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The structure and function of human serum albumin in the normal and diseased states

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THE STRUCTURE AND FUNCTION OF HUMAN SERUM ALBUMIN IN THE NORMAL AND DISEASED STATES

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Approved by

First Reader _____

Olga Gursky, Ph.D.
Professor of Physiology and Biophysics

Second Reader _____

Shobini Jayaraman, Ph.D.
Senior Research Scientist

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ABSTRACT

Human serum albumin (HSA) is the major protein in human serum and an important carrier of free fatty acids and small-molecule drugs. Various post-translationally modified forms of HSA, including glycated and oxidized HSA, are implicated in a variety of diseases, including cardiovascular disease and diabetes.

In this study, the structure and stability of HSA was investigated through circular dichroism and fluorescence spectroscopy. The features of fatty acid-free HSA, fatty acid-bound HSA, and post-translationally modified protein forms, including cystenylated, glycated, oxidized, and glycoxidized HSA, were compared. The pro- or anti-oxidant effect of these various forms was studied through examining the oxidation of LDL by Cu ions in the presence of each of these forms of HSA.

The results showed that fatty acid binding did not significantly alter the structural, stability or anti-oxidant properties of HSA. However, all post-translational modifications of HSA reversed its effect on LDL oxidation: unmodified HSA was clearly anti-oxidant, while modified HSA acted as a pro-oxidant by decreasing the lag phase of LDL oxidation. To our knowledge, this effect has not been reported previously. The biochemical and structural basis underlying this general effect and its relevance to various oxidation processes *in vivo* remains to be determined in the future. As the

modified forms of HSA studied here are implicated in a variety of disease states, these studies could suggest significant changes to the functional properties of human serum albumin resulting from these post-translational modifications.

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LIST OF ABBREVIATIONS

ANS	1-anilinoaphthalene-8-sulfonate
CD	Circular dichroism
FA	Fatty acid
FFA	Free Fatty Acid
FRET	Förster Resonance Energy Transfer
Gd HCl	Guanidine Hydrochloride
HDL	High Density Lipoprotein
HSA	Human Serum Albumin
HSA-1	Human Serum Albumin bound to fatty acids
HSA-2	Human Serum Albumin unbound to fatty acids
IDL	Intermediate Density Lipoprotein
LDL	Low Density Lipoprotein
ROS	Reactive Oxygen Species
VLDL	Very-Low Density Lipoprotein

CHAPTER 1: INTRODUCTION

Human serum albumin (HSA), the most common protein in human serum, has many important physiological roles including the maintenance of colloid osmotic pressure, the transport of fatty acids,[1] the maintenance of blood pH,[2] and an anti-oxidant function.[3] Albumin also has a central role in transporting many pharmacological substances throughout the body, including ibuprofen, warfarin, and many other drugs.[4] Thus, the study of the structure and function of HSA has many wide-ranging implications.

1.1 The structure of human serum albumin

HSA is a 66.5kD, 585 amino acid polypeptide composed of three homologous domains thought to originate from a triplicated ancestral gene.[5] Each domain of HSA can be divided into subdomain A (containing six α-helices) and subdomain B (containing four α-helices). HSA has ~68% α-helical content, some random coil secondary structure, and essentially no β-sheet secondary structure[6]; Figure 1A shows a cartoon depiction of albumin color-coded by secondary structure.[7]

In 1992, He and Carter first published the crystal structure of albumin determined to 2.8 Å resolution.[8] More recently published crystal structures are of albumin complexed with various small molecules, including simple fatty acids such as myristate (Figure 1B).[9]

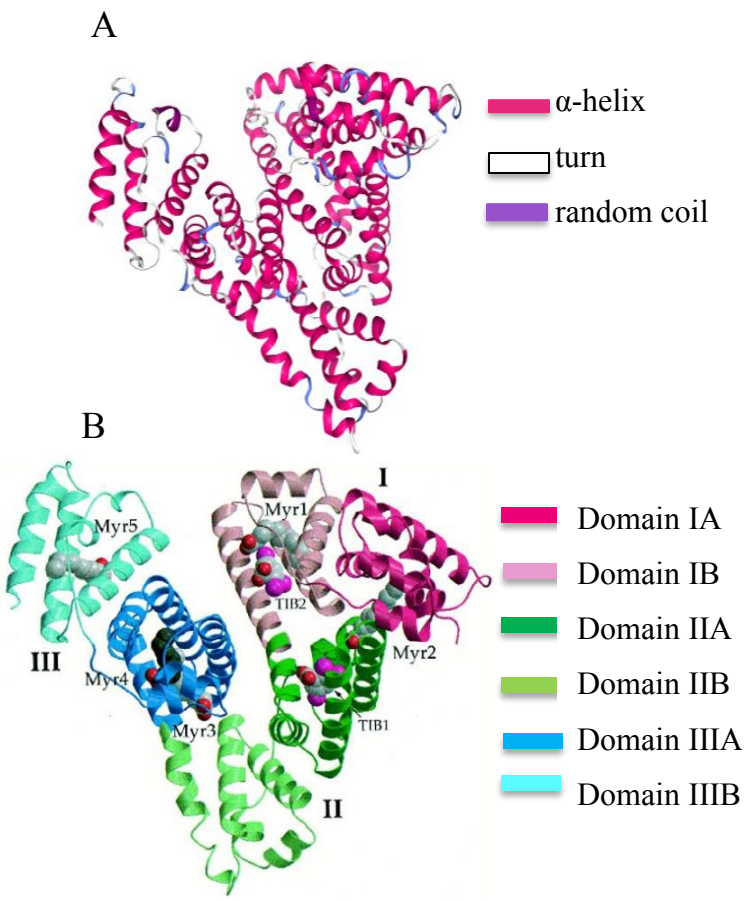

Figure 1.1: The 3-D Structure of HSA

(1A) This ribbon diagram shows the crystal structure of HSA generated from the PDB which depicts the high α-helical content of the protein.[7]
(1B) A depiction of HSA illustrating myristate bound at five major fatty acid binding pockets, adapted from Mandelkow et al.[9] Protein domains are color-coded.

1.1.1 Fatty acid binding sites

One of albumin's main functions is to carry fatty acids (FAs) and other small hydrophobic molecules through the blood. Albumin contains at least seven moderate-to-high-affinity binding sites for fatty acids, with various sources differing in their

descriptions of some of the lower affinity sites.[1,10-12] Figure 1B shows a ribbon diagram of albumin with bound molecules of myristate in five of its fatty acid binding sites[9].

As albumin composes around ~60% of all proteins in human serum,[1] knowledge of its fatty acid binding sites has been used to design drugs that can circulate through plasma bound to albumin at these sites. Also, since albumin circulates through plasma with a half-life of 19 days, it is an especially attractive potential drug carrier. One drug utilizing this mechanism, detemir, is a myristic acid derivative of insulin used to treat diabetes.[13] Treatment with detemir as an insulin analogue avoids the typical insulin peak seen with insulin replacement therapy, because detemir circulates bound to albumin and thus maintains protracted activity for hours after delivery.[13]

1.1.2 Drug binding sites of HSA

In 1975, Sudlow et al. described two drug binding sites of albumin now called Sudlow sites I and II, in domains IIA and IIIA, respectively.[14,15] Both of these binding sites are located in hydrophobic cavities within their subdomain.[6] Sudlow site I is able to bind a variety of ligands due to flexibility in the binding site shape; one important ligand for this site is the drug warfarin.[16] Sudlow site II also binds a variety of molecules, especially aromatic carboxylates such as benzodiazepines.[14] Regions of the Sudlow sites also correspond to two of the seven commonly described high-affinity fatty acid binding sites of albumin.[17]

1.1.3 The free thiol of Cys-34

Albumin contains 35 cysteine residues which form 17 disulfide bridges, with the free thiol of Cys-34 able to participate in redox reactions.[18] This thiol can be oxidized by the formation of a disulfide bond with a free cysteine residue or glutathione, forming a form of albumin called non-mercapto-albumin (HNA), which is distinguished from the reduced form human mercapto-albumin (HMA). Cys-34 can also be irreversibly oxidized to form sulfinic acid (Cys-SO_2H) or sulfonic acid (Cys-SO_3H). HSA has 6 methionine residues, which can also undergo oxidation.[17-8]

The free thiol of Cys-34 comprises about 80% of the free thiols present in the plasma.[19] As this residue is relatively far from the Sudlow I and II drug binding sites, it is an attractive binding target for new drug design.[20]

1.1.4 Glycation of HSA

Glycation, the non-enzymatic addition of a carbohydrate to a protein or lipid, is one potential modification of HSA in plasma. HSA can undergo glycation via the addition of reducing sugars to the amine groups of lysine or arginine residues or the N-terminus of the protein.[21]

Early stage glycation occurs when a primary amine group of HSA is attacked by a nucleophilic reducing sugar, resulting in Schiff base formation and the linearization of the reducing sugar. Then, the 2-OH group of the Schiff base can be oxidized to a ketone, forming the ketoamine Amadori product. This product can become further modified via a variety of processes, including oxidation. These modifications often result in the

formation of intermediate products that can react with positively charged arginine and lysine residues. This can ultimately cause cross-linking of the albumin protein that leads to significant disruptions in the protein's structure.[21]

The measurement of levels of glycated albumin in the serum of diabetic patients has become a common means of examining glycemic control. Diabetes refers to a family of disorders in which insulin signaling pathways are disrupted, causing unusually high serum glucose levels. Especially when diabetes is under poor glycemic control, and blood glucose levels are consistently aberrantly high, increased amounts of albumin become glycated, which can affect protein function.[21]

1.1.5 Aromatic residues of HSA

HSA contains one tryptophan residue, Trp-214, in subdomain IIA.[6] This residue is located near the Sudlow I site. Therefore, local changes in the environment of this residue may result in changes to this binding site.

