, thus inhibiting uptake of the inhibitor. In contrast, it can be seen from Figure 2.4 that whilst the presence of SJA6017 had no significant effect on the development of advanced lens opacification, it significantly affected earlier stages of lens opacification (Figure 2.5). This effect would not appear to be due to SJA6017 restricting the uptake of Ca^{2+} by porcine lens as the presence of the inhibitor had no significant effect on the ability of extra-lenticular Ca^{2+} to induce changes in the total Ca^{2+} of porcine lenses (Figure 2.6A). In combination, these results strongly suggest that the ability of SJA6017 to retard the observed porcine opacification is due to its ability to inhibit calpains, which have been activated in response to elevated levels of lens Ca^{2+} .

The levels of opacification seen in porcine lenses in the presence of 30 mM extra-lenticular Ca^{2+} (Figure 2.4B') corresponds to that observed in advanced cortical cataract (Biswas et al., 2004). The total Ca^{2+} content of these lenses was of the order of 16.0 $\mu\text{moles Ca}^{2+} (\text{gm wet lens weight})^{-1}$, which corresponds to a value of approximately 24 millimoles $\text{Ca}^{2+} (\text{Kg lens water})^{-1}$ and compares to the value of 22 millimoles $\text{Ca}^{2+} (\text{Kg lens water})^{-1}$ recently reported for advanced age-related cortical cataract in human lenses (Tang et al., 2003b). This comparability of Ca^{2+} levels at similar stages of cataract, suggests that there may be similarities in the levels of calpain activation shown by human and porcine lenses in the disorder. Current understanding is that calpain 2 is probably the only major calpain activated in human cataractogenesis (section 1.5.3) and it is interesting to note that, at the onset of porcine opacification observed in the present study (10 mM extra-lenticular Ca^{2+}), the total lens Ca^{2+} was of the order of 5.5 $\mu\text{moles Ca}^{2+} (\text{gm wet lens weight})^{-1}$, which

corresponds to a lens water Ca^{2+} concentration of approximately 8.2 mM. It is well established that millimolar levels of the ion lead to the direct activation of calpain 2, whose normal *in vitro* activity is tightly regulated by a variety of factors (section 1.5.4; Goll et al., 2003). Moreover, it can be seen from Figure 2.5 that SJA6017 significantly inhibited the onset of porcine opacification and SJA6017 is known to strongly inhibit calpain 2 activity (Fukiage et al., 1997).

Taking these results overall, it would seem that they provide a basis for further investigations. The porcine lens may be a useful model for investigating some aspects of human cataractogenesis. SJA6017 is clearly taken up by porcine lenses and shows considerable efficacy in reducing their opacification. It has previously been suggested that calpain inhibitors could be developed as anti-cataract agents for use in cases where surgery is not an option, and in the treatment of diabetic cataract as a complement to conventional diabetes therapy (Biswas et al., 2003). SJA6017, or its derivatives, may be suitable for development in such capacities. Indeed, supporting this suggestion, analogues of SJA1067 have recently been shown to be efficacious in inhibiting nuclear cataract in a rat lens culture model (Nakamura et al., 2003).

2.6 ACKNOWLEDGMENTS

The author would like to gratefully thank Dr. James Wallace for his helpful discussions with regards to the statistical analysis of data and Messrs. Azuma and Inoue of Senju Pharmaceuticals, Japan, for their generous donation of the calpain inhibitor SJA6017 used in this work. We would also like to thank Mr Kevin Pritchard for his technical assistance with the photography in this investigation.

CHAPTER 3

A theoretical investigation into the potential of calpain 2, domain V, to form lipid interactive secondary structure

Biswas, S., Harris, F., Daman, O A., Wallace, J., Phoenix, D. A. (2001) A theoretical investigation into the role of amphiphilic α -helical structure in the lipid interactions of m-calpain. Abstracts of the Association for Research in Vision and Ophthalmology, Investigative Ophthalmology and Visual Science **42**, p 5877, No 4710-B729; Daman, O. A., **Biswas, S., Harris, F., Wallace, J. and Phoenix, D. A.** (2001) A theoretical investigation into the lipid interactions of m-calpain. *Mol. Cell. Biochem.*, **223**, 159-163.

3.1 ABSTRACT

Calpain 2 is a calcium-dependent heterodimer whose activation appears to be modulated by membrane interaction involving a segment, G₁₇TAMRILGG, in domain V of the enzyme's smaller subunit. Here, sequence analysis of calpain 2 from a number of mammalian species showed domain V from each to possess a segment, centred on the TAMRIL sequence, with the potential to form a lipid interactive α -helix. These α -helical segments showed high levels of homology between species and comparable DWIH values, *circa* 7.0, indicating high membrane interactive potentials. Additionally, these α -helices were found to possess comparable hydrophobic moments ($\langle \mu_H \rangle$) of the order of 0.5, which identified them as candidate oblique orientated α -helices. Consistent with this result, hydropathy plot analysis showed each α -helix to possess a significant N \rightarrow C hydrophobicity gradient whilst graphical analysis demonstrated that each α -helix possessed strong structural similarities to the known oblique orientated α -helix of the influenza HA2 peptide. Based on these results, it is suggested that an early stage in the membrane activation of calpain 2 may be oblique angled penetration of the membrane by α -helical structure in domain V of the enzyme.

3.2 INTRODUCTION

As described in chapter 1 the membrane activation model for calpain 2 is that most generally accepted. Membrane interaction is a preliminary event in this model and previous studies have shown that a C-terminal segment, G₁₇TAMRILGG, in domain V of the enzymes smaller subunit is required for these interactions (Crawford *et al.*, 1990). A previous sequence analysis of domain V from porcine calpain 2 revealed no potential for either amphiphilic β -sheet or amphiphilic α -helix formation (Crawford *et al.*, 1996), the major elements of membrane interactive secondary structure found in proteins (Phoenix *et al.*, 2002). Here, the sequence of domain V from calpain 2 is analysed using several recently developed techniques for the detection of protein segments with membrane interactive potential. The primary structures of domain V from porcine, human, bovine and rabbit calpain 2 (table 2.1.) are analysed using the DWIH (depth weighted insertion hydrophobicity) algorithm of Roberts *et al.*, (1997). This algorithm identifies protein segments with the potential to form membrane interactive α -helices and provides a novel quantitative measure of this potential. These calpain 2 sequences are also analysed according to extended hydrophobic moment plot methodology (Harris *et al.*, 2000; Eisenberg *et al.*, 1984), which identifies protein segments with the potential to form oblique orientated α -helices. These α -helices possess highly specialised structure / function relationships and are characterised by the possession of hydrophobicity gradients, which facilitate membrane penetration at a shallow angle (Brasseur, 2000). Supported by graphical analysis (Hennig, 1999) and hydropathy plot analysis (Kyte and Doolittle, 1982), the results obtained from these sequence analyses showed that each calpain 2 examined possesses a domain V segment, centred on the TAMRIL sequence, with the potential

to form a lipid interactive oblique orientated α -helix. It is predicted that such structure may play a role in the membrane activation of calpain 2.

3.3 EXPERIMENTAL

The sequence of the HA2 influenza peptide, and those of domain V from human, bovine, porcine and rabbit calpain 2 (tables 3.1 and 3.2.) were obtained from the "SWISSPROT" data bank, (<http://ca.expasy.org/sprot/>, accessed 13.12.03) and tested for homology using the Match-box Web Server 1.3 facility for protein sequence alignment (http://www.sciences.fundp.ac.be/biologie/bms/matchbox_submit.shtml, accessed, 13.12.03). The ability of these calpain 2 domains to form lipid interactive α -helical structure was then investigated using the depth-weighted inserted hydrophobicity (DWIH) algorithm of Roberts *et al.*, (1997) and the extended hydrophobic moment plot analysis of Harris *et al.*, (2000).

3.3.1 DWIH analysis

The DWIH algorithm provides a measure of membrane interactive potential by maximising the sequestration of hydrophobic residues within a hydrophobic compartment. The window size is not fixed but optimised to identify the maximum window size, which could potentially interact with the membrane. A statistical significance for the measure is obtained by Monte-Carlo techniques, which take into account the significance of the amino acid residues present (uniform test) and the significance of the arrangements of the amino acid residues around the α -helix (random test). The DWIH algorithm was used to analyse the calpain 2 domains shown

in table 3.1 for the ability to form lipid interactive α -helical structure (tables 3.2 and 3.3).

3.3.2 Extended hydrophobic moment plot methodology

This methodology first uses hydrophobic moment analysis (Eisenberg *et al.*, 1982a) to identify putative α -helical sequences and to quantify their amphiphilicity. Essentially, the hydrophobic moment measures the structured partitioning of hydrophilic and hydrophobic residues in a regular repeat structure of period θ and hence can provide a measure of α -helical amphiphilicity (Eisenberg *et al.*, 1984). To determine the hydrophobic moment, the hydrophobicities of consecutive residues in a putative α -helical sequence are treated as vectors with positive values implying that a residue is hydrophobic. A vector with magnitude proportional to the hydrophobicity of amino acid, k , is drawn from the α -helical long axis towards the central α -carbon of the amino acid residue. The hydrophobic moment is defined by:

$$\mu(\theta) = \left\{ \left[\sum_{k=1}^l H_k \sin(k\theta) \right]^2 + \left[\sum_{k=1}^l H_k \cos(k\theta) \right]^2 \right\}^{1/2}$$

where $\mu(\theta)$ is the hydrophobic moment of a sequence of length l residues. H_k is the hydrophobicity of residue k , and is usually quantified according to the normalised consensus hydrophobicity scale of Eisenberg *et al.*, (1982b). The usual analytical approach is to consider a window, with a length of eleven amino acid residues, progressing along the amino acid sequence and for each window, the associated hydrophobic moment at 100° is calculated. Windows with maximal values of $\mu(100^\circ)$

are taken as the most likely candidates for amphiphilic α -helix formation. The amphiphilicity of an α -helix thus identified is given by the more usual form of the hydrophobic moment, $\langle \mu_H \rangle$, which is defined by $\mu(100^\circ) / 11$. For the calpain 2 domains shown in Table 3.2, $\langle \mu_H \rangle$ and the corresponding mean hydrophobicity, $\langle H \rangle$, were computed and these results are presented in Table 3.3. With these data, extended hydrophobic moment plot methodology then attempts to classify putative α -helical sequences as either globular, transmembrane, surface active or oblique orientated. Accordingly, the values of $\langle \mu_H \rangle$ and $\langle H \rangle$ computed for the windows identified in Table 3.2 were used as coordinate pairs to represent their parent α -helices as data points (Figure 3.1) on the extended form of the hydrophobic moment plot diagram (Harris *et al.*, 2000; Eisenberg *et al.*, 1984). For comparative purposes, the HA2 peptide, which is known to form a lipid interactive oblique orientated α -helix (Brasseur, 2000), was similarly analysed (Tables 3.2 and 3.3) and plotted (Figure 3.1).

3.3.3 Hydropathy plot analysis and graphical analysis

Wingen/Winpep software from Google (Hennig, 1999) was used to perform hydropathy plot analysis on the m-calpain common segment, GTAMRILGGVI (Table 3.2), using the hydrophobicity scale of (Kyte and Doolittle, 1982) and a seven-residue window (Figure 3.2). This software was also used to model this latter sequence and that of the HA2 peptide as two-dimensional axial projections, taken perpendicular to the helical long axis and assuming an amino acid periodicity of 100° (Figure 3.3).

