CHARACTERIZATION OF THE STRUCTURE OF α-CRYSTALLIN WITH THE SYNTHETIC CROSS-LINKER 3,3'-DITHIOBIS(SULFOSUCCINIMIDYL PROPIONATE)

by

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A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Chemistry

Under the Supervision of Professor David L. Smith

Lincoln, Nebraska

December 2003

DISSERTATION TITLE

Characterization of the Structure of Alpha-Crystallin with the Synthetic Cross-linker 3,3'-Dithiobis(sulfosuccinimidyl propionate)

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Despite numerous efforts, the three-dimensional structure of the lens protein α-crystallin remains elusive. In this study, the structure of α-crystallin was investigated using the cross-linker 3,3-dithiobis(sulfosuccinimidyl propionate), DTSSP. Cross-linking reagents can react with side chains of amino acids that are within close proximity. Digestion of the cross-linked proteins and mass spectrometric analysis of the resulting peptides can identify the cross-linked residues, thus providing insight into the folded structures of proteins. However, our initial analysis of α-crystallin cross-linked with DTSSP was difficult because many ions in the mass spectra could not be attributed to the expected reactions of DTSSP.

To better understand its reactivity, products from the reaction of DTSSP with several model peptides were analyzed by HPLC electrospray ionization mass spectrometry. Several products not previously reported were identified. Sources for these unexpected products were traced to reaction of DTSSP with ammonium ions in the buffer, to reaction of contaminants present in the commercial DTSSP reagent, and to

unexpected reactivity of DTSSP with serine and tyrosine residues. In addition, the collision-induced-dissociation of peptides modified by DTSSP showed that certain DTSSP-peptide adducts easily undergo in-source fragmentation to give additional, unexpected ions. This study of the reactions of DTSSP with model peptides revealed the major types of ions that are likely to be found in proteolytic digests of proteins cross-linked with DTSSP, therefore facilitating the identification of residues in α-crystallin that were modified by DTSSP.

Examination of the modifications of αA and αB treated with DTSSP showed multiple modifications of several lysines in both subunits, suggesting that some regions of αA and αB were readily accessed by DTSSP. However, the lack of modification at other lysines in the hydrophobic regions of αA and αB suggests that the accessibility of the cross-linker was limited. In addition, an intermolecular cross-link was detected between the C-termini of αA and αB. Evidence that these regions are within 12 Å in the folded protein supports some of the structural models proposed for α-crystallin.

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LIST OF ABBREVIATIONS

SEC.....size exclusion chromatography

ESIMS.....electrospray ionization mass spectrometry

DTSSP.....3,3'-dithiobis(sulfosuccinimidyl propionate)

Da.....daltons

RP-HPLC.....reversed-phase HPLC

MS.....mass spectrometry

ESI.....electrospray ionization

Q-ToF.....quadrupole time-of-flight

ToF.....time-of-flight

FWHM.....full-width half-maxium

CID.....collision-induced dissociation

NHS-ester.....*bis*-*N*-hydroxysuccinimidyl ester

DTT.....dithiothreitol

MALDI.....matrix-assisted laser desorption

FTMS.....fourier transform mass spectrometry

TFA.....trifluoroacetic acid

CA.....carbonic anhydrase

BSA.....bovine serum albumin

gfp.....glu-fibrino peptide

α-MSH.....α-melanocyte stimulating hormone

GGYR.....G-5386

LeuEnk.....leucine enkephalin

cyc.....cyclized

ac.....acetylated

FA.....formic acid

eV.....electron volts

DCC.....N,N'-dicyclohexyl carbodiimide

EDTA.....ethylenediaminetetracetic acid

NaN_3.....sodium azide

SDS-PAGE.....sodium dodecyl sulfate polyacrylamide gel electrophoresis

ME.....mercaptoethanol

GuanCl.....guanidine hydrochloride

MES.....morpholionethane sulfonic acid

ACKNOWLEDGEMENTS

I am eternally grateful to my advisers, Dr. David Smith and Dr. Jean Smith. Thank you for taking me into your lab and for teaching me only a fraction of what you know about mass spectrometry and lens crystallins.

I would like to thank Dr. Patrick Dussault, Dr. David Berkowitz and Dr. Marjorie Lou for taking the time to serve on my graduate committee.

I would also like to thank Dr. Ron Cerny and Dr. Kurt Wulser for their guidance and support. Thank you for answering my incessant questions. My time in the basement wouldn't have been nearly as fun without your friendship. I also appreciate the help of Smith Lab members both past (Dr. Stacy Hanson, Ashraf Raza, Dr. John Engen) and present (Jiong Yu, Dr. Venamin Lapko, Dr. Patrick Wintrode).