1.2 Oxidative processes in plasma

Cardiovascular disease is the leading cause of death in the United States; a major cause of cardiovascular disease is atherosclerosis, the narrowing of arteries due to plaque buildup.[22] It has long been known that high levels of reactive oxygen species (ROS) such as H_2O_2 in the blood can contribute to the development of atherosclerosis through a variety of mechanisms including disrupting cell membrane integrity, promoting ECM protein production, and promoting inflammation.[23] One particularly important step in the

formation of atherosclerotic plaques occurs when ROS oxidize lipoprotein particles in the blood, as described below.

1.2.1 Lipid circulation via lipoprotein transport

While albumin is the primary vehicle in the blood carrying small hydrophobic molecules such as free fatty acids, the primary method of lipid circulation is via lipoproteins, which are macromolecular assemblies of specific proteins (apolipoproteins) and various lipids. These assemblies differ in terms of particle size, density, protein and lipid composition and function. They are named according to their density: Very Low, Intermediate, Low, and High-Density Lipoproteins (VLDL, IDL, LDL, and HDL), with HDL containing the highest protein:lipid ratio.[24]

VLDL is assembled in the liver from the assembly of apolipoprotein B, phospholipids, triglycerides, and cholesterol. These particles then circulate through the bloodstream, where lipoprotein lipase hydrolyzes triglycerides, decreasing the lipid content of VLDL. This process converts VLDL to IDL as the protein:lipid ratio of the assembly increases. Under normal physiological conditions, ~50% of IDL particles are taken up by the liver before they become LDL; the rest continue to lose triglyceride content in the bloodstream and are termed LDL when their cholesterol content is higher than their triglyceride content.[25]

While LDL particles are responsible for bringing cholesterol from the liver to the peripheral tissues, High-Density Lipoproteins (HDL) perform the opposite function: the transport of cholesterol back to the liver.[26] The ratio of LDL/HDL is used by many as an

indicator of potential cardiovascular disease.[26] One reason for this is the problems that can result from a surfeit of LDL, especially oxidized LDL, in the blood.

1.2.2 The deleterious process of LDL oxidation

The potential relevance of oxidized LDL to the development of atherosclerotic plaques has been studied since it was noted in the 1980's that the incubation of macrophages with oxidized LDL produces cholesterol buildup, while the incubation of macrophages with unmodified LDL does not.[27] Oxidized LDL refers to a form of LDL in which the triglyceride, cholesterol, and apolipoprotein components of the assembly become oxidized by a variety of potential mechanisms. The oxidation of apolipoprotein components typically generates modifications including altered cysteine, methionine, lysine, and arginine residues, which can cause abnormally cross-linked polypeptides.[28] The oxidation of lipid components leads to the formation of molecules including fatty acid peroxides and hydroxides, hydrocarbons, 7-ketocholesterol, and other products of cholesterol oxidation.[28]

Because of these alterations, oxidized LDL is taken up by macrophages at a faster rate than normal LDL. This can lead to the build-up of foam cells, macrophages with a high lipid content, along the arterial wall. This buildup directly contributes to the formation of atherosclerotic plaques, which mature as foam cells secrete pro-inflammatory cytokines. Eventually, a fibrous cap forms over the plaque; the rupture of this cap can lead to blood clotting and dangerous arterial occlusion.[28]

Anti-oxidant substances in blood can oppose this process by reducing the amount of free ROS in the circulation. In particular, the ability of plasma albumin to undergo oxidation, as described in 1.1.3, means that albumin can be thought of as a buffer in plasma to regulate the levels of reactive oxidative species in the blood, reducing the harmful effects of these substances. The free thiol of Cys-34 comprises about 80% of the free thiols present in the plasma, giving HSA a central role in regulating the levels of ROS in plasma.[18]

To investigate the anti-oxidant function of HSA, we performed *in vitro* studies to determine the potential effect of unmodified HSA, fatty acid-bound HSA, and various post-translationally modified forms of HSA on the oxidation of LDL by copper.

CHAPTER 2: METHODS

2.1 Protein samples

Samples of HSA-1 (catalog # A9511), HSA-2 (catalog # A1887), and glycated albumin (catalog # A8301) used in this study were purchased from Sigma Aldrich. Stock solutions of albumin were prepared at 5 mg/mL protein concentrations in 10 mM phosphate buffer (PB), pH 7.5-7.7, and these samples were dialyzed extensively against PB to remove preservatives.

The concentration of protein samples was measured with absorbance spectroscopy, measuring the absorption of UV light at wavelength $\lambda = 280$ nm. The absorbance intensity was compared with a value generated from Beer's Law, $A = \varepsilon cl$, using the protein concentration c mg/mL, the cell path length l cm, and the known extinction coefficient of albumin $\varepsilon = 35219\ M^{-1}\ cm^{-1}$.[29]

2.2 Circular dichroism spectroscopy

2.2.1 An introduction to CD spectroscopy

Circular dichroism (CD) spectroscopy is a well-known method used to investigate the secondary structure of proteins. In a CD experiment, circularly-polarized light is passed through a sample, and the differential degree of absorption of left- and right-handed circularly polarized light is determined. This difference can be substantial for any chiral sample, including polypeptides (as all amino acids except glycine exhibit chirality). In particular, in the far-UV region (190-250 nm), the primary chromophore in a protein is

the peptide bonds of the polypeptide backbone.[30] Therefore, far-UV CD spectra report on the secondary structure of proteins.

Regular structural features of the protein backbone lead to characteristic features in far-UV CD spectra: an α-helical structure is indicated by two minima at 208 nm and 222 nm and a maximum at 192 nm; the β-pleated sheet CD spectrum is characterized by a minimum at 217 nm and a maximum at 198 nm; the random coil structure is indicated by a minimum at ~200 nm.[30-32] Figure 2 depicts the typical CD spectra of polypeptides exhibiting a fully α-helical, β-pleated sheet, or random coil secondary structure.[30] Of course, most proteins consist of varying degrees of these different secondary structure components, resulting in a signal that can be seen as a combination of the contributions of these various structural components. In particular, albumin has been characterized to be about 60% α-helical in content, with very little β-pleated sheet, and most of the rest of the protein consisting of a random coil secondary structure. [33]

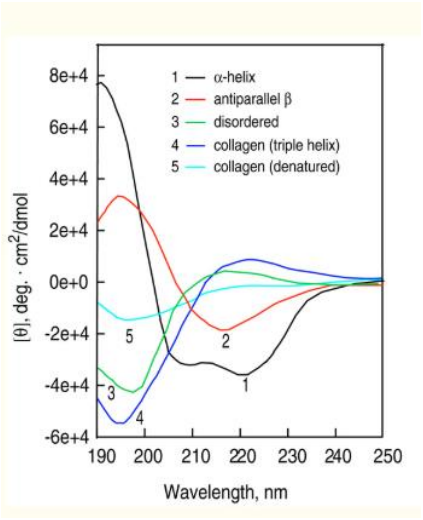

Figure 2.1: Circular dichroism spectra characteristic for protein secondary structures

Here are the CD spectra of model peptides with secondary structure consisting of entirely one type. Adapted from[30].