3.4 RESULTS AND DISCUSSION

It has been suggested that the enzymatic activity of calpain 2 may be modulated by membrane interaction involving the small subunit of the protein (section 1.5.3.1). Experimental results obtained for porcine calpain 2 have shown that domain V of the protein's small subunit possesses a C-terminal segment with the sequence TAMRIL, which is required for calpain 2 – lipid interaction (Arthur and Crawford, 1996). Here, theoretical analyses were used to investigate the possibility that this lipid interaction may involve amphiphilic α -helical secondary structure. The primary structure of domain V of calpain 2 was derived for a number of mammalian species and analysis of these sequences showed that they possessed high levels of homology (> 90%) with each sequence possessing a C-terminal TAMRIL segment corresponding to that found in porcine calpain 2 (Table 3.1). These primary structures were then analysed according to the DWIH methodology of Roberts *et al.*, (1997) and for each sequence analysed, a putative α -helical region was identified (Table 3.2). These α -helical regions showed high levels of homology and possessed the common sequence GTAMRILGGVI, which can be seen from Table 3.2 to include the TAMRIL segment identified by Crawford *et al.*, (1996). Based on these results, we suggest that the TAMRIL segment may contribute to an amphiphilic α -helical region, which is present in domain V of each calpain 2 analysed. This suggestion is reinforced by the results of a further analysis of the domain V primary sequences shown in table 3.2. The hydrophobic moment methodology of Eisenberg *et al.*, (1982a) identified putative lipid interactive sequences in each case, which showed 100% overlap with those identified by DWIH analysis.

Table 3.1 Sequence homology of domain V from various mammalian m-calpains

| m-calpain | Domain V sequence |
|------------------|--|
| Human | MFLVNSFLKGGGGGGGGGGGLGGGLGNVLGGLISGA GGGGGGGGGGGGGGGGGGGGGTAMRILGGVI |
| Rabbit | MFLVNSFLKGGGGGGGGGGGLGGGLGNVLGGLISGAG GGGGGGGGGGGGGGAGGGGTAMRILGGVI |
| Porcine | MFLVNSFLKGGGGGGGGGGGLGGGLGNVLGGLISGA GGGGGGGGGGGGGGGGGGGGGTAMRILGGVISAI |
| Bovine | MFLVNSFLKGGGGGGGGGGGLGGGLGNVLGGLISGA GGGGGGGGGGGGGGGGGGGGGTAMRILGGVISAI |

Table 3.1 shows the primary sequence of domain V from calpain 2 of various mammalian species. Multiple sequence alignment, as described in section 3.3, showed these domains to possess 80% homology.

Table 3.2 α -helical regions identified in the primary structures of calpains

| Protein or peptide | Domain V sequence |
|--|----------------------------------|
| Domain V from human calpain 2 | GGG <u>GTAMRILGGVI</u> |
| Domain V from rabbit calpain 2 | GGG <u>GTAMRILGGVI</u> |
| Domain V from porcine calpain 12 | GGG <u>GTAMRILGGVISAI</u> |
| Domain V from bovine calpain 2 | GGG <u>GTAMRILGGVISAI</u> |
| Influenza virus, fusion peptide HA2 | GLFGA IAGFIENGWEGMIDG |

Table 3.2 shows putative α -helical regions, which were identified in the primary structure of various calpain 2 domain V species, and the HA2 viral fusion peptide. These sequences were identified according to either the hydrophobic moment methodology of Eisenberg *et al.*, (1982a) (shown in bold) or the DWIH methodology of Roberts *et al.*, (1997) (shown underlined), all as described in sections 3.3.1 and 3.3.2.

Table 3.3 Hydrophobic moment and DWIH parameters for α -helical regions identified in calpains

| Protein | Parameters derived from hydrophobic moment analysis | | Parameters derived from DWIH analysis | | |
|--------------------------|---|---------------------|---------------------------------------|--------------------------|-------------------------|
| | $\langle \mu_H \rangle$ | $\langle H \rangle$ | DWIH | Uniform significance (%) | Random significance (%) |
| Human calpain 2 | 0.46 | 0.47 | 6.16 | 1.2 | 0.5 |
| Rabbit calpain 2 | 0.46 | 0.47 | 6.16 | 1.2 | 0.5 |
| Porcine calpain 2 | 0.57 | 0.54 | 7.91 | 0.3 | < 0.001 |
| Bovine calpain 2 | 0.57 | 0.54 | 7.91 | 0.3 | < 0.001 |
| Influenza HA2 | 0.23 | 0.48 | - | - | - |

For the putative α -helical regions shown in table 3.2 table 3.3 shows values of $\langle H \rangle$ and $\langle \mu_H \rangle$, which were computed according to the hydrophobic moment methodology of Eisenberg *et al.*, (1982a) and, DWIH values and associated parameters, which were computed according to the methodology of Roberts *et al.*, (1997), all as described in sections 3.3.1 and 3.3.2.

For each putative α -helical region identified by hydrophobic moment analysis, the values of $\langle \mu_H \rangle$ and $\langle H \rangle$ were computed (Table 3.3) and these values then used to plot data points (Figure 3.1) on the extended hydrophobic moment plot diagram (Harris *et al.*, 2000; Eisenberg *et al.*, 1984). It can be seen from Figure 3.1 that these data points lie in the area delineating candidate oblique orientated α -helices, proximal to that of the HA2 peptide, which is known to form such an α -helix. These observations suggest that the calpain 2 segments analysed may form oblique orientated α -helices. This suggestion is reinforced by the results of hydrophathy plot analysis of these sequences, (Figure 3.2), which showed that there was a progressive increase in hydrophobicity in moving from the N-terminus to the C-terminus (Figure 3.2), suggesting the ability to form an α -helix with an asymmetric distribution of hydrophobicity along the α -helical axis.

Figure 3.1. Hydrophobic moment plot analysis of protein segments

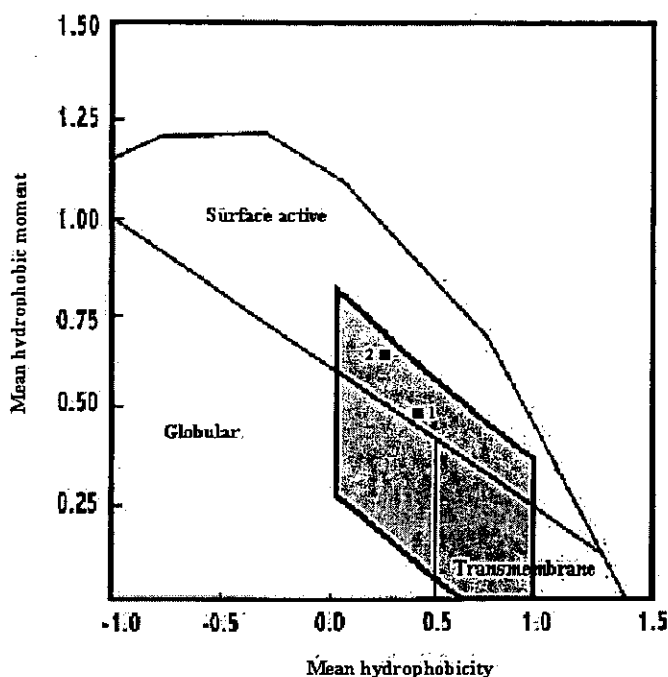


Figure 3.1 shows the conventional hydrophobic moment plot diagram of Eisenberg *et al.*, (1984) with an overlaid grey region delineating candidate oblique orientated α -helices (Harris *et al.*, 2000). The sequences shown in table 3.2 were plotted on the diagram according to their $\langle \mu_H \rangle$ and corresponding $\langle H_0 \rangle$ values (table 3.3), all as described in section 3.3.2. The data point representing the calpain 2, domain V segment, GTAMRILGGVI (1), can be seen to lie in the grey region, proximal to that representing the HA2 peptide (2), indicating that the segment may be a candidate for oblique orientated α -helix formation.

Figure 3.2. Hydropathy plot analysis of protein segments.

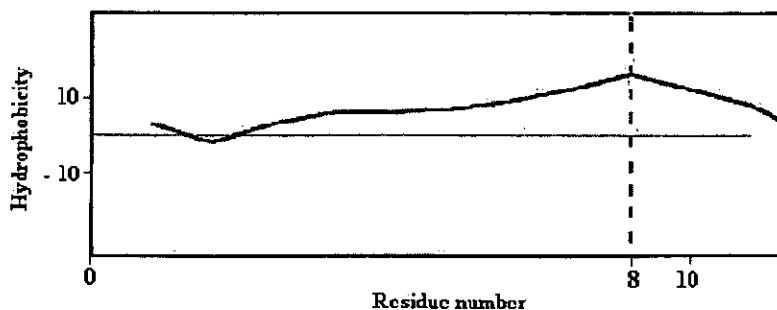


Figure 3.2. shows an hydropathy plot analysis of the calpain 2, domain V segment, GTAMRILGGVI, performed using a seven residue window and the software of Hennig (1999), all as described in section 3.3.3. It can be seen that hydrophobicity progressively increases along the length of the segment with a maximal value centred on the C-terminal glycine, residue 8.

Similar asymmetric distributions of hydrophobicity, or hydrophobicity gradients, are found in the α -helices of viral fusion peptides such as HA2, and these α -helices possess highly specialised structure / function relationships; their hydrophobicity gradients facilitating membrane penetration at a shallow angle and the promotion of membrane fusion (Brasseur, 2000). It has been established that both the nature and the spatial organisation of the amino acid residues forming oblique orientated α -helices is important for their biological activity (Nievea and Agirre, 2003; Fujii, 1999; Decout *et al.*, 1999). The DWIH measure of the predicted α -helical regions (Table 3.2) have been computed and the results show that both the uniform and random significance of these measures is $\ll 5\%$, indicating that both the nature and the order of the amino acid residues forming these α -helices is highly significant. To investigate this observation further, the sequence common to the predicted domain V α -helical regions, GTAMRILGGVI, and that of the HA2 peptide (Table 3.2), were taken and represented as a two dimensional projections (Figure 3.3). It can be seen from figure 3.3 that both sequences exhibit structural similarities with each possessing a narrow hydrophilic face, which is rich in glycine residues and a wide hydrophobic face, which is rich in bulky amino acid residues. Moreover, it can be seen for the GTAMRILGGVI projection that the bulky residues, leucine and isoleucine are preponderant in the C-terminal region of the α -helix. This localisation of strongly hydrophobic amino acid residues is structurally consistent with the higher levels of hydrophobicity predicted for the C-terminal region of the segment (Figure 3.2) and possession of an hydrophobicity gradient (Phoenix *et al.*, 2002; Brasseur, 2000).

Figure 3.3. Two-dimensional axial projections of protein sequences

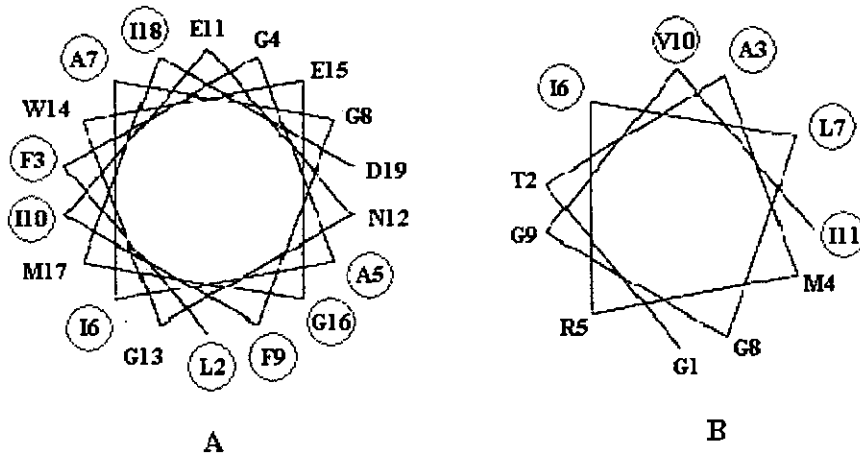


Figure 3.3. shows the primary structures of: (A) the influenza peptide, HA2, a known oblique orientated α -helix former and (B) the putative oblique orientated α -helix forming segment identified in calpain 2, domain V (table 3.2), represented as two-dimensional axial projections using the software of Hennig, (1999), all as described in section 3.3.3. Annotated numbers represent the relative locations of amino acid residues within protein primary structure and hydrophobic residues are circled. It can be seen that each α -helix possesses a glycine rich polar face and a wide hydrophobic face rich in bulky amino acid residues. In the case of the GTAMRILGGVI segment these residues, isoleucine (6) and leucine (7 and 11) can be seen to be localised in the C-terminal region of the α -helix.