A big thank you goes out to the members of my family: my parents, Chuck and Patsy Madinger; my brother, Chip Madinger, his wife Julie and their children, Alex and Maura; and my sister, Trisha DuBinok and her husband, Jeff DuBinok. You guys were a bottomless supply of moral support.

Finally, I would like to thank my husband, Marcus, for his love and support. You are the source of my motivation and strength. I would not have been able to accomplish this without you.

For Marcus

CHAPTER 1

INTRODUCTION AND BACKGROUND: α-CRYSTALLIN AND SYNTHETIC CROSS-LINKERS

1.1 BACKGROUND AND SIGNIFICANCE

1.1.1 The Human Lens

The mammalian lens is an avascular tissue that is composed of two cell types: epithelial cells and fiber cells (Figure 1.1). The epithelial cells are found along the anterior of the lens and differentiate into fiber cells, which compose the major part of the lens. New fiber cells are laid down as concentric layers on previously formed fiber cells. Thus, fiber cells at the lens nucleus are the oldest, while the cortex contains the newest cells (Berman 1991).

As demonstrated in Figure 1.2, the lens is located between the iris and the retina of the mammalian eye. Since the function of the lens is to focus images on the retina, lens transparency is required for proper vision. The high protein content of the lens contributes to its transparency: proteins comprise approximately 35% of the lens, while the other 65% of the lens is water. More than 90% of these proteins are structural proteins, called crystallins (Bloemendal 1977). It is the close packing of these crystallins that provides the refractive index needed for proper light focusing (Delaye and Tardieu 1983).

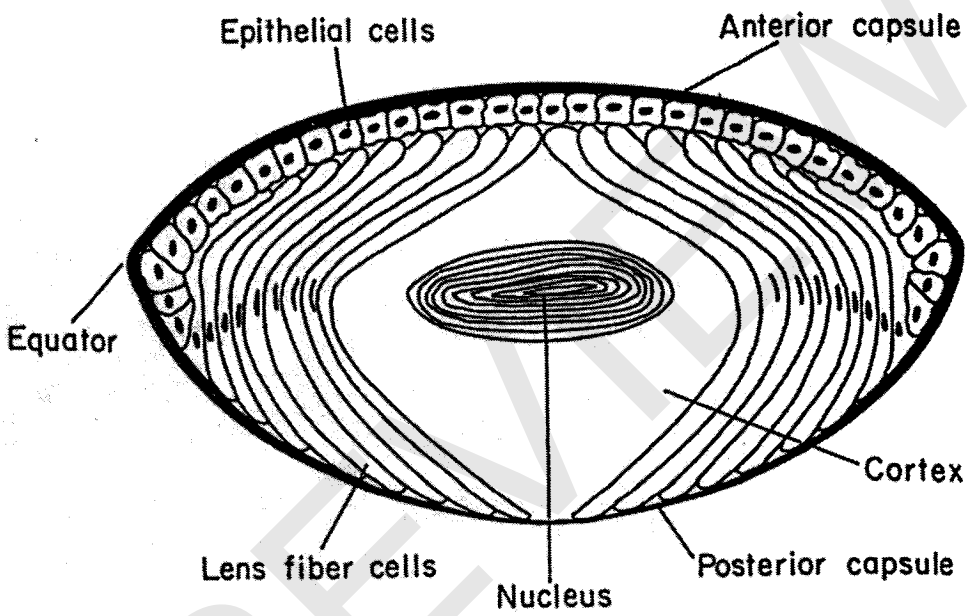

Figure 1.1 - Cross-sectional diagram of a mammalian lens (Berman, 1991).