Since CD spectroscopy is a useful tool to measure protein secondary structure, the measurement of a CD signal during heating of a sample can be used to monitor protein unfolding from a native into a random coil structure to thereby study protein stability. In particular, measuring ellipticity at one wavelength over the course of a thermal melt can provide useful information about the potential loss of secondary structure characteristics that cause a signal at that wavelength. For a protein with significant α-helical structure, this can be done by measuring ellipticity at 222 nm over the course of a melt, since a difference in absorption of left and right-circularly polarized light for protein samples at this wavelength is almost entirely due to the α-helical content of the protein's secondary structure.

2.2.2 Method for CD spectroscopy in this study

CD Spectroscopy was performed with an AVIV-450 spectrometer. Unless otherwise stated, albumin with a concentration of 0.1 mg/mL was dissolved in 10 mM PB at pH 7.7 at room temperature and was placed in a 0.1 mm path length cell. Far-UV CD spectra were recorded at room temperature from 190 nm to 250 nm. The α-helical content of a protein was estimated from the measured value of the molar residue ellipticity at 222 nm, $[\Theta_{222}]$, with the equation % α-helix = $[(-[\Theta]_{222} + 3{,}000)/39{,}000] \times 100$.[33]

For thermal denaturation (melting) experiments, CD signal, which was reported as ellipticity (Θ), was recorded at 222 nm. Heating was performed at a rate of 2 °C per min from 25 °C to 90 °C, followed by cooling to room temperature at the same rate.

2.3 Fluorescence spectroscopy

2.3.1 An introduction to fluorescence spectroscopy

Fluorescence spectroscopy is another well-known method used to investigate protein structure. In a fluorescence spectroscopy experiment, fluorophores absorb radiation at a specific wavelength and then, after a time interval called the fluorescence lifetime, radiation from a lower energy level (longer wavelength) is emitted. Due to their electronic structure, aromatic side chains of proteins absorb UV light more than any other protein residues; phenylalanine residues do so weakly, so the major features of albumin that respond to UV excitation are the Trp-214 residue and tyrosine residues. Emission at 295 nm is selectively absorbed by tryptophan residues, and 280 nm excitation is absorbed by both tryptophan and tyrosine residues.[34]

Therefore, the emission spectra of HSA at 295 nm excitation can be used to examine the local environment of the Trp-214 residue. When albumin is in the folded state, Trp-214 is buried in an internal hydrophobic pocket, though when albumin unfolds, this arrangement is disrupted. When this occurs, the maximum of the fluorescence emission typically undergoes a red shift from ~335 nm to 350 nm, which reflects an increased polarity near Trp-214 resulting from its increased solvent exposure upon unfolding. Thus, the change in the ratio of fluorescence intensity at 335 nm to 350 nm can be used to assess the environment of Trp-214 and the degree to which albumin is in its native state or an unfolded state.[34]

Fluorescent probes can also be used to study the conformational state of a protein; in particular, the fluorescent dye 1-anilinoaphthalene-8-sulfonate (ANS) is known to bind to large hydrophobic pockets of proteins.[35] In 1954, Stryer showed that in the presence of buffer, this dye fluoresces weakly, but when bound to a hydrophobic pocket of apohemoglobin, an increased emission is produced at wavelength $\lambda = 450$ nm.[36]

When ANS binds to a hydrophobic pocket of HSA, excitation of Trp-214 at $\lambda = 295$ nm can lead to a transfer of energy to ANS and a fluorescence emission maximum at $\lambda \sim 450$ nm.[37] The mechanism for effect is Förster resonance energy transfer (FRET), a useful method to test the spatial proximity of specific pairs of fluorophores. FRET occurs if the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor.[34] Then the light emitted by the donor is absorbed by the acceptor and re-emitted at a lower energy (shorter wavelength). This happens when the donor and a fluorophore acceptor are within approximately 10 nm of each other (the

distances are different for different donor-acceptor pairs), yet at longer distances the energy transfer cannot occur. In an ANS-HSA FRET experiment, the intrinsic fluorescence of the Trp-214 residue of HSA (emission maximum at 340 nm) functions as the donor and ANS as the acceptor (emission maximum at 450 nm).[37]

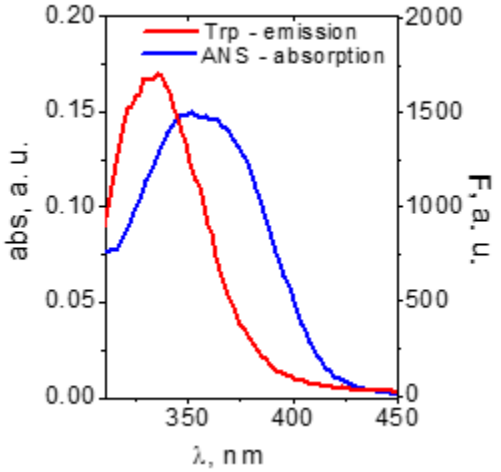

Figure 2.2: FRET from Trp to ANS is facilitated by the overlap in the absorption spectrum of ANS and the emission spectra of Trp

Here the emission spectrum for Trp-214 of HSA (excitation wavelength λ = 295 nm) is plotted next to the absorption spectrum of ANS. The clear spectral overlap between Trp emission and ANS absorption at ~350 nm allows for FRET from Trp-214 to ANS.

2.3.2 Fluorescence spectroscopy experiments in this study

Fluorescence spectroscopy experiments, excluding the Guanidine Hydrochloride (Gd HCl) denaturation experiments, were performed using a Varian Cary Eclipse spectrofluorometer with a 5 mm path length cell. Experiments were performed using 0.1 mg/mL of protein dissolved in 10 mM PB at pH 7.7. To measure the intrinsic fluorescence of HSA-1 and HSA-2, an excitation wavelength of λ_{ex} = 280 nm was used to measure the contributions of tyrosine and tryptophan residues, and λ_{ex} = 295 nm was used to measure the contribution of tryptophan alone. Emission spectra were recorded from 310 nm to 500 nm.

For fluorescence experiments using HSA and ANS, an excitation wavelength of λ_{ex} = 295 nm was used and emission spectra were recorded from 310 to 650 nm. First, samples of ANS were dissolved alone in 10 mM PB at pH 7.7, and the low intensity emission spectra for ANS was recorded. Then, ANS at varying concentrations in PB was added to samples of HSA at 0.1 mg/mL at pH 7.7. The sample was excited at λ_{ex} = 295 nm (to directly excite ANS), and the emission spectra were recorded from 310 to 650 nm.

2.3.3 Gd HCl denaturation experiments

The denaturation of proteins with Gd HCL is a useful means to study protein stability. In these experiments, the loss of protein structure upon increasing denaturant concentrations is monitored at a constant temperature.

0.1 mg/mL samples of HSA-1 and HSA-2 were incubated overnight at room temperature with 1 - 6 M Gd HCl in 10 mM PB at pH 7.7. A TECAN fluorescence plate

reader was used to measure the fluorescence intensity of these samples. Excitation at ƛ = 295 nm was used to measure emission at 335 nm and 350 nm.

2.4 LDL oxidation experiments

2.4.1. LDL oxidation by Cu^{+}

The oxidation of LDL by transition metals such as Copper is a commonly studied *in vitro* method of LDL oxidation. This reaction proceeds with three defined phases: the lag phase, the propagation phase, and the saturation phase. During the lag phase, free radicals are destroyed by anti-oxidants. Then, during the lag phase, a peroxidation chain reaction occurs in which unsaturated fatty acids contained within LDL are oxidized to hydroperoxides. This causes an increase in the level of conjugated dienes in the sample, which is significant because the absorbance of an LDL sample at 234 nm is typically low but is greatly increased with the formation of conjugated dienes. Therefore, the level of absorbance at 234 nm can be measured to track the kinetics of this reaction.[38,39]

2.4.2. Method for LDL oxidation experiments in this study

LDL (apoB concentration 0.1 mg/mL) in 10 mm PB at pH 7.5 was equilibrated at 37 °C. Then, 10 μm of $CuSO_4$ was added to initiate the oxidation reaction. The reaction kinetic was monitored using a Varian Cary Eclipse spectrofluorometer with a 5 mm path length cell by measuring the absorbance at 234 nm at 37 °C. LDL oxidation in the

presence of albumin was performed under identical conditions with 0.1 mg/ml of albumin.

CHAPTER 3: EFFECTS OF FATTY ACID BINDING ON THE STRUCTURE OF HUMAN SERUM ALBUMIN

3.1 Effects of free fatty acid binding on the secondary structure and thermal stability of HSA analyzed by far-UV CD spectroscopy

Far-UV CD spectra were recorded for 0.1 mg/mL albumin samples to examine the secondary structure of HSA with and without fatty acids bound, termed HSA-1 and HSA-2, respectively. As shown in Figure 3A, the spectra of HSA-1 and HSA-2 both show characteristic aspects of an α-helical signal: minima at 208 nm and 222 nm and a maximum at 192 nm. There was no significant difference found between the spectra for the FA-bound and unbound forms of HSA. Both have an α-helical content of ~60%, calculated as described in 2.2.2. Therefore, the secondary structure of HSA was not significantly changed upon free fatty acid binding.