Studies on the HA2 peptide and others of this α -helical class have shown that the positional preferences of glycine residues are core to the function of these α -helices (Fujii, 1999). It has been suggested that the presence of glycine residues within these α -helical arrangements is required to maintain the balance between hydrophilicity and amphiphilicity needed for membrane interaction (Harris et al., 2000) and we speculate that this could be the case for our predicted α -helical regions.

In conclusion, it is suggested that the lipid interactions of each calpain 2 analysed involves an amphiphilic α -helical region in domain V, which includes the TAMRIL segment identified by Arthur and Crawford, (1996). These α -helical regions show a number of structural resemblances to proteins and peptides, which associate

with lipid / membranes *via* oblique orientated α -helices. Based on these results, it is suggested that an early stage in the membrane activation model proposed for calpain 2 may be oblique angled penetration of the membrane by α -helical structure in domain V of the enzyme. Clearly, these results form a basis for future investigations into the role of lipid / membranes in the activation of calpain 2 and such investigations are undertaken in chapters 4 and 5.

CHAPTER 4

Biophysical investigation into the potential of domain V, calpain 2, to form lipid interactive secondary structure

Phoenix, D. A., Dennison, S., Harris, F., Hauß, T., Dante, S., Brandenburg, K. and Biswas, S (2003) Investigations into the membrane interactions of m-calpain, an enzyme implicated in diabetic cataractogenesis. 4th International Symposium on Diabetes Mellitus and its Complications: From Molecular Biology to Clinical Medicine. Preston. p18; Biswas, S., Daman, A., Harris, F. and Phoenix, D A. (2001) *A theoretical investigation into the lipid interaction of m-calpain*. Proceedings of the 4th European Symposium of the Protein Society, Protein Science **10**, (suppl 1), p 149, No. 367; Biswas, S., Harris, F. and Phoenix, D. A. (2001) The lipid interactions of m-calpain. Abstracts of the Biochemical. Society, 675th meeting, p39, No 51.

4.1 ABSTRACT

In chapter 3 it was predicted that a lipid interactive oblique orientated α -helix, formed by a GTAMRILGGVI segment in domain V of calpain 2 may feature in the membrane activation of the enzyme. Here, FTIR conformational analysis shows a peptide homologue of this segment, VP1, to be predominantly β -sheet in free solution but to adopt α -helical structure in the presence of DMPC (20%) and DMPS (65%). The peptide interacted strongly with monolayers formed from either of these lipids ($\Delta SP = 5.0 - 5.5 \text{ mN m}^{-1}$) at surface pressures mimetic of naturally occurring membranes (30 mN m^{-1}) but by apparently different mechanisms in each case. VP1 interacted primarily with the headgroup region of DMPC monolayers but was able to penetrate the membrane core region of DMPS monolayers. Additionally, the presence of ionic agents reduced the ability of VP1 to penetrate DMPS monolayers by approximately 50%. The peptide also showed strong haemolytic ability (half-maximal haemolysis = 1.45 mM) and based on these combined results, it is suggested that anionic lipid facilitates haemolysis and membrane penetration of VP1 *via* mechanisms that are consistent with its adoption of oblique orientated α -helical structure. These results strongly support the predicted use of such structure by domain V of calpain 2 in the membrane activation model of the enzyme (chapter 3).

4.2 INTRODUCTION

A wide variety of biophysical systems have been developed to study the interactions of proteins / peptides with membranes and these may be generally subdivided into those that use model membranes and those that use naturally occurring membranes. The most commonly used systems based on naturally occurring membranes are whole cell lytic systems, which are cells possessing an internal biomarker that can be quantitatively determined when externalised, thus providing a measure of protein mediated membrane lysis. To study the interactions of proteins with prokaryotic membranes, bacterial cells have been genetically engineered to produce enzymes or proteins that are chosen for their ability to serve as biomarkers of bacteriolysis (Harris et al., 2003). However, to study the interactions of proteins / peptides with eukaryotic membranes, the most widely used approach is haemolytic analysis in which proteins under study are incubated with erythrocytes. The haemoglobin of these cells acts as a naturally occurring biomarker of protein-mediated erythrocyte lysis and is readily quantified spectrophotometrically when released from the cell (Brandenberg et al., 2002; Harris and Phoenix, 1997).

Liposomes are frequently used as model membranes and provide a good physical mimic of the whole-cell membrane systems found *in vivo*. Such lipid bodies are often used to provide a lipid / water interface, mimicking the amphiphilic environment of a membrane surface, when studying the conformational behaviour of proteins in the presence of membranes. The other major model used to mimic naturally occurring membranes is the monolayer system, which possesses a number of advantages over other membrane models. Monolayers allow chosen parameters to be widely varied, including: the nature of the lipid molecules forming the monolayer, the

composition of the subphase, and a variety of incidental factors such as pH and temperature. This choice permits a variety of *in vivo* situations to be mimicked and moreover, monolayers generally possess stability and well-established planar geometry where the lipid molecules have a specific orientation, assisting the interpretation of lipid / monolayer interactions (Maget-Dana, 1999). However, perhaps the major advantage of monolayer use in membrane studies is that their surface pressure can be adjusted to choice and an initial surface pressure of 30 mN M^{-1} is known to be mimetic of naturally occurring membranes (Demel, 1974). Thus, with an appropriate choice of lipid(s), a variety of specific *in vivo* membrane situations can be mimicked and used to investigate differing physiological functions of membrane interactive proteins and peptides.

In chapter 3, theoretical analyses predicted that domain V of calpain 2 possessed a segment, GTAMRILGGVI, with the potential to form a lipid interactive oblique orientated α -helix, which may feature in the membrane activation model of the enzyme. Here, FTIR spectroscopy is used to study the conformational behaviour of a peptide homologue of this segment, VP1, in the presence of zwitterionic and anionic liposomes. Langmuir-Blodgett troughs were used to investigate the ability of the peptide to interact with monolayers formed from these respective lipid types whilst the interactions of VP1 with naturally occurring membranes are studied using haemolytic analysis.

4.3 MATERIALS AND METHODS

4.3.1 Reagents

The peptide VP1 with sequence GTAMRILGGVI was supplied by PEPSYN, University of Liverpool, UK, produced by solid-state synthesis and purified by HPLC to a purity of greater than 99%. The peptide was stored as a stock solution (10 mM) in 10% (v/v) ethanol at 4°C. Human erythrocytes were supplied by the Royal Preston Hospital, UK. Dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylserine (DMPS) and all solvents, which were of spectroscopic grade, were purchased from Sigma (UK).

4.3.2 Preparation of phospholipid small unilamellar vesicles

Small unilamellar vesicles (SUV's) were prepared according to Keller et al., (1992). Essentially, lipid / chloroform mixtures were dried with nitrogen gas and hydrated with aqueous N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid], (HEPES) at pH 7.5 to give final phospholipid concentrations of 50 mM. The resulting cloudy suspensions were sonicated at 4°C with a Soniprep 150 sonicator (amplitude 10 milli-decibels) until clear suspensions resulted (30 cycles of 30 seconds), which were then centrifuged (15 min, 3000 g, 4°C).

4.3.3 FTIR conformational analyses of VP1

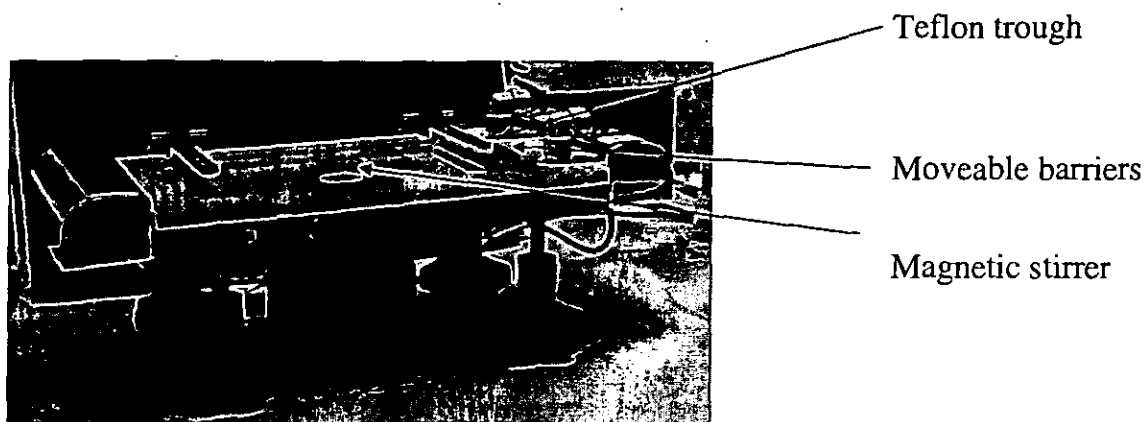
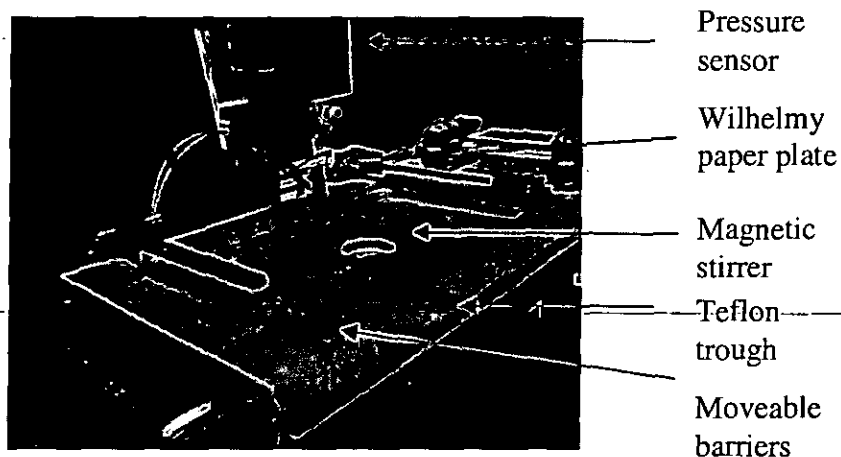
To give a final peptide concentration of 1 mM, VP1 was solubilised in 50 mM aqueous HEPES (pH 7.5) or suspensions of SUV's, which were formed from either: DMPS, DMPC or DMPE, prepared as described above. Samples of solubilised peptide were spread on a CaF₂ crystal, and the free excess water was evaporated at room temperature. The single band components of the VP1 amide I vibrational band (predominantly C=O stretch) was monitored using an FTIR '5-DX' spectrometer (Nicolet Instruments, Madison, WI, USA) and for each sample, absorbance spectra produced (figure 4.1). These spectra were analysed and for those with strong absorption bands, the evaluation of the band parameters (peak position, band width and intensity) was performed with the original spectra, if necessary after the subtraction of strong water bands. In the case of spectra with weak absorption bands, resolution enhancement techniques such as Fourier self-deconvolution (Kauppinen et al., 1981) were applied after baseline subtraction with the parameters: bandwidth, 22-28 cm⁻¹, resolution enhancement factor, 1.2-1.4 and Gauss / Lorentz ratio of 0.55. In the case of overlapping bands, curve fitting was applied using a modified version of the CURFIT procedure written by D. Moffat (National Research Council, Ottawa, Canada). An estimation of the number of band components was obtained from deconvolution of the spectra, curve fitting was then applied within the original spectra after the subtraction of baselines resulting from neighbouring bands. Similar to the deconvolution technique, the bandshapes of the single components are superpositions of Gaussian and Lorentzian bandshapes. Best fits were obtained by assuming a Gauss fraction of 0.55 - 0.6. The CURFIT procedure measures the peak areas of single band components and after statistical evaluation, determines the relative percentages of

primary structure involved in secondary structure formation. For VP1, relative levels of α -helical structure (1650 cm^{-1} - 1655 cm^{-1}) and β -sheet structures (1625 cm^{-1} - 1640 cm^{-1}) were computed and are shown in Table 4.1.