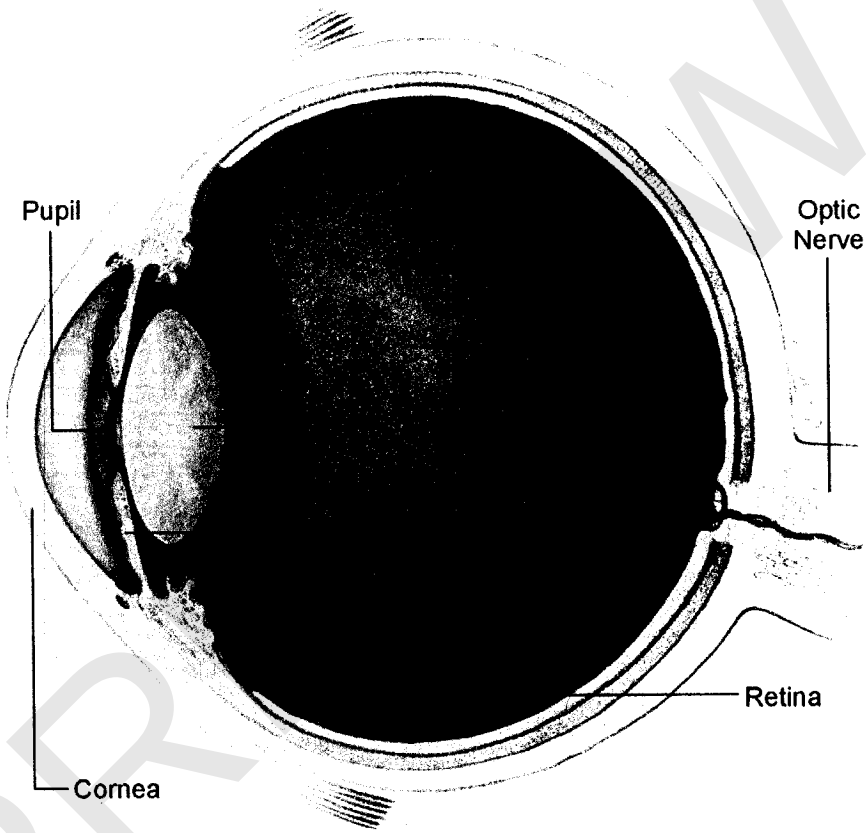

Figure 1.2 - Diagram of the human eye (National Eye Institute, National Institutes of Health; http://www.nei.nih.gov/photo/eyean/).

1.1.2 The Heteroaggregate, α-Crystallin

The lens crystallins are organized into three main classes (α, β and γ) based upon structural homologies and the size of aggregates they form. Size exclusion chromatography (SEC) demonstrates that α-crystallins are the largest of the three crystallins (Figure 1.3). The molecular weight of α-crystallin ranges from 300 kDa to 1.2 MDa. The average molecular weight of human α-crystallin is 800 kDa (Horwitz 1992).

As a heteroaggregate, α-crystallin is composed of approximately 40 subunits of two 20 kDa proteins: αB (Kramps et al. 1977; Dubin et al. 1990; Miesbauer et al. 1994) and αA (de Jong et al. 1975; Takemoto and Emmons 1991; Miesbauer et al. 1994). The ratio of these subunits varies with both species and age. For instance, the ratio of αA to αB in human lenses is 2:1 for a fetal lens and 3:2 for a middle aged lens (Ma et al. 1998) whereas the ratio of αA to αB in bovine lenses is 3:1 (Siezen et al. 1978). Since the primary structure of αB has more basic residues than the primary structure of αA, these subunits can be fractionated by reversed-phase HPLC (RP-HPLC) (Smith et al. 1991). The UV trace of the separation of α-crystallin with a C_4 column is presented in Figure 1.4. Hydrophilicity prediction profiles indicate that the subunits of α-crystallin are amphiphilic: αA and αB both have a hydrophobic N-terminus and a hydrophilic C-terminus (Puri et al. 1983).

1.1.2.1 The Function of α-Crystallin

As indicated, α-crystallins maintain lens transparency by contributing to the refractive index of lenses (Delaye and Tardieu 1983). The first indication that α-