Then, to examine the relative stability of HSA-1 and HSA-2, a CD melt was performed with ellipticity [Θ] measured at ƛ = 222 nm. Given that the signal at 222 nm for an α-helical protein should be a minimum of the far-UV spectrum, measurements were taken at this value to monitor the disruption of secondary structure during heating. There was no significant difference in the melting curves of the fatty acid-bound and unbound forms of HSA. Therefore, thermal stability of HSA did not significantly change upon free fatty acid binding.

Also, samples of HSA-1 and HSA-2 both showed hysteresis. One likely explanation for this is that proteins often aggregate upon thermal denaturation; such

aggregation will not be reversed upon cooling of the sample. Thus, both FA-bound and unbound forms of HSA showed irreversible unfolding during the thermal melt.

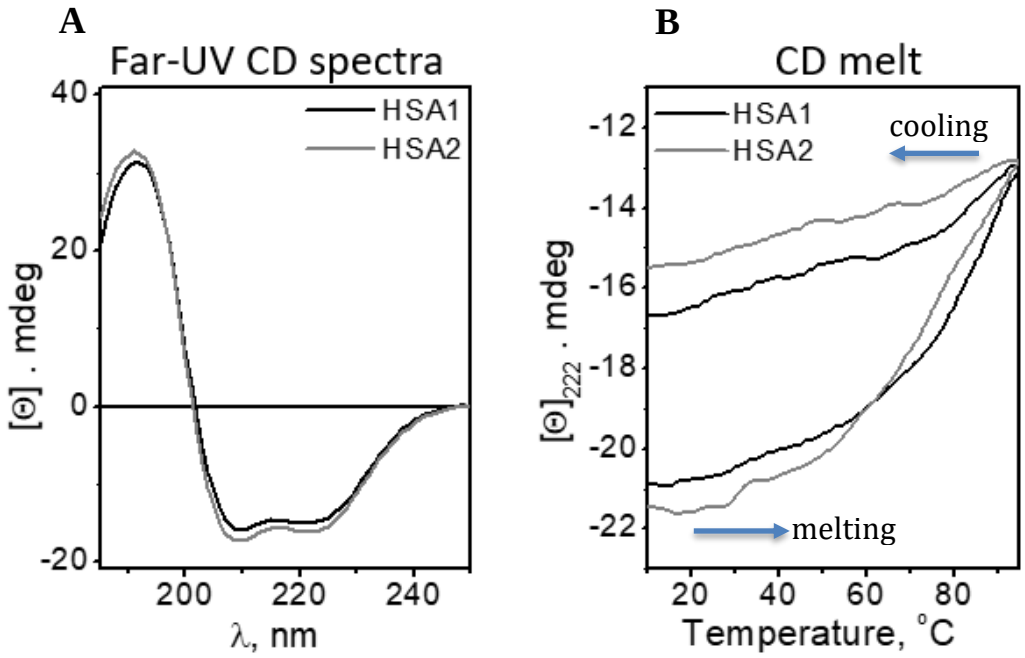

Figure 3.1: The effect of fatty acid binding on the secondary structure and stability of HSA

(3A): Far-UV CD spectroscopy performed with 0.1 mg/mL albumin dissolved in PB as described in 2.2.2. The spectra show no significant difference between HSA-1 (with bound FA) and HSA-2 (without FA).
(3B): 0.1 mg/mL samples of HSA-1 and HSA-2 were heated and then cooled at a rate of 2 °C/min while the CD signal at 222 nm was measured.

3.2 Effects of free fatty acid binding on the environment of Trp-214 in HSA analyzed by intrinsic Trp fluorescence

The intrinsic fluorescence of fatty acid-bound and fatty acid-free forms of HSA was measured to see if there were differences in the local environment of the aromatic residues of HSA-1 and HSA-2. Figure 3.2 shows the emissions spectra generated for samples of HSA-1 and HSA-2 with an excitation wavelength of either ƛ = 280 nm (Figure 3A) or ƛ = 295 nm (Figure 3B). Excitation at ƛ = 280 nm was performed to measure the intrinsic Trp fluorescence of the protein due to tyrosine and tryptophan residues, and excitation at ƛ = 295 nm was performed to solely excite Trp-214. For excitation at both wavelengths, the unbound form of HSA had an emission maximum ~1.5 times that of the fatty-acid bound form of HSA, and the wavelength of this emission maximum was the same for HSA-1 and HSA-2. This difference in emission may result from various factors which require further investigation. However, the observation that the wavelength of the maximal fluorescence remained invariant indicates that the polarity near Trp-214 (and hence, its solvent exposure) did not significantly change upon FA binding.

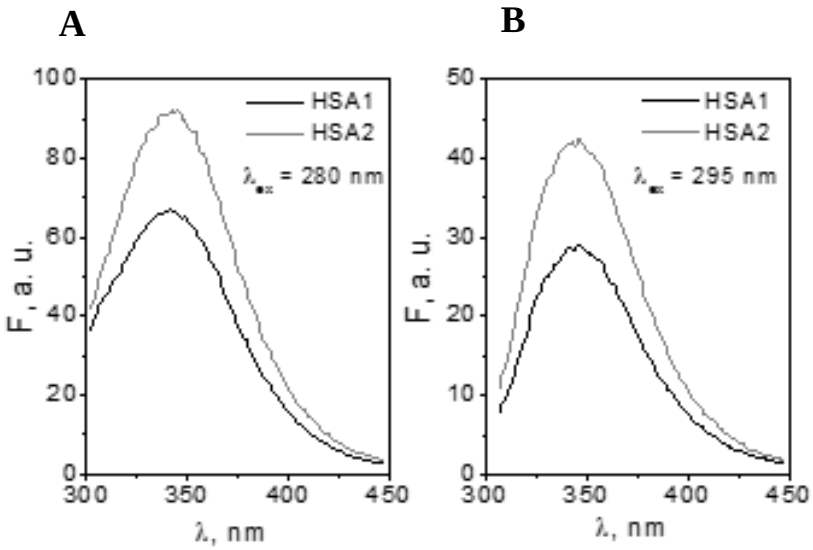

Figure 3.2: The effect of fatty acid binding on the intrinsic fluorescence of HSA

Intrinsic fluorescence experiments performed as described in 2.3.2; 0.1 mg/mL of albumin was dissolved in 10 mM PB at pH 7.7, and excitation at λ_{ex} = 280 nm (A) and λ_{ex} = 295 nm (B) was performed to measure the fluorescence intensity of FA-bound albumin (HSA-1) and FA-unbound albumin (HSA-2).

3.3 Effect of free fatty acid binding on structural stability of HSA at an ambient temperature analyzed by chemical denaturation

A guanidine hydrochloride (Gd HCl) denaturation study was performed to compare by an independent method the stability of HSA-1 and HSA-2. Samples of HSA-1 and HSA-2 were incubated overnight at room temperature with various concentrations of Gd HCl dissolved in standard buffer. After this incubation period, the fluorescence emission of these samples due to excitation at λ = 295 nm was measured. The ratio of emission at 335 nm / 350 nm was measured to determine the relative degree of exposure of the Trp-214 residue to the solvent environment, as described in 2.3.2. There was no

significant difference in the sigmoidal curves generated for the bound and unbound form of HSA (Figure 3.3); this suggests that both forms have similar stability.

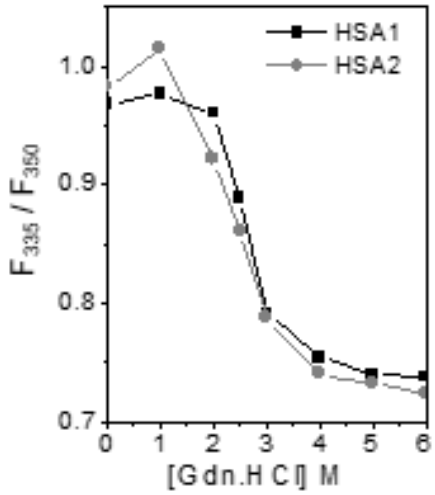

Figure 3.3 The chemical denaturation of FA-bound (HSA-1) and unbound (HSA-2) albumin

0.2 mg/mL samples of HSA-1 and HSA-2 were dissolved in 10 mM Pb at pH 7.7 and incubated with 1 -6 M Gd HCl. The plots of 335 nm / 350 nm emission intensity as a function of Gd HCl concentration for HSA-1 and HSA-2 both show a clear sigmoidal shape, indicating a cooperative protein unfolding with a transition midpoint circa 2.5 M Gd HCl.