4.3.4 Monolayer studies on VP1

All monolayer equipment was supplied by NIMA (UK). Surface tension was monitored by the Wilhelmy plate method using a microbalance (Demel, 1974). Studies were conducted using a $5 \times 15\text{ cm}$ Teflon trough containing 80 ml of buffer subphase (10 mM Tris buffer, pH 7.5).

Figure 4.1 Langmuir-Blodgett trough for monolayer studies



The trough was equipped with moveable barriers, which responded to the microbalance and could be adjusted to maintain monolayers at either constant surface pressure or constant surface area. Monolayers were formed by spreading pure phospholipids (10 mM) in chloroform, compressed to give a surface pressure of 30 mN m⁻¹ and then maintained at constant area. Stock VP1 was added to the subphase *via* a reservoir extending into the subphase, which was continuously stirred by a magnetic bar and VP1 – monolayer interactions were recorded as changes in monolayer surface pressure (Figure 4.2)

4.3.5 Haemolytic assay of VP1

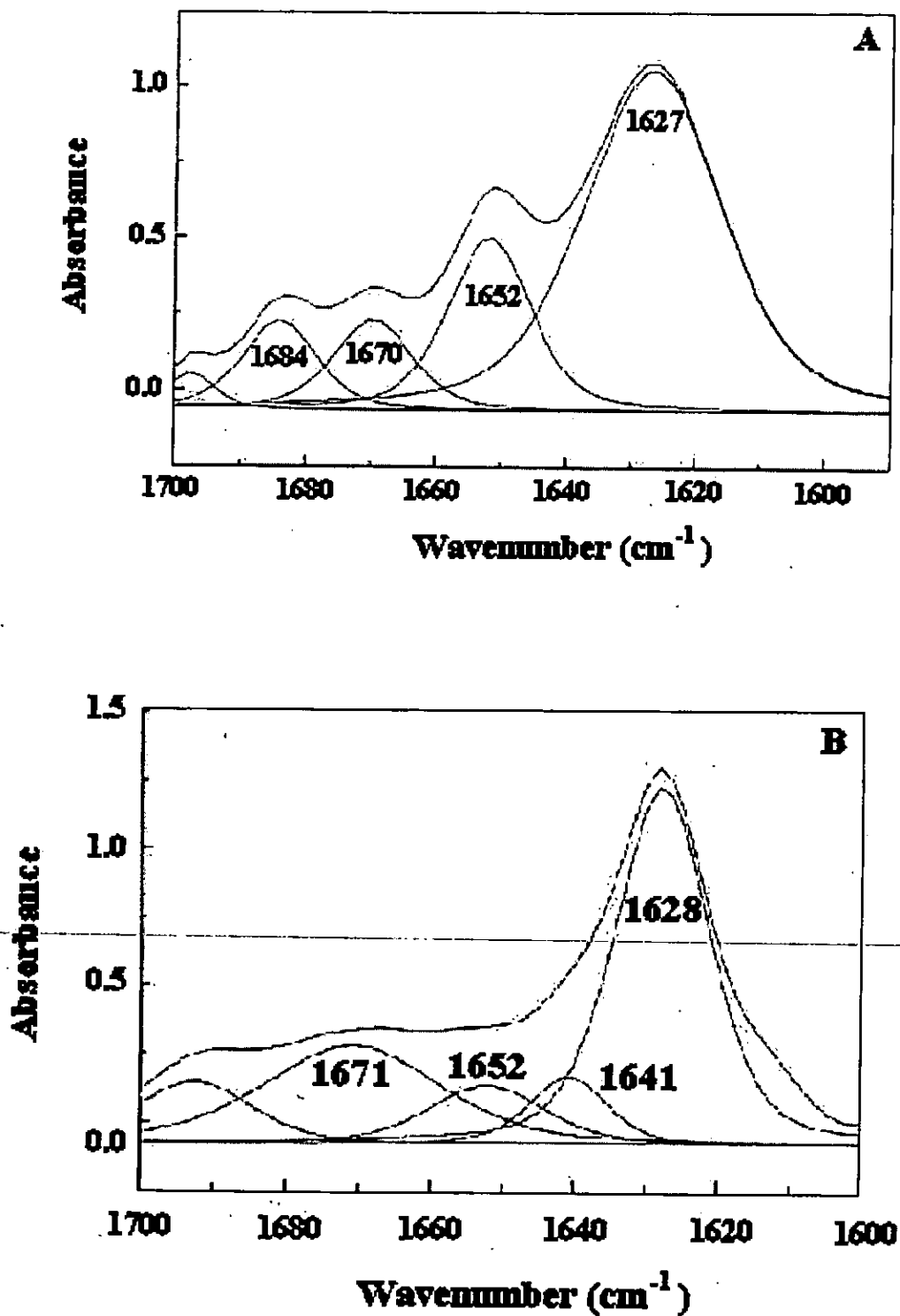
Haemolytic assay was conducted according to (Harris and Phoenix, 1997). Essentially, packed red blood cells were washed three times in Tris Buffered Sucrose (TBS; 0.25M sucrose, 10mM Tris, pH 7.5) and resuspended in the same medium to give an initial blood cell concentration of approximately 0.05 gm%(w/v). For the haemolytic assay, this concentration was adjusted such that incubation with 0.1% (v/v) Triton X-100 for 1 hour produced a supernatant with $A_{416} = 1.0$ and this was taken as 100% haemolysis. Varying amounts of VP1 were solubilised in aliquots (1 ml) of blood cells at assay concentration and the resulting mixtures incubated at room temperature with gentle shaking. After 1 hour the suspensions were centrifuged at low speed (1500g, 15 min, 25°C) and the A_{416} of the supernatants determined. In all cases, levels of haemolysis were determined as the % haemolysis relative to that of Triton-X100 and the results recorded (Figure 4.3). The addition of buffer instead of peptide gave a basal lysis of less than 1 % in all cases.

4.4 RESULTS AND DISCUSSION

Theoretical analyses have predicted that a lipid interactive oblique orientated α -helix, formed by a GTAMRILGGVI segment in domain V of calpain 2 may feature in the membrane activation of the enzyme (chapter 3). Here, this prediction was investigated using a variety of biophysical techniques to study the conformational behaviour and lipid interactive properties of a peptide, VP1, corresponding to the GTAMRILGGVI segment of calpain 2.

FTIR conformational analysis of VP1 showed that in aqueous solution, the peptide was predominantly formed from β -sheet structures, exhibiting less evidence of α -helical structure (Figure 4.2A). However, confirming theoretical predictions, these analyses showed the peptide to adopt α -helical structure in the presence of DMPC (20%) and DMPS (65%) (Table 4.1; Figure 4.2B and 4.2C), suggesting that the GTAMRILGGVI segment requires the anisotropic environment of a lipid interface to adopt α -helical structure.

Figure 4.2 FTIR conformational analysis of VP1 in the presence of lipid



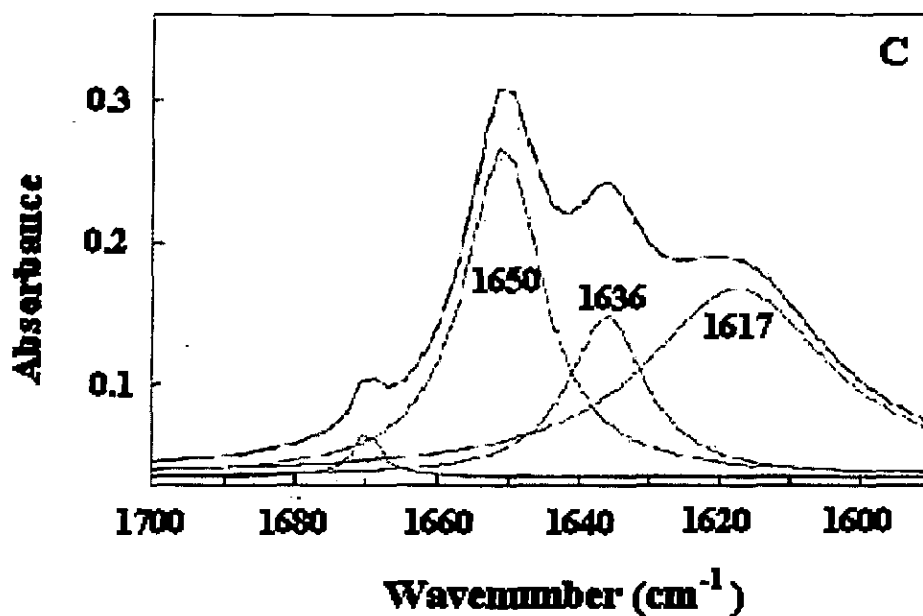


Figure 4.2 shows spectra representing FTIR conformational analyses of VP1 in aqueous buffer (A), in the presence of DMPC (B) and in the presence of DMPS (C). Annotated numbers indicate band peak absorbances and for each spectrum, the relative percentages of VP1 α -helical secondary structure (1650 cm^{-1} - 1652 cm^{-1}) were computed. In the presence of DMPC these were 20% and in the presence of DMPS, 65%. All FTIR analyses were performed as described in section 4.3.3.