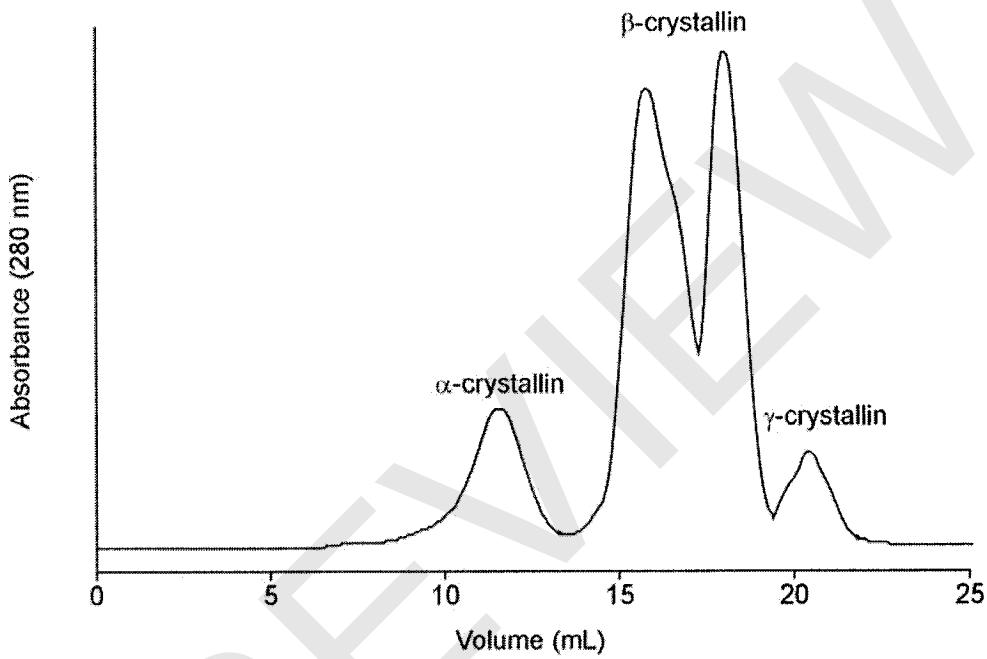

Figure 1.3 - Size exclusion separation of human, water-soluble lens crystallins.

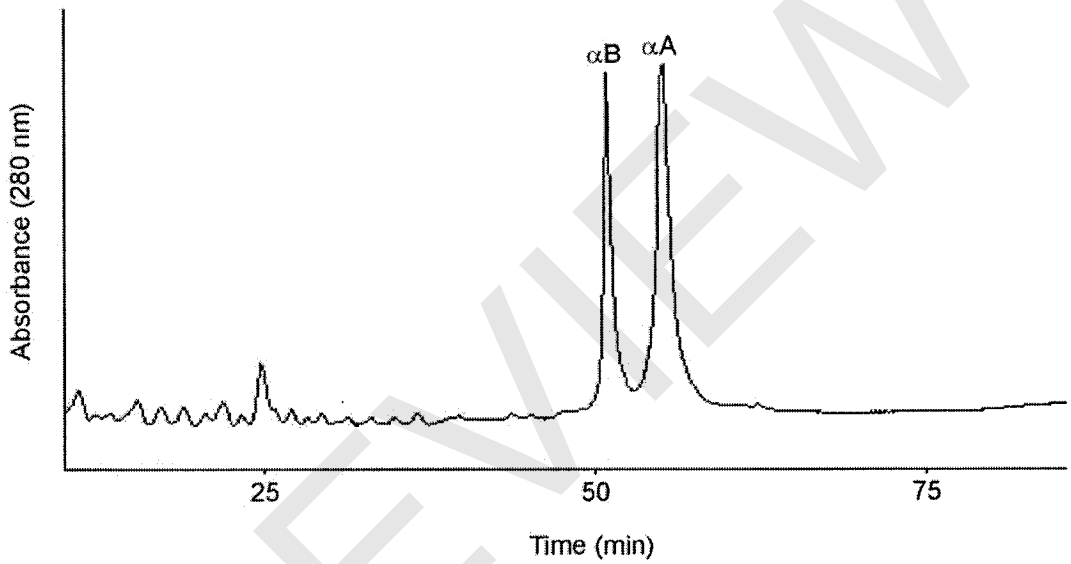

Figure 1.4 - C_4 reversed phase chromatogram of a-crystallin. Analysis by ESIMS (data not shown) indicated that αB eluted at 51 min (39% acetonitrile, 0.1% trifluoroacetic acid) while αA eluted at 55 min (44% acetonitrile, 0.1% trifluoroacetic acid).

crystallin may have an additional function came in 1982 when the sequence homology between bovine α-crystallin and the molecular chaperones, the small heat shock proteins (sHSPs), was established (Ingolia and Craig 1982). In 1992, the ability of bovine α-crystallin to suppress the thermally induced aggregation of various proteins was reported (Horwitz 1992). Since then, the ability of α-crystallin to protect other proteins from stress-induced aggregation has been extensively studied *in vitro* (Raman and Rao 1994; Farahbakhsh et al. 1995; Raman et al. 1995; Wang and Spector 1995). However, denatured enzymes bound to α-crystallin only regain activity in the presence of other chaperones (Wang and Spector 2000). Because it is unable to independently reactivate denatured proteins, the activity of α-crystallin is described as "chaperone-like".

Since the chaperone-like activity of α-crystallin reduces the light scattering induced by the aggregation of denatured proteins, α-crystallin may play an important role in preventing cataract formation. While the hydrophobic residues of α-crystallin have been implicated in its binding of denatured proteins (Raman and Rao 1994), the exact mechanism of the chaperone-like activity of α-crystallin is still unknown. To gain insight into the mechanism of its chaperone-like activity, numerous studies regarding the structure of α-crystallin have been conducted.

1.1.2.2 The Structure of α-Crystallin

Because of its heterogeneity, attempts to crystallize α-crystallin have been unsuccessful and the aggregate is too large for high resolution NMR spectroscopy (Groenen et al. 1994). Therefore, a three-dimensional structure of α-crystallin has not