3.4 ANS binding to HSA-1 and HSA-2

To examine potential differences in tertiary structure for HSA-1 and HSA-2, the hydrophobic probe 1-anilinonaphthalene-8-sulfonate (ANS) was used to examine the hydrophobic cavities of albumin. Samples containing 20 μm ANS and 0.1 mg/mL albumin in standard PB were excited at either ƛ = 360 nm or ƛ = 295 nm to test either direct ANS emission or the emission of ANS induced by the Forster resonance energy transfer (FRET) from Trp-214, respectively.

Figure 3.4A shows the fluorescence emission spectra of ANS bound to albumin and excited at λ = 360 nm; the emission signal due to ANS alone is weak, especially when compared to the signal from ANS bound to albumin (the black and dark gray lines). In the bound form, ANS shows specific spectral characteristics as compared to unbound ANS, including a ~65 nm red-shift (indicating decreased polarity upon ANS transfer from solution to the HSA binding site) and significant increase in emission intensity (due to decreased quenching of ANS by the solvent). Comparing the emission due to ANS bound to HSA-1 or HSA-2, there was no change in the emission wavelength of ANS (λ_{em} = 475 at λ_{ex} = 360 nm). This means that there is no significant difference in the polarity of the ANS binding site between the fatty acid-bound and FA-free forms of HSA. Nevertheless, there was a significant difference in emission intensity, where ANS bound to HSA-1 has an emissions intensity 1.5 times that of ANS with HSA-2. The exact cause for this intensity difference is unclear. However, the overall spectral similarity for ANS bound to HSA-1 and HSA-2 suggests that ANS does not directly compete with the fatty acids bound to HSA-1 at the same binding site.

These samples of ANS and albumin were also used to measure FRET from Trp to ANS using λ_{ex} = 295 nm to excite Trp-214. Figure 3.4B and 3.4C show the representative FRET data for HSA-1 and HSA-2 in standard buffer. First, as expected, the control sample of ANS in buffer did not show any significant emission at this excitation wavelength. However, upon ANS binding to either HSA-1 or HSA-2, we only saw ANS emission at ~ 450 nm, with a complete loss of the Trp emission signal at 340 nm (Figure

3.4B and C). Therefore, we observed a nearly complete energy transfer from Trp to ANS, so the ANS binding site(s) must be close to Trp-214 in both HSA-1 and HSA-2.

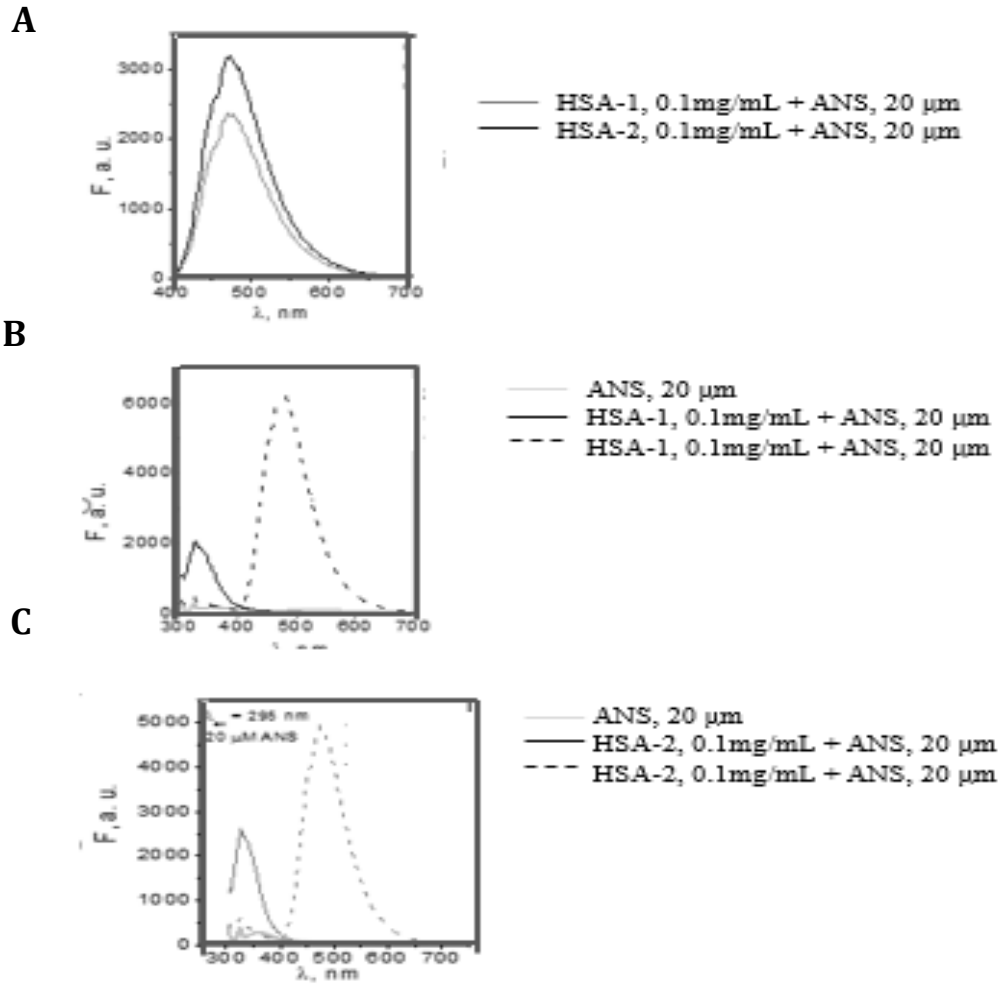

Figure 3.4: The effect of fatty acid binding on hydrophobic regions of albumin

Fluorescence experiments were performed as described in 2.3.2. 20 μm ANS was dissolved in 10 mM PB at pH 7.7, both alone and with 0.1 mg/mL of HSA-1 and HSA-2. Panel A shows direct emission from ANS at λ_{ex} = 360 nm. Panels B and C show FRET from Trp-214 to ANS using λ_{ex} = 295 nm. FRET experiments using HSA-1 and HSA-2 are shown in panels B and C, respectively.

In summary, our studies indicated structural and stability properties of FA-bound and FA-free forms of HSA. CD spectra showed no significant differences in secondary

structure between HSA-1 and HSA-2. Stability studies using thermal and chemical denaturation showed no significant changes in HSA stability upon fatty acid binding. Studies of the intrinsic Trp emission of HSA and indirect emission via bound ANS showed differences in fluorescence intensity between HSA-1 and HSA-2 but no clear indication of specific structural differences. We conclude that fatty acid binding to HSA does not cause any substantial changes in the protein structure and stability.

3.5: LDL oxidation in the presence of HSA-1 or HSA-2

Next, we studied the potential effects of fatty acid binding on one of the functions of albumin, which is its ability to act as an anti-oxidant. To measure the effects of fatty acid binding on the anti-oxidant properties of HSA, copper-induced oxidation of low-density lipoprotein (LDL) was performed in the absence or in the presence of albumin. LDL particles containing 0.1 mg/mL apoB were dissolved in standard buffer, and then $CuSO_4$ was added. The kinetics of the resulting oxidation reaction were monitored at 37 °C by measuring the absorbance of UV light at 234 nm, as the conjugated dienes formed by this reaction absorb at this wavelength.[38] After measuring the rate of this reaction with just Cu and LDL, the experiment was run again with 0.1 mg/mL of either HSA-1 or HSA-2 added. As the solid black and grey curves of Figure 3.5 show, the addition of HSA-1 or HSA-2 to this reaction slowed the oxidative process. Therefore, albumin with or without fatty acids bound can have an anti-oxidant effect on LDL.

In particular, the delay in LDL oxidation upon addition of albumin occurs during the lag phase of this reaction. This suggests that the anti-oxidant effect of HSA is due to

the decreased interactions of the Cu^{2+} ions and the LDL. This could result from Cu^{2+} binding to HSA instead of LDL. In fact, HSA has a Cu^{2+} binding site formed by the N-terminal residues.[6]

Importantly, our results show that there is no significant difference between the anti-oxidant effects of HSA-1 or HSA-2, suggesting that the anti-oxidant effect of albumin on LDL is not significantly influenced by the fatty acid binding to albumin. This suggests that the binding of fatty acids to albumin does not interfere with the interaction between albumin and Cu2+ ions.