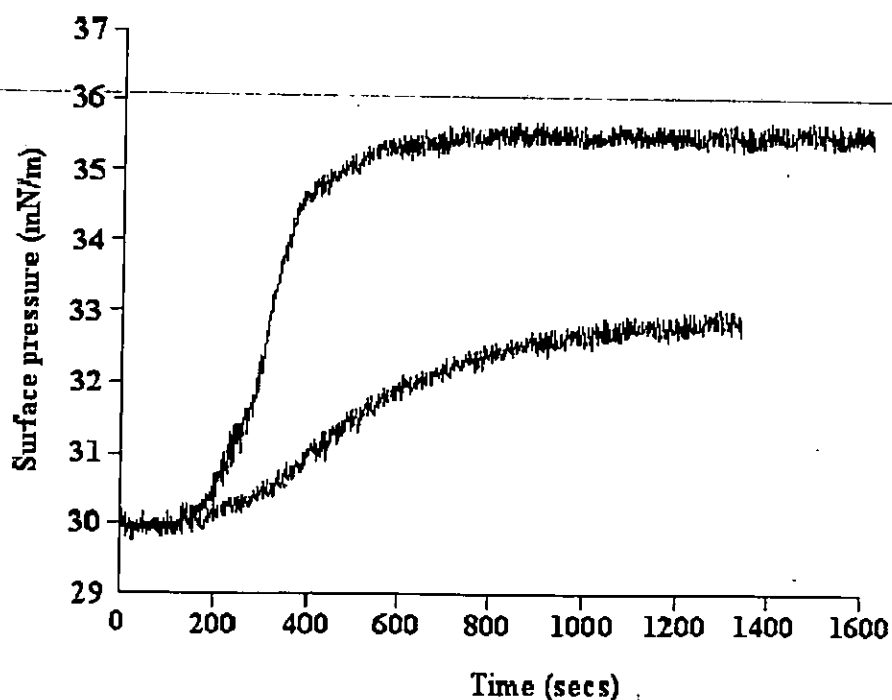
Table 4.1 VP1 secondary structural contributions in the presence of lipid

| lipid | % α -helix | % β -sheet |
|-------|-------------------|------------------|
| - | - | 92 |
| DMPS | 65 | 32 |
| DMPC | 20 | 61 |

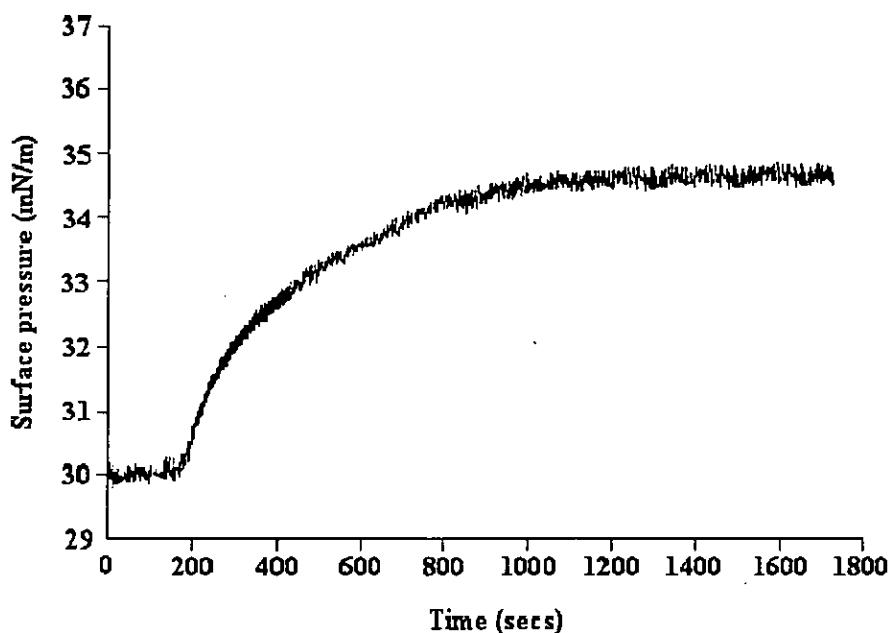
Table 4.1 shows the relative levels of α -helical structure and β -sheet structure determined in VP1, a peptide homologue of the putative oblique orientated α -helix forming segment identified in domain V of calpain 2 (Table 2.1). The peptide was either: in aqueous solution or in the presence of lipid. Conformational analysis of VP1 was performed using FTIR spectroscopy and the resulting spectra (Figure 4.1) used to determine relative levels of secondary structure as described in section 4.3.3.

VP1 adopted relatively low levels of α -helical structure (20%) (Table 4.1; Figure 4.2B) in the presence of DMPC monolayers yet interacted strongly with these monolayers, inducing a final surface pressure change of 5.0 mN m^{-1} (Figure 4.3). Previous studies have shown that this peptide has only a minor effect on the lipid phase transition temperature and fluidity of DMPC membranes (Brandenberg et al., 2002), indicating that the primary interaction of VP1 with DMPC monolayers may be with the headgroup region rather than penetration of the hydrophobic acyl chain region. Taken in combination, these results suggest that the interactions of VP1 with DMPC membranes is unlikely to involve oblique orientated α -helix formation. Indeed, it seems likely that these interactions may involve β -sheet structure, which was the predominant secondary structural type shown by the peptide in the presence of DMPC membranes (61%) (Table 4.1; Figure 4.2B).

Figure 4.3 The monolayer interactions of VP1



A



B

Figure 4.3 shows the typical time course for interactions of VP1 (final concentration $20 \mu\text{M}$) with lipid monolayers at an initial surface pressure of 30 mN m^{-1} , taken to represent that of naturally occurring membranes. Figure 4.3A shows the interaction of VP1 with DMPS monolayers (upper curve) where it can be seen that the peptide reaches maximal levels of penetration ($\Delta \text{SP} = 5.5 \text{ mN m}^{-1}$) after 600 secs. Figure 4.3A also shows that when 100 mM NaCl was introduced into the subphase of the VP1 – DMPS monolayer system (lower curve), the maximal levels of peptide – monolayer interaction were reduced by approximately 50% ($\Delta \text{SP} = 2.6 \text{ mN m}^{-1}$) whilst the time taken to achieve these levels was 1200 secs. Figure 4.3B shows that the ability of VP1 to penetrate DMPC monolayers ($\Delta \text{SP} = 5.0 \text{ mN m}^{-1}$) is comparable to that of the peptide to penetrate DMPS monolayers but the time taken to achieve these levels was increased to 1200 secs. All monolayer experiments were conducted as described in section 4.3.4.

VP1 induced a final surface pressure change of 5.5 mN m^{-1} in DMPS monolayers, indicating a capacity to interact with these monolayers that is comparable to that shown with DMPC monolayers but the use of differing mechanisms would appear to be involved. FTIR conformational analysis showed that VP1 is predominantly α -helical (65%, table 4.1; Figure 4.2C) in the presence of DMPS membranes whilst previous studies have shown that the peptide interacts strongly with the acyl chain region of DMPS membranes (Brandenberg et al, 2002). Moreover, it can be seen from figure that the kinetics of VP1 interaction with DMPS and DMPC

monolayers show marked differences (Figure 4.3). With DMPC monolayers, the presence of the peptide led to a slow progressive increase in monolayer surface pressure, which showed hyperbolic type kinetics and a maximum surface pressure change after 1200 secs (Figure 4.3). In contrast, VP1 induced a rapid, almost linear rise in the surface pressure of DMPS monolayers over 200 secs, which increased to a maximum surface pressure change after 600 secs. VP1 possesses a sole positively charged arginine residue, which suggests that electrostatic interactions between this residue and the anionic headgroup region of DMPS monolayers may contribute to the enhanced rates of interaction observed between the peptide and these monolayers. This suggestion was strongly supported when 100 mM NaCl was included in the DMPS monolayer subphase (Figure 4.3). It can be seen from figure 4.3 that the high ionic strength of this salinated subphase has greatly decreased the rate, and levels, of VP1 - DMPS monolayer interaction with maximal monolayer surface pressure changes of 2.6 mN m^{-1} occurring after 1200 secs. Taken overall, these results clearly show that the membrane interactions of VP1 involve α -helix formation by the peptide and anionic lipid.

To investigate the ability of VP1 to interact with naturally occurring membranes, the haemolytic potential of the peptide was studied. VP1 was found to be strongly haemolytic with an half-maximal lytic dose of 1.45 mM (Figure 4.4) and when taken with both the DMPS monolayer data shown in figure 4.2 and the theoretical analyses of chapter 3, these results suggest a basic model for VP1 haemolytic action. According to this model: positively charged VP1 preferentially targets anionic lipid headgroups at the erythrocyte membrane interface. In this anisotropic environment, VP1 adopts α -helical structure and the hydrophobicity gradient possessed by this α -helical structure then facilitates erythrocyte membrane

penetration at a shallow angle, leading to the destabilisation of membrane lipid packing and haemolysis. To assist this form of membrane penetration, it seems likely that the arginine residue of the VP1 α -helix would engage the snorkelling mechanism (Segrest et al., 1990). This mechanism has been shown to enhance the levels of membrane penetration achieved by many membrane interactive proteins and peptides (Strandberg and Killian, 2003) and alleviates the energetically unfavourable positioning of the positive charges carried by arginine and lysine residues within the hydrophobic membrane core region. In the case of the VP1 α -helix, the snorkelling mechanism would allow the arginine residue of this α -helix to extend its long hydrophobic alkyl chain, permitting deeper α -helix penetration of the erythrocyte membrane's hydrophobic core region yet still allowing the arginine's positively charged moiety to interact with anionic lipid headgroups.

Figure 4.4 The haemolytic action of VP1

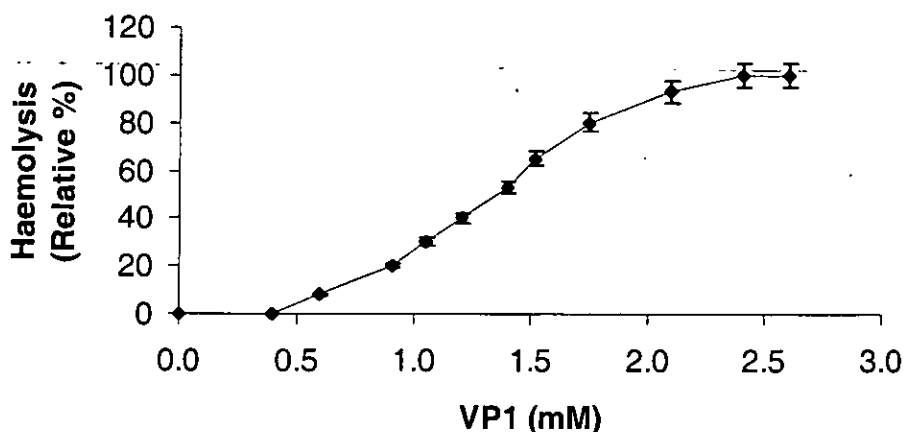


Figure 4.4 shows the haemolytic curve of VP1 when incubated with human erythrocytes. It can be seen that a threshold concentration of 0.4 mM VP1 was required for haemolysis to occur whilst a concentration of 2.4 mM, VP1 led to 100% lysis of erythrocytes ($LD_{50} = 1.45$ mM). The % haemolysis was determined in triplicate, all as described in section 4.3.5, and the mean % haemolysis plotted as a function of VP1 concentration with error bars representing \pm SD ($n=5$).

It can be seen from figure 4.4 that VP1 exhibits a sigmoidal type relationship between levels of haemolysis and VP1 concentration, which requires a threshold VP1 concentration of 0.4 mM. This relationship implies that under the experimental conditions used here, VP1 haemolytic action involves some form of cooperative action between molecules of the peptide. A number of peptides, which appear to form oblique orientated α -helices (Harris et al., 2002), have previously been reported to exhibit haemolytic ability with sigmoidal kinetics and cooperative mechanisms of action (Harris and Phoenix, 1997). It was suggested by Harris and Phoenix, (1997) that these latter peptides may utilise a “carpet” type mechanism (Dennison et al., 2004) in their haemolytic action; a mechanism, which could also explain the sigmoidal kinetics of VP1 haemolysis. Using this mechanism, erythrocyte membrane destabilisation by VP1 would lead to membrane lesions whose levels increase with rising peptide concentration. At VP1 concentrations below 0.4 mM, the accumulating effects of these lesions would not be sufficient to lead to significant erythrocyte cell lysis. However, as VP1 concentration increases above this threshold peptide level, progressively greater cooperative interaction between the formation of VP1-mediated membrane lesions would lead to increasingly higher levels of erythrocyte cell lysis until at 2.5 mM peptide (Figure 4.4), total haemolysis would occur. It is interesting to note that the peptides studied by Phoenix and Harris (1997) contrast with VP1 in that the former peptides were haemolytic at micromolar concentrations and showed no requirement for anionic lipid in their membrane interactions (Harris et al., 1997). It is well established that erythrocyte membranes possess an asymmetric distribution of anionic lipids with the extracytoplasmic leaflet depleted in such lipids (Op den Kamp, 1979) and it may therefore be that the relatively high levels of VP1 required for haemolysis reflect the relative inaccessibility of anionic lipid to the peptide.