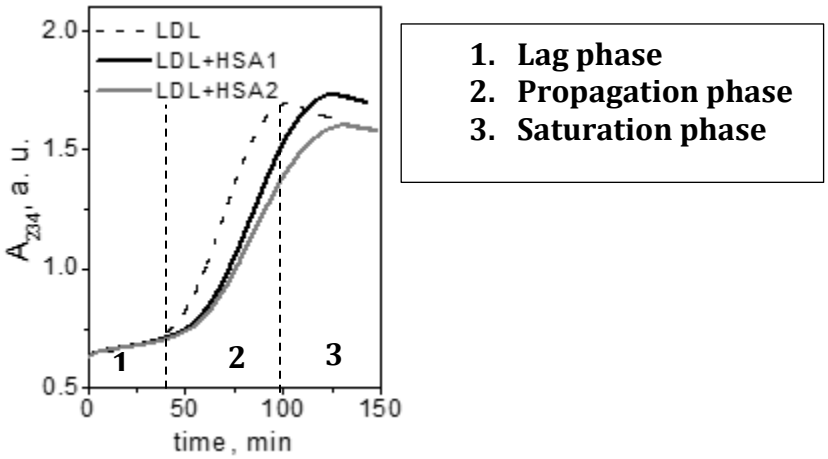

Figure 3.5: The effect of HSA-1 and HSA-2 on LDL oxidation

LDL containing 0.1 mg/mL protein was dissolved in 10 mm PB at pH 7.5; for experiments run with albumin, 0.1 mg/mL of either HSA-1 or HSA-2 were added. At time t=0, 10 μm of $CuSO_4$ was added, and the UV absorbance at 234 nm was measured as a function of time.

CHAPTER 4: EFFECTS OF POST-TRANSLATIONAL MODIFICATIONS ON THE STRUCTURE OF HUMAN SERUM ALBUMIN

4.1 Effects of cystenylation of HSA on the structure and anti-oxidant properties of albumin

The free thiol group of Cys-34 in human serum albumin performs a significant anti-oxidant role by reducing levels of free radicals in plasma. Therefore, we modified Cys-34 in a controlled reaction with glutathione and analyzed the impact of this modification on the structure and function of HSA.

The Cys-34 residue is located on the protein surface and is exposed to solvent, as shown in Figure 4.1. To examine structural and functional effects of modifying this residue, we incubated HSA samples with glutathione and then performed structural studies to compare these samples to the unmodified HSA-1 and HSA-2. We also considered possible functional effects of this cystenylation by examining the oxidation of LDL in the presence of cys-HSA as compared to in the presence of the unmodified protein.

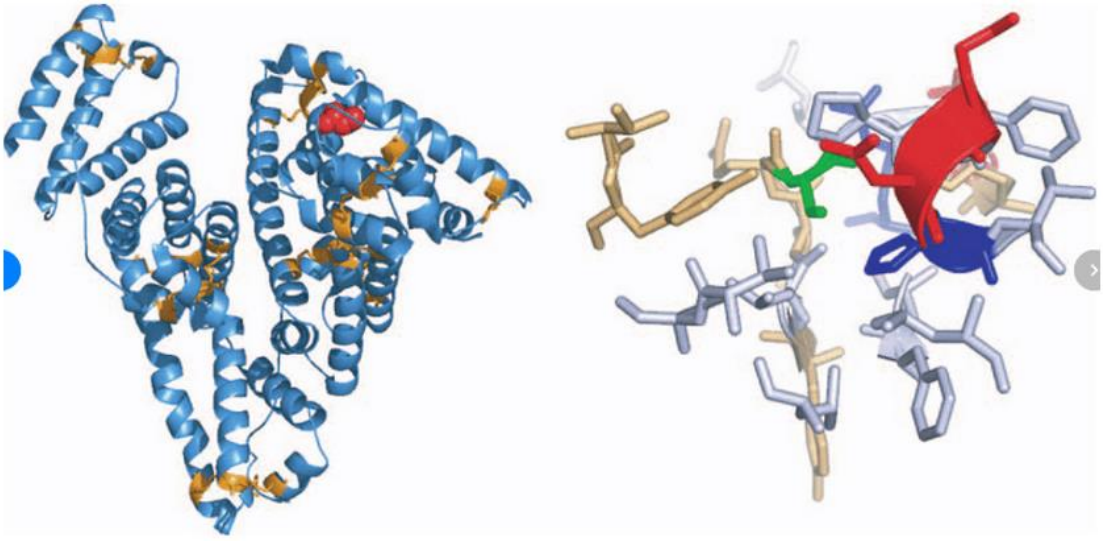

Figure 4.1: The location of the free Cys-34 residue in HSA

(A)The crystal structure of HSA is shown, with disulfide bonds in yellow and the free Cys-34 residue in red.
(B) The local structure of the region of subdomain IA containing Cys-34 is shown, with the free cysteine again in red. Adapted from[40].

4.1.1 The cystenylation process

A similar process was performed to separately produce cystenylated samples of HSA-1 and HSA-2. 100 mM glutathione was added to 5 mg/mL of HSA in 10 mM PB, pH 7.5, and the reaction mixture was incubated at 50 °C for 30 minutes. Then, the mixture was extensively dialyzed against standard buffer.

4.1.2 Secondary structure and stability of cystenylated albumin

First, the secondary structure of cystenylated albumin (cys-HSA) was compared with that of unmodified HSA. Far-UV CD spectra were recorded for 0.1 mg/mL of cys-HSA-1 and cys-HSA-2. The CD spectra for cys-HSA-1 and cys-HSA-2 both show a

significant loss of α-helical secondary structure as compared to unmodified HSA-1 and HSA-2. Further study will be required to determine the precise reason for this change, as the cystenylation process required incubation of the sample at 50 °C for thirty minutes, and this could cause denaturation and aggregation of the albumin protein, which would then decrease the α-helical character of the CD signal of the sample. Therefore, this CD change is probably due to some combination of the change in protein structure due to cystenylation and the aggregation of the HSA sample during the modification process.

Next, a CD melt was performed with ellipticity [Θ] measured for ƛ = 222 nm for the cys-HSA-1 sample (Figure 4.2B). As with the melting data for unmodified albumin, this sample showed hysteresis, probably resulting from protein aggregation upon cooling. Comparison of this data with the melting data for HSA-1 shows no significant difference in stability between the two samples.

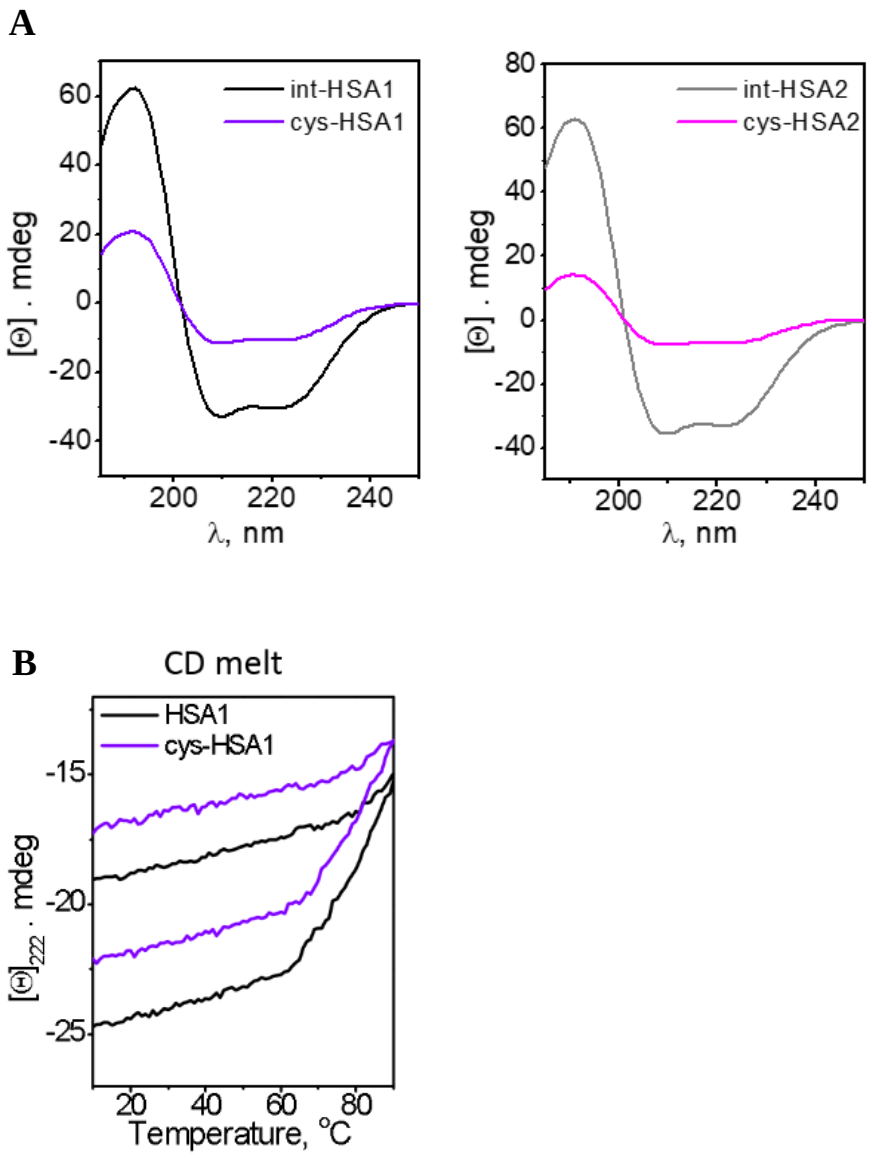

Figure 4.2: The effect of cystenylation on the secondary structure and stability of HSA