In conclusion, these studies have shown that VP1, a peptide homologous to the GTAMRILGGVI segment of domain V in calpain 2, was able to form lipid interactive α -helical structure. Anionic lipid was shown to enhance the levels of α -helicity adopted by VP1 and appeared to facilitate membrane penetration by the peptide *via* mechanisms that were consistent with its adoption of oblique orientated α -helical structure. It is also speculated that VP1 may achieve deeper levels of membrane penetration by use of the snorkelling mechanism (Phoenix et al., 2002; Segrest et al., 1992). It has previously been shown that calpain 2 activity is modulated by the presence of anionic lipid (Arthur and Crawford, 1996) and in combination, these results clearly support the role predicted for domain V of calpain 2 in the membrane activation model of the enzyme (chapter 2).

4.5 ACKNOWLEDGEMENTS

I would like to thank Dr. Klaus Brandenburg for his performance of the FTIR spectroscopic analyses included within this study.

Chapter 5

Investigation into the role of the calpain 2 small subunit in the lipid / membrane activation of the enzyme.

5.1 ABSTRACT

Calpain 2 is a heterodimeric enzyme whose *in vivo* requirement for Ca^{2+} appears to be lowered by lipid / membrane interactions. Colorimetric assay showed calpain 2 to require 4 mM Ca^{2+} for 100% proteolytic activity, as defined by this assay, but to show negligible activity in the presence of 1 mM Ca^{2+} . In contrast, calpain 2 showed significant proteolytic activity at this 1 mM Ca^{2+} level, in the presence of either: DMPI, DMPS, DMPC or DMPE, with this activity ranging between 37% and 77% of the protein's full enzymatic activity. The large subunit of calpain 2 (LS-calpain 2) is proteolytically active in the absence of the calpain 2 small subunit and when assayed under conditions corresponding to those used for calpain 2, LS-calpain 2 was found to require the presence of 6 mM Ca^{2+} for 100% proteolytic activity. However, in the presence of either: DMPI, DMPS, DMPC or DMPE, LS-calpain 2 showed no evidence of reduced Ca^{2+} requirements for proteolytic activity and based on these combined results, it is suggested that the lipid activation of calpain 2 requires the presence of this enzyme's small subunit. In addition, when compared to zwitterionic lipid under corresponding conditions, anionic lipid induced an approximate twofold enhancement of calpain 2 activity (70% - 77% as compared to 37% - 49%) and a similar enhancement in average rates of anionic lipid monolayer interaction (changes in surface pressure (ΔSP) = 1.5×10^{-3} mN M^{-1} sec^{-1} at 10 mN M^{-1} as compared to $\Delta\text{SP} = 5.0 \times 10^{-4}$ mN M^{-1} sec^{-1} at 10 mN M^{-1}). It is suggested that an electrostatically driven affinity for anionic lipid may help lower the Ca^{2+} requirement for calpain 2 activation.

5.2 INTRODUCTION

Calpain 2 is a heterodimeric enzyme with the large subunit housing the enzyme's active site and it is generally believed that *in vivo* lipid / membranes lower the enzyme's Ca^{2+} requirement for activation (section 1.5.3.1; Biswas et al., 2004). It was found nearly a decade ago that the large subunit of calpain 2 (LS-calpain 2) possesses full catalytic activity in the absence of the enzyme's small subunit (Yoshizawa et al., 1995). More recent studies have identified a Ca^{2+} -regulated lipid binding site in domain III of the calpain 2 large subunit (Tompa et al., 2001) and it was suggested that this site may mediate the role of lipid binding in calpain 2 activation (Hood et al., 2003; Goll et al., 2003). These results clearly suggest that the small subunit of calpain 2 may not be required for the enzyme's activation. However, sequence analysis (chapters 2) and biophysical studies (chapter 3; Crawford et al., 1996) have suggested that segments in domain V, and thereby the small subunit, of calpain 2 may participate in the lipid / membrane activation of the enzyme. Supportive of this suggestion, recent studies on mice have shown that the homozygous disruption of *CAPN4*, the allele for this subunit, results in embryonic lethality, clearly suggesting that the small subunit of calpain 2 is both an essential and integral part of the enzyme's function (Arthur et al., 2000; Zimmerman et al., 2000). Reinforcing these observations, more recent studies have shown that a novel tissue specific form of the subunit is expressed in humans, suggesting biological diversification by this small subunit and thereby, implying functional relevance (Schad et al., 2002).

Here, it is attempted to ascertain whether the small subunit of calpain 2 is essential or dispensable for the enzyme's activation. Colorimetric assay is used to

determine the Ca^{2+} requirement for both calpain 2 and LS-calpain 2 to proteolyse casein and then the ability of a range of lipids to lower these Ca^{2+} requirements is investigated. The results show lipid is able to lower the Ca^{2+} requirements for calpain 2 activation but not that of LS-calpain 2. Based on these results, it is suggested that the presence of the calpain 2 small subunit is required to facilitate the lipid activation of the enzyme. It is also shown that anionic lipid has an enhanced ability to lower the Ca^{2+} requirements of calpain 2 activation when compared to zwitterionic lipid. Based on lipid monolayer studies, it is suggested that this ability could be related to an electrostatically driven preference for anionic lipid by the enzyme.

5.3 MATERIALS AND METHODS

5.3.1 Reagents

Dimyristoyl phosphatidylserine, dimyristoyl phosphatidylcholine, all solvents, which were of spectroscopic grade, N, N-dimethylated casein and LS-calpain 2 (the enzymatically active 80 kDa subunit, derived from rabbit skeletal muscle) were purchased from Sigma (UK). A recombinant form of calpain 2, derived from the rat enzyme, was purchased from Calbiochem (UK). Both calpains were stored in enzyme buffer [20 mM imidazole, pH 7.5; 1 mM EGTA; 1 mM EDTA; 5 mM 2-mercapto ethanol] at 4°C.

5.3.2 The preparation of phospholipid small unilamellar vesicles

Small unilamellar vesicles (SUV's) were prepared according to the method of Keller *et al.*, (1992). Essentially, phospholipid / chloroform mixtures were dried with nitrogen gas and hydrated with enzyme buffer to give a final phospholipid concentration of 2.0 mM. The resulting cloudy suspensions were sonicated at 4°C with a Soniprep 150 sonicator (amplitude 10 microns) until clear suspensions resulted, which were then centrifuged (15 min, 3000 g, 4°C). The resulting cleared suspensions were taken as stock solutions for immediate use.

5.3.3 Enzyme assay conditions

Enzymatic activity was assayed using a modified form of the colorimetric assay developed by Kawashima *et al.*, (1984): Either LS-calpain 2 or calpain 2, in 250 µl of enzyme buffer [20 mM imidazole, pH 7.5; 1 mM EGTA; 1 mM EDTA; 5 mM 2-mercapto ethanol], was added to: 250 µl of enzyme buffer: 100 µl of 50 mM cysteine solution; 100 µl of 4% (w/v) N, N-dimethylated casein in buffer [0.5 M imidazole, pH 7.5]; 200 µl of deionised water and 100 µl of CaCl₂ that varied between 10 mM, 20 mM, 30 mM, 40 mM, 50 mM or 60 mM. Each of these mixtures was then incubated for 1 hour at 30°C, the reaction stopped by the addition of 1.0 ml 5% (w/v) of Trichloroacetic acid, the whole centrifuged at low speed (1500 g, 15 min, 30°C). After centrifugation, 400 µl of the supernatant was added to: 100 µl of 0.7 M Na₂CO₃ in 0.1 M NaOH; 100 µl of 2 M iodoacetic acid in 2M NaOH and 2.0 ml of: 0.5% (w/v) CuSO₄, 1.0% (w/v) K Na tartrate and 1.5% (w/v) Na₂CO₃ in 0.1 M NaOH; in the ratio 1:1:50 (v/v). The mixture was left for 10 min, 200 µl of 1M Folin-Phenol reagent added, the whole left for 1 hour and the absorbance at 750 nm

measured, all at 30°C. These absorbance values were used as relative measures of enzymatic activity.

5.3.4 Units of enzymatic activity

Using the above protocol (section 5.3.3), levels of either LS-calpain 2 or calpain 2 were arbitrarily chosen and used to determine the respective minimum levels of Ca^{2+} that permitted the enzymatic activity of these proteases. The above protocol (section 5.3.3) was then used to determine the quantity of either LS-calpain 2 or calpain 2 that produced an absorbance change of 1.0 when assayed with casein as substrate for one hour in the presence of the minimum level of Ca^{2+} required for enzymatic activity. In each case, this quantity of enzyme was defined as possessing one unit of enzymatic activity and an absorbance change of 1.0 taken to represent 100% enzyme activity as described by Kawashima.

5.3.5 The lipid activation of LS-calpain 2 and calpain 2

As control experiments, levels of either LS-calpain 2 or calpain 2, which possessed one unit of enzyme activity (section 5.3.4), were assayed as described in section 5.3.3. Similar experiments were performed except that the addition of 250 μl of enzyme buffer was replaced by the addition of 250 μl of enzyme buffer containing SUV's of either DMPS or DMPC, prepared as described in section 5.3.2. In all cases, experiments were conducted in triplicate and the mean absorbance determined. This mean absorbance was used to express enzyme activity as a percentage relative to the standard that an absorbance of 1.0 represented an enzymatic activity of 100%. (section

5.3.4). The relative % enzymatic activity was then plotted as a function of the final Ca^{2+} concentration used under the assay conditions of section 5.3.3 with error bars representing \pm SD (Figures 5.1 and 5.2).

5.3.6 Monolayer interactions of calpain 2

All monolayer equipment was supplied by NIMA (UK). Surface tension was monitored by the Wilhelmy plate method using a microbalance (Demel, 1974, Figure 4.1). Studies were conducted using a 5×15 cm Teflon trough containing 80 ml of either 10 mM Tris (pH 7.5) or this buffer supplemented with NaCl (final concentration 100 mM). The trough was equipped with moveable barriers, which responded to the microbalance and could be adjusted to maintain monolayers at constant surface area. Monolayers were formed by spreading pure phospholipids (10 mM) in chloroform onto the subphase, followed by compression to give the desired final surface pressure. 20 μmol . Stock calpain 2 was introduced to the subphase *via* a reservoir extending below the monolayer and the subphase was continuously stirred by a magnetic bar. Calpain 2 – lipid monolayer interactions were monitored *via* changes in monolayer surface pressure and recorded as a function of time (figure 5.3).

5.4 RESULTS AND DISCUSSION

It has previously been reported that lipid is able to lower the Ca^{2+} requirements of calpain 2 for activation but no corresponding studies on LS-calpain 2 seem to have been conducted. Clearly, a comparison of such studies may provide information on the participation / non-participation of calpain subunits in the lipid activation of calpain 2 and such a comparison is undertaken here.

Colorimetric assay of LS-calpain 2 (section 5.3.5) showed that the enzyme required Ca^{2+} levels of 6 mM for 100% activity (figure 5.1), which was defined as an

absorbance change of 1.0 after an incubation of one hour with casein as substrate (section 5.3.4). However, LS-calpain 2 exhibited no significant activity at Ca^{2+} concentrations ≤ 5 mM (Figure 5.1). These experiments were repeated in the presence of either: DMPI, DMPS, DMPC or DMPE. No significant LS-calpain activity was detected at Ca^{2+} concentrations ≤ 5 mM, showing that these lipids had no significant ability to reduce the Ca^{2+} levels required for activation of the enzyme (Figure 5.1). It has been suggested that domain III of calpain 2 may play a role in the lipid-mediated reduction of the Ca^{2+} levels required for activation of the enzyme (Hood et al., 2003; Goll et al., 2003). Clearly, LS-calpain 2 also possesses domain III but the results shown in Figure 5.1 show that under our experimental conditions, the ability of this domain to interact with lipid does not lead to reductions in the Ca^{2+} requirements of LS-calpain 2 for activity. These results clearly suggest that additional factors are involved in the lipid-mediated reduction of Ca^{2+} requirements for calpain 2 activation.

Figure 5.1 The lipid activation of LS-calpain 2

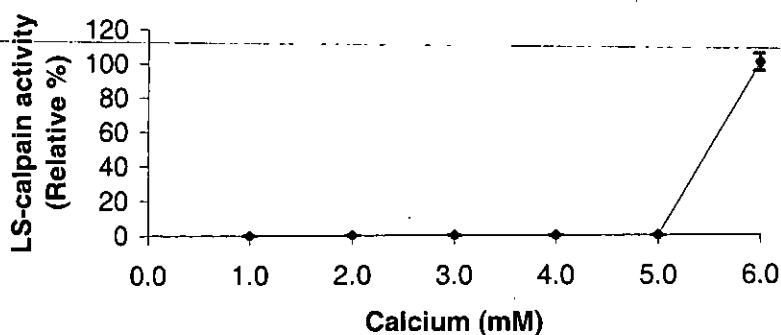


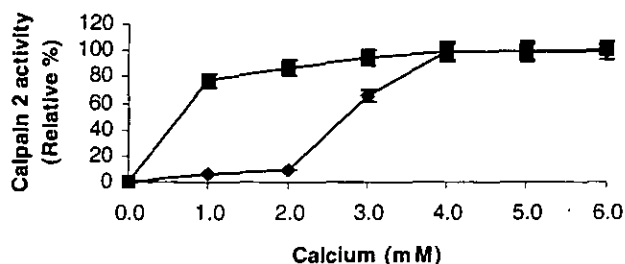
Figure 5.1 shows LS-calpain 2 activity as a function of Ca^{2+} concentration when assayed with casein as substrate, all as described in section 5.3.3. The enzyme required Ca^{2+} levels of 6 mM for 100% activity and exhibited no significant activity at Ca^{2+} concentrations ≤ 5 mM. Corresponding assays were performed in the presence of either: DMPI, DMPS, DMPC or DMPE and it was found that these lipids had no significant ability to reduce the levels of Ca^{2+} required for LS-calpain 2 activity, therefore producing data points coincident with those of the corresponding assay of LS-calpain 2 assay in the absence of lipid. LS-calpain 2 activity was expressed as the mean relative % activity of triplicate determinations and Ca^{2+} levels as the final Ca^{2+} concentration used under assay conditions, all as described in section 5.3.5, with error bars representing \pm SD. (n=5)

Colorimetric assay of calpain 2 (section 5.3.5) showed that the enzyme required the presence of 4 mM Ca^{2+} to exhibit 100% activity with casein as substrate (Figures 5.2A - 5.2D). As Ca^{2+} levels were decreased, the proteolytic activity of calpain 2 rapidly decreased and at Ca^{2+} concentrations ≤ 2 mM, the activity of the enzyme was $< 10\%$ (Figures 5.2A - 5.2D). At 1 mM Ca^{2+} , the enzyme showed negligible proteolytic activity (6%) but the activity of the enzyme was increased to 77% in the presence of DMPI, 70% in the presence of DMPS, 49% in the presence of DMPC and 37% in the presence of DMPE (Figures 5.2A - 5.2D). As shown in figure 5.2, these lipid-mediated effects decreased with increasing Ca^{2+} until at 4 mM Ca^{2+} , no significant effect of lipid on the activity of calpain 2 was observed. These experiments clearly show that lipid is able to mediate reductions in the Ca^{2+} requirements of calpain 2 for activity. Moreover, when these results are taken with those described above for corresponding experiments on LS-calpain 2 (Figure 5.1), they strongly suggest that the presence of the calpain 2 small subunit is required for the lipid-mediated activation of this latter enzyme.

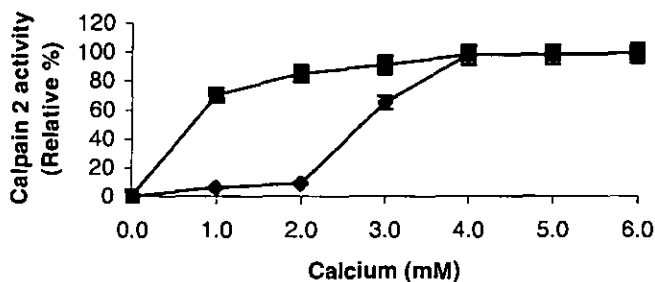
Comparison of Figures 5.2A and 5.2B to Figures 5.2C and 5.2D show that the proteolytic activity of calpain 2 is up to approximately twofold enhanced in the presence of DMPI and DMPS when compared to that in the presence of DMPC and DMPE (70% - 77% as compared to 37% - 49%). These results clearly suggest that calpain 2 may have a preference for association with anionic lipid and to study this suggestion, the interactions of the enzyme with lipid monolayers were observed. Calpain 2 showed high levels of interaction with both DMPS and DMPC monolayers at an initial surface pressure of 10.0 mN M^{-1} (Figure 5.3). Moreover, the final surface pressure changes induced by calpain 2 in both cases were comparable at 6 mN M^{-1} and 5 mN M^{-1} respectively (Figure 5.3), suggesting that monolayer insertion by

Figure 5.2 The lipid activation of calpain 2

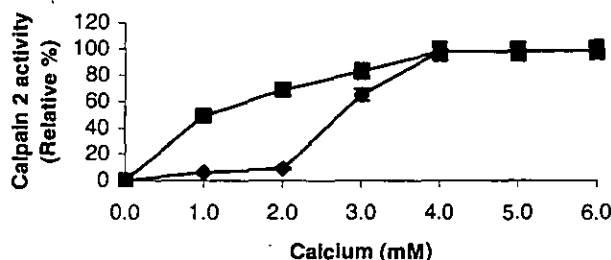
A.



B.



C.



D.

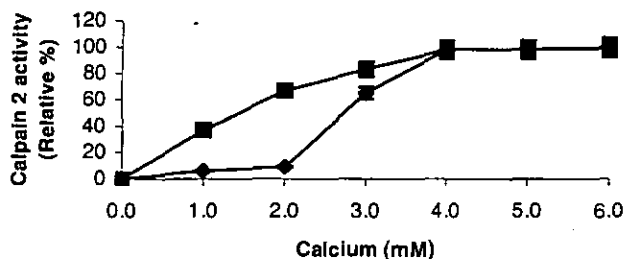


Figure 5.2 shows calpain 2 activity plotted as a function of Ca^{2+} concentration when assayed with casein as substrate, all as described in section 5.3.3, either in the presence (■) or absence (◆) of: DMPI (Figure 5.2A), DMPS (Figure 5.2B), DMPC (Figure 5.2C), DMPE (Figure 5.2D). It can be clearly seen from these figures that in the absence of lipid, calpain 2 shows negligible activity at Ca^{2+} concentrations ≤ 2 mM. However, the enzyme shows significant proteolysis at Ca^{2+} concentrations ≤ 2 mM when in the presence of each lipid

studied, which at 1 mM Ca^{2+} ranged between 37% -77%. It can also be seen that, at these Ca^{2+} concentrations, the proteolytic activity of calpain 2 was generally greater in the presence of anionic lipid (70% -77%, Figures 5.2A and 5.2B) when compared to zwitterionic lipid (37% - 49%, Figures 5.2C and 5.2D). Calpain 2 activity = the mean relative % activity \pm SD. (n=5)

the enzyme was hydrophobicity driven. However, the kinetics of these monolayer interactions showed fundamental differences. The interaction of calpain 2 with DMPC monolayers were relatively slow with final surface pressure changes reached after approximately 10,000 sec (Figure 5.3B), giving an average rate of surface pressure change of 5.0×10^{-4} mN M^{-1} sec^{-1} . In contrast, the rates of interaction of calpain 2 with DMPS monolayers were much higher with final surface pressure changes reached after a period of 4000 sec (Figure 5.3A), giving an average rate of surface pressure change of 1.5×10^{-3} mN M^{-1} sec^{-1} .

In combination, these results suggest that electrostatic interactions between cationic regions of calpain 2 and DMPS headgroups accelerate the rates of interaction between the enzyme and this lipid when compared to those observed between calpain 2 and DMPC monolayers. Strongly supporting this suggestion, it was also found that when 100 mM NaCl was included in the subphase of DMPS monolayers, the high ionic strength of the salt reduced the rates, and levels, of surface pressure change shown between calpain 2 and these monolayers by approximately 40% (lower curve, Figure 5.3A). Hence the overall results clearly suggest that, whilst calpain 2 has a general hydrophobicity-driven ability to interact with lipid, the enzyme has an electrostatically-driven preference for interaction with anionic lipid. Such a preference for anionic lipid could explain the enhanced ability of such lipid to reduce the levels of Ca^{2+} required for calpain 2 activation observed in Figure 5.2. Moreover, it was suggested above that the calpain 2 small subunit, and thereby the GTAMRILGGVI segment of domain V, may be required for the lipid / membrane activation of the

enzyme. It would seem significant that this segment was also shown to exhibit a preference for anionic lipid in chapter 4, further supporting the view that this segment may participate in the lipid-mediated activation of calpain 2.

Figure 5.3 The monolayer interactions of calpain 2

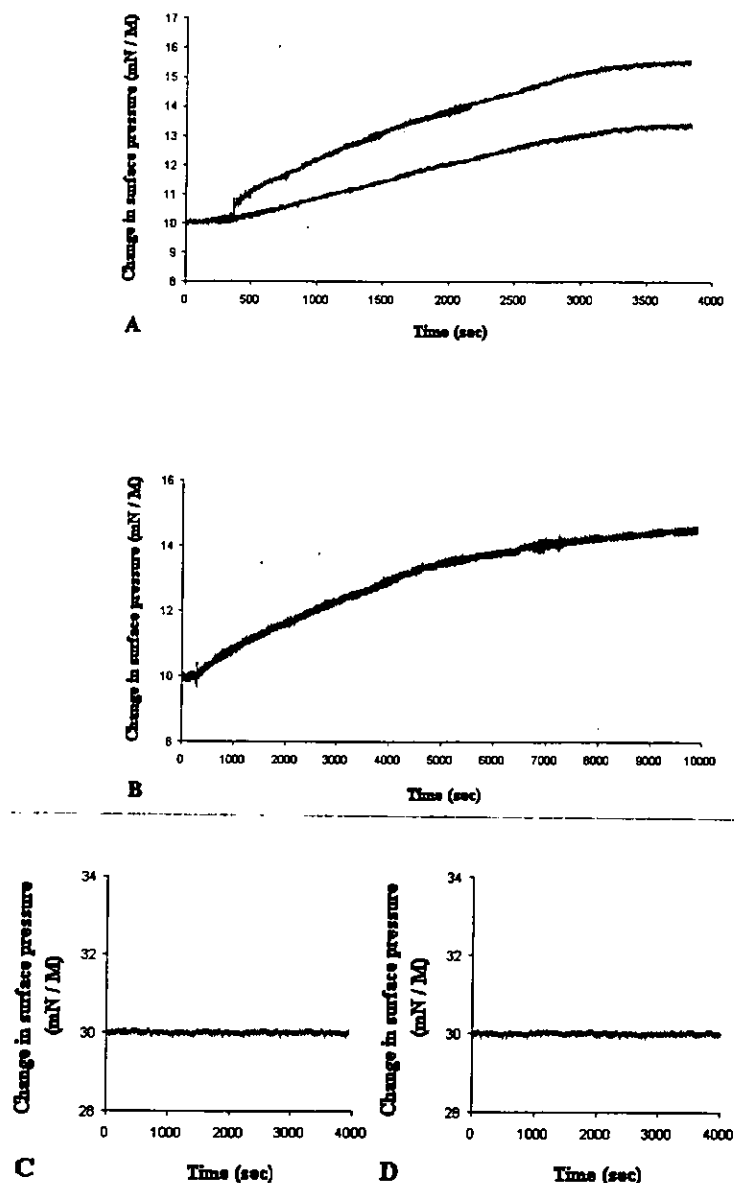


Figure 5.3A and 5.3B show the interactions of calpain 2 with monolayers at an initial surface pressure of 10.0 mN M^{-1} , all as described in section 5.3.6. The enzyme showed comparable final surface pressure changes with DMPS monolayers (upper curve, Figure 5.3A) and DMPC monolayers (Figure 5.3B), suggesting a major role for hydrophobic forces in these interactions. However, when compared to DMPC monolayers (Figure 5.3B) the accelerated rates of changes in surface pressure shown by calpain 2 with DMPS monolayers (upper curve, Figure 5.3A), suggests an electrostatically driven preference for anionic lipid. Supporting this suggestion, 100 mM NaCl reduced the levels, and rates, of surface pressure changes associated with calpain 2 - DMPS interactions (lower curve, Figure 5.3A) by