(A) Far-UV CD spectra were recorded with 0.2 mg/mL cys-HSA-1 (purple) and cys-HSA-2 (pink) dissolved in PB. These spectra were compared with those for HSA-1 and HSA-2 (black) shown in Figure 3.2.
(3B): 0.2 mg/mL of cys-HSA-1 was heated and then cooled at a rate of 2 °C/min while the CD signal at 222 nm (purple) was measured. This melting curve was compared with that for HSA-1 (black).

4.1.3 Chemical denaturation of cystenylated albumin with Gd HCl

Next, a chemical denaturation experiment was performed using Gd HCl to compare the stability of cys-HSA-1 and cys-HSA-2 samples under the same conditions as those used for the chemical denaturation experiments for HSA-1 and HSA-2. Comparing native FA-bound and FA-free forms of HSA with cystenylated FA-bound and cystenylated FA-free forms of HSA, there is no significant difference in structural stability. This result is in concordance with the result of the CD melt, which also showed no significant difference in structural stability following this post-translational modification.

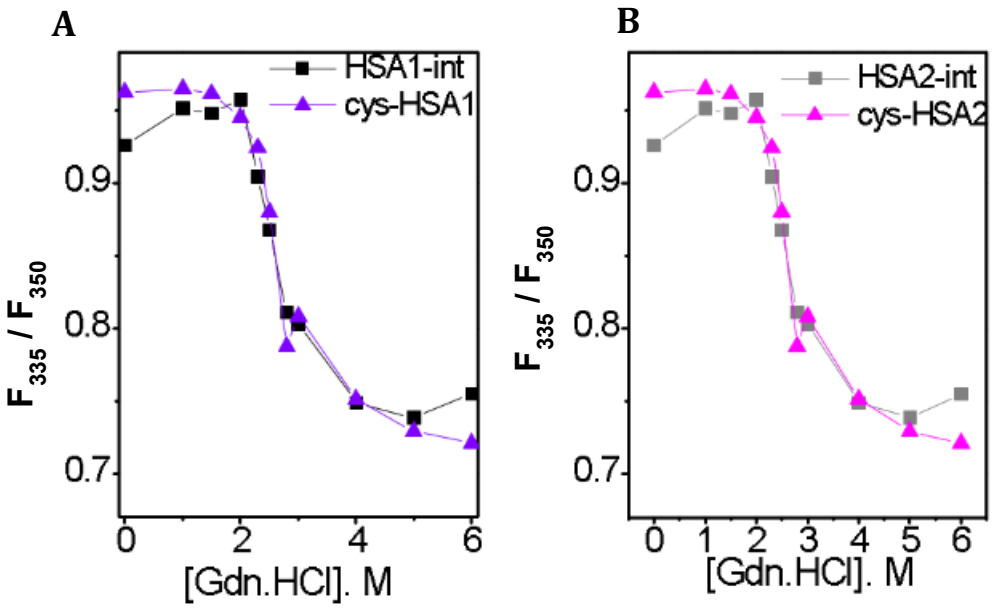

Figure 4.3: The chemical denaturation of cystenylated HSA

0.2 mg/mL samples of cys-HSA-1 and cys-HSA-2 in 10 mM PB at pH 7.7 contained 0 -6 M Gd HCl, as described in 2.3.3. The plots of 335 nm / 350 nm emission intensity as a function of Gd HCl concentration are shown for intact and cystenylated HSA-1 (A) and HSA-2 (B). The plots show a clear sigmoidal shape with a transition midpoint circa 2.5 M Gd HCl, which is similar for all HSA samples.

4.1.4 The effects of cystenylation on the anti-oxidant properties of HSA

We performed the oxidation of LDL in the presence of cys-HSA-1 and cys-HSA-2 to see if the cystenylation of the protein affects its anti-oxidant function. This reaction was performed exactly as described in 2.4.2 using the modified forms of the albumin protein. Figure 4.4 shows the results of LDL oxidation in the presence of cys-HSA-1 (A) and cys-HSA-2 (B) as compared with oxidation in the presence of unmodified albumin and without any albumin added. In contrast to unmodified HSA-1 and HSA-2, which delay the oxidation of LDL, both modified forms of HSA have a pro-oxidant effect.

Both cys-HSA-1 and cys-HSA-2 seemed to significantly decrease the lag phase. It is difficult to determine the exact cause of this change; however, since the effect of HSA-1 and HSA-2 seems to be a delay of the lag phase due to copper binding to HSA, it is clear that this effect does not occur in the presence of cys-HSA-1 and cys-HSA-2. Therefore, this result suggests that there is reduced copper binding to the cystenylated forms of HSA as compared to the unmodified forms. This could be due to the irreversible thermal denaturation of the protein sample during the cystenylation process or due to a change in HSA structure caused by the post-translational modification itself, which affects the copper binding site.

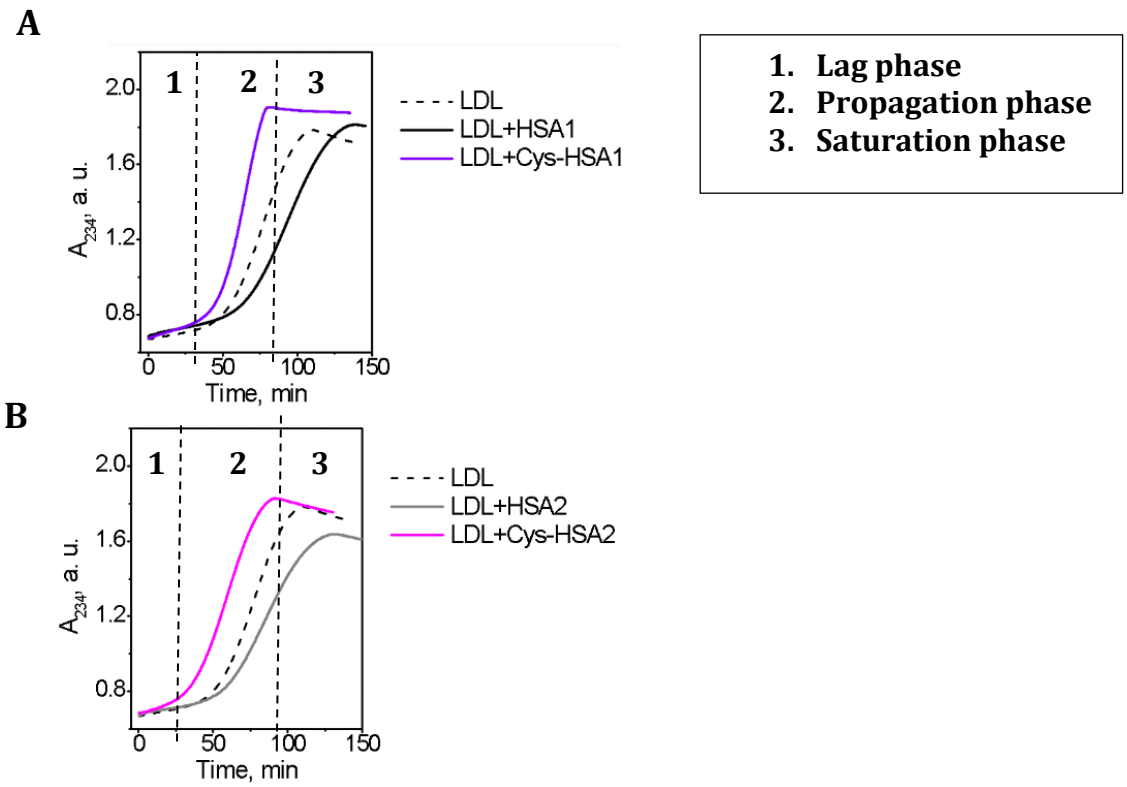

Figure 4.4: The effect of cystenylation on the anti-oxidant properties of HSA

LDL oxidation reactions were performed using the same procedure as described in 3.5: 0.1 mg/mL of apoB in LDL was dissolved in 10mm PB at pH 7.5, and for experiments run with cystenylated albumin, 0.1 mg/mL of cys-HSA-1 (A) and cys-HSA-2 (B) were added. Then, 10 µm of $CuSO_4$ was added. The absorbance of 234 nm UV light was measured as a function of how much time had passed since the Copper Sulfate was added.