circa 40%. In contrast to these previous results, Figures 5.3C and 5.3D show DMPS and DMPC monolayers respectively, at an initial surface pressure of 30.0 mN M^{-1} , mimetic of naturally occurring membranes. Calpain 2 was introduced into the subphase of these monolayers but showed no ability to introduce changes in their surface pressures. In combination, these results suggest that lipid-packing density may be important to the lipid interactions of calpain 2.

In summary, these results strongly support a role for the calpain 2 small subunit in the lipid / membrane mediated activation of the enzyme. However, it is interesting to note that in the present study, this enzyme showed no evidence of interaction with monolayers of either DMPS or DMPC at surface pressure of 30 mN M^{-1} , which is representative of naturally occurring membranes (Figure 5.3C). Whilst these results do not preclude the enzyme from membrane interaction, they suggest that lipid-packing density is important to the lipid interactions of the enzyme. Indeed, it is noticeable that many of the studies advocating calpains to be membrane interactive have been performed using lipid vesicles. Due to their high curvature, these bodies possess lower lipid packing densities in their surface regions and it may be that such bodies feature in the lipid activation or biological activity of calpain 2.

Consistent with this suggestion, Yudin *et al.*, (2000) recently showed that calpain 2, and calpain 1, were strongly associated with the acrosomal reaction of *Cynomolgus* macaque sperm. These enzymes were localised to a region between the acrosomal vesicle membrane and the plasma membrane prior to this reaction and strongly associated with the acrosomal shroud after. It appears that calpain participation in this reaction involves the promotion of fusion between the acrosomal membrane with the sperm plasma membrane. Interestingly, the promotion of membrane fusion is an activity strongly associated with oblique orientated α -helical structure; protein architecture, which chapter 3 predicted was likely to be formed by the GTAMRILGGVI segment.

CHAPTER 6

Final discussion and future work

6.1 CONCLUDING DISCUSSION AND FUTURE WORK

The precise physiological role(s) of calpains in the lens are not fully understood but it has been postulated that following differentiation, calpains remain in mature fibre cells to degrade damaged lens proteins that accumulate during the life of the lens. On this basis, two hypotheses to explain the role of calpains in cataract have been proposed. In the first, loss of lens calpain activity leads to pathologically elevated levels of damaged proteins, reduced lens optical performance and cataract (David and Shearer, 1989). However, it clearly emerges from our review of the literature in chapter 1 that the unregulated calpain-mediated proteolysis of crystallins makes a contribution to cataract in aged lenses and calpain inhibitors have been shown to be able to retard these processes. These observations are clearly inconsistent with the above hypothesis and rather, support the alternative hypothesis that the pathologically-mediated over-activation of calpains cause the accumulation of partially-degraded lens proteins, thereby contributing to age-related and other forms of cataract (David and Shearer, 1989). This latter hypothesis is also consistent with our studies on porcine lenses, which showed that the pathological elevation of Ca^{2+} in these lenses produced an almost linear response in levels of cataract, which, moreover, could be reduced by calpain inhibitors (chapter 2). These latter studies also suggested that the over-activation of calpain 2 may contribute to porcine cataract, which has previously been shown to contribute to cataract in other animals and appears to be the major contributor to human cataract (chapter 1).

It has been proposed that lipid / membranes may mediate the activation of calpain 2 by lowering the enzyme's Ca^{2+} requirements for proteolysis, although there is some debate as to whether the enzyme's small subunit participates in this process

(chapter 1). The present study has shown dimeric calpain 2 to be membrane interactive and that lipid is able to reduce the Ca^{2+} requirements of this form of the enzyme for activation but shows no corresponding ability with monomeric calpain 2. This latter form of calpain 2 is a fully active protease comprising the intact large subunit of calpain 2 but lacking the small subunit of the enzyme and clearly, these combined results strongly support a role for this subunit in the lipid / membrane activation of calpain 2 (chapter 5). Consistent with this suggestion, the combined results of chapter 4 and 5 have suggested that a segment, GTAMRILGGVI, in domain V of the calpain small subunit may play a role in the in the lipid / membrane activation of the enzyme. Based on the combined data of these two chapters, a model for the membrane interaction of this domain V segment is presented: in figure 6.1. Incorporating the model of Figure 6.1, and the data of chapter 5, the following sequence of event is suggested to occur in the lipid activation of dimeric calpain 2: The cytosolic enzyme is first translocated (chapter 1) to the membrane / lipid bodies (chapter 5). In the presence of anionic lipid, activation of the enzyme proceeds (chapter 5) and the segment, GTAMRILGGVI, in domain V of the calpain 2 small subunit forms an oblique orientated α -helix (chapter 3 and chapter 4). The hydrophobicity gradient possessed by this α -helix acts in conjunction with snorkelling interactions between its arginine residue and anionic lipid to achieve angled insertion into the membrane / lipid surface and penetration to the level of the hydrophobic acyl chain region (Figure 6.1). These interactions lead to further conformational changes in the calpain 2 small subunit that result in the activation of the enzyme at lower Ca^{2+} levels.

It is envisaged that future work will have two major foci. Membrane penetration by the GTAMRILGGVI segment will be further investigated using

Figure 6.1 The lipid / membrane interaction of the GTAMRILGGVI segment

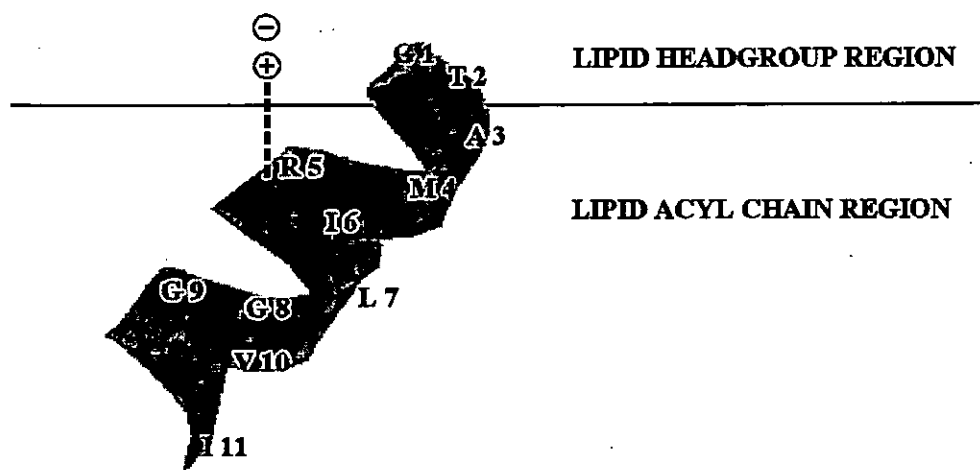


Figure 6.1 shows a schematic representation of the lipid surface / membrane interaction of the calpain 2 segment, GTAMRILGGVI, in which the segment is represented as a ribbon structure. Due to its hydrophobicity gradient, the segment is depicted as penetrating the lipid surface / membrane in an oblique orientation with its strongly hydrophobic C-terminal region in the hydrophobic lipid acyl chain region. The angle of membrane penetration was arbitrarily chosen as 45° but most tilted peptides adopt orientations between 30° and 60° (Brasseur et al., 2000). Also represented is snorkelling by the segment's arginine residue with the positively charged sidechain of the residue extending up to interact with negatively charged moieties in the lipid headgroup region. Figure 6.1 is not to scale but geometrical interpretation of previously presented data (Stopar et al., 2003) shows that the angled arrangement of the GTAMRILGGVI α -helix in relation to the membrane surface could allow snorkelling by the segment's arginine sidechain.

neutron lamellar diffraction and a series of deuterated peptide homologues of the segment. These studies can indicate the relative bilayer locations of the deuterated residues and simple geometry will allow an estimate of the angle of penetration used by the segment. The other major focus of future studies will be on the membrane interactions of the lone dimeric calpain 2 small subunit. Clones of genes for both this intact subunit and, this subunit lacking most of the GTAMRILGGVI segment, are now available. FTIR spectroscopic analysis and monolayer studies on the membrane interactions of the proteins expressed by these genes will provide information on the

role(s) played by the GTAMRILGGVI segment in the lipid interactions of the calpain 2 small subunit, and thereby, possibly the enzyme's lipid / membrane activation mechanism.

As a final comment, the mainstay of the present treatment of cataract is surgery. Cataract surgery was described as long ago as 300 BC in the treatise of 'Susruta', from the ancient Indian civilisation (Biswas et al., 2001). A revolution in the field of cataract surgery was initiated by Harold Ridley, who in 1948 designed the intra-ocular lens implant, and Charles Kelman, who in 1967 developed phaco-emulsification surgery, the ophthalmic version of keyhole surgery (Olsen et al., 2003). Modern cataract surgery, although very successful in dramatically reversing the visual debility induced by cataract (Rubin *et al.*, 1993) is a highly skilled surgery with expensive and sophisticated instrumentation requiring considerable investment in manpower and technology. Even with an excellent success rate, there is a 1:300 incidence of severe complications resulting in permanent blindness (Masket, 1992; Powe et al., 1994). As with many other medical conditions where surgical treatment has been replaced over time by drug-based medical therapy, which is easily administered, a medical option to treat or prevent cataract is perhaps the 'Holy Grail' of cataract treatment. Significant advances are continuously being made in the development of membrane permeable calpain inhibitors, which are suitable for topical administration in the eye. Research into the molecular genetics of cataract is also underway and advances have been made in understanding the genetic basis of congenital cataract. This may lead to the introduction of novel medical therapies, possibly involving gene manipulation, to prevent or treat cataract (Francis et al., 1999). Continued progress in this direction could lead to the non-surgical treatment of

cataract – which would perhaps be the next revolution and the Holy Grail of cataract treatment

CHAPTER 7

References

7.1 REFERENCES

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CHAPTER 8

Publications list

8.1 PUBLICATIONS LIST

Papers

Biswas, S., Harris, F. and Phoenix, D. A. Calpains: targets of cataract prevention? (2004) *Trends Mol. Med.* In press.

Biswas, S., Harris, F., Singh, J and Phoenix, D. A. (2003) Calpains and Diabetic cataractogenesis. *Mol. Cell. Biochem.* In press.

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