4.2 Effects of oxidation of HSA on the structure and anti-oxidant properties of albumin

Oxidation is one of the most common post-translational modification of albumin in the diseased state. Therefore, we investigated the potential structural and functional consequences of the potential structural and functional consequences of oxidizing FA-bound and FA-free HSA through exposure to hydrogen peroxide.

4.2.1 The oxidation of albumin

11.3 μL of 0.3% hydrogen peroxide was added to 10 mL of 2 mg/mL HSA in 10 mM PB, pH 7.5, and the reaction mixture was incubated at 40 ℃ for 1 hour. Then, the mixture was extensively dialyzed against standard buffer.

4.2.2 Structural studies of oxidized HSA

The secondary structure of oxidized HSA-1 (ox-HSA-1) and oxidized HSA-2 (ox-HSA-2) was studied utilizing far-UV CD spectroscopy following the method described in 2.2.2. The CD spectra for the oxidized samples (Figure 4.5A) suggested a reduction in α-helical content as compared to that for unmodified HSA-1 and HSA-2. However, the HSA oxidation experiment involved incubation of the sample for one hour at 40 ℃. Therefore, there is the potential for aggregation of the protein sample during this experiment; such aggregation would cause a change in CD signal of the sample, which may or may not reflect changes in the helical content. Therefore, it is impossible to determine the degree of the change in protein structure due to the oxidation of the sample.

A CD melt was then performed following the method in 2.2.2 to examine the stability of oxidized HSA-1 (Figure 4.5B) and oxidized HSA-2 (Figure 4.5C). The results for these samples were not significantly different than those for unmodified HSA-1 and HSA-2, and the oxidized samples showed hysteresis, demonstrating irreversible unfolding due to heating.

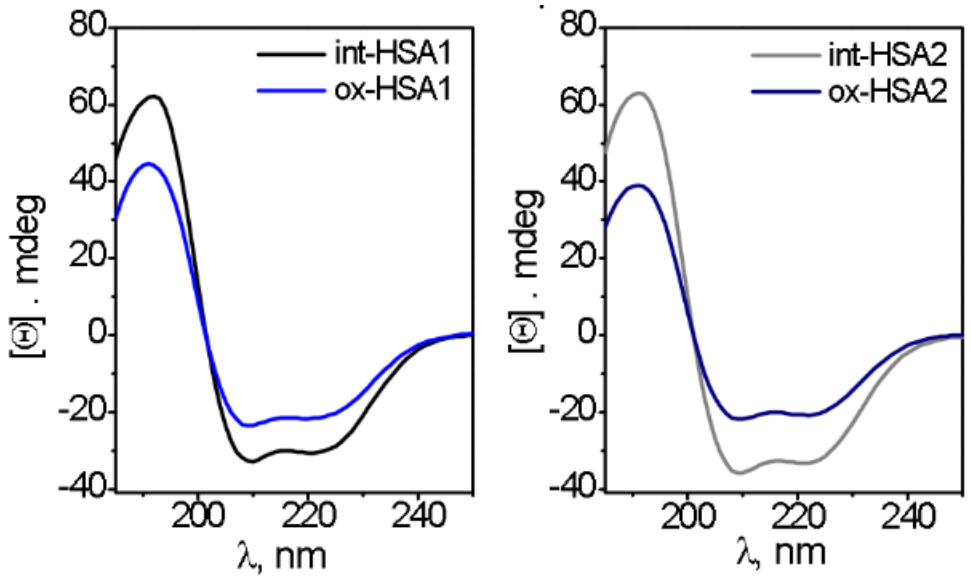

Figure 4.5: The effect of oxidation on the secondary structure of HSA

Far-UV CD spectra were recorded with 0.2 mg/mL ox-HSA-1 (blue) and ox-HSA-2 (dark blue) dissolved in PB. These spectra were compared with those for HSA-1 (black) and HSA-2 (grey).

4.2.3 The effect of oxidation on the anti-oxidant property of HSA

LDL oxidation experiments were performed with the ox-HSA samples to compare the results to those found for the unmodified HSA samples. The results of these experiments are shown in Figure 4.6. Interestingly, unlike unmodified HSA, which

served as an anti-oxidant for LDL, oxidized HSA showed a pro-oxidant effect by decreasing the lag phase in LDL oxidation. This effect was similar for HSA-1 and HSA-2 in that FA binding did not influence the pro-oxidant or anti-oxidant properties of HSA. The molecular basis for this effect of HSA on LDL oxidation needs further investigation.

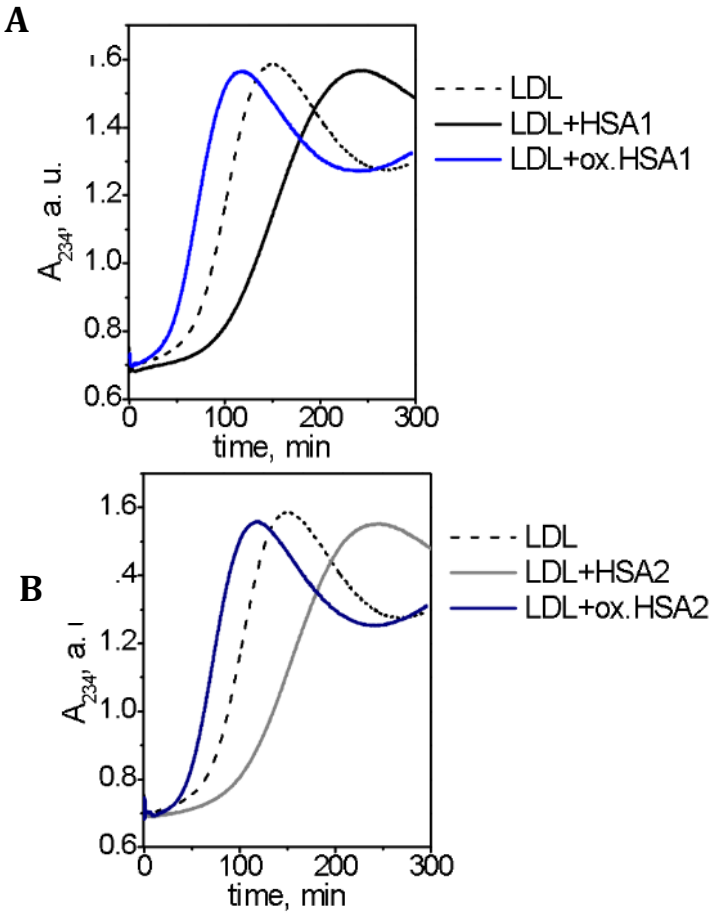

Figure 4.6: The effect of oxidation on the anti-oxidant properties of HSA

LDL oxidation reactions were performed using the same procedure as described in 3.5: 0.1 mg/mL of apoB in LDL was dissolved in 10mm PB at pH 7.5, and 0.1 mg/mL of ox-HSA-1 (A) and ox-HSA-2 (B) were added. Then, 10 μm of $CuSO_4$ was added. The absorbance of 234 nm UV light /was measured as a function of how much time had passed since the Copper Sulfate was added.

4.3 Effects of glycation and glycoxidation of HSA on the structure and anti-oxidant properties of albumin

Samples of HSA glycated in vitro, with 1-5 mol hexose (as fructosamine) per mol albumin, were purchased from Sigma Aldrich. The structure of glycated HSA-1 (gly-HSA-1) was studied utilizing the same techniques as those used to study the structure of the previous HSA samples.

4.3.1 Structural studies of glycated albumin

The CD spectra of the glycated sample (Figure 4.7A) showed no significant difference in secondary structure as compared to the unmodified HSA sample. Therefore, the α-helical content of the glycated sample is very similar to that of unmodified albumin. The CD melt of the glycated sample showed a small low-temperature shift when compared to the CD melt of the unmodified protein, suggesting a small decrease in stability upon glycation. The significance of this effect and its molecular origin needs further investigation. difference when compared to the CD melt of the unmodified protein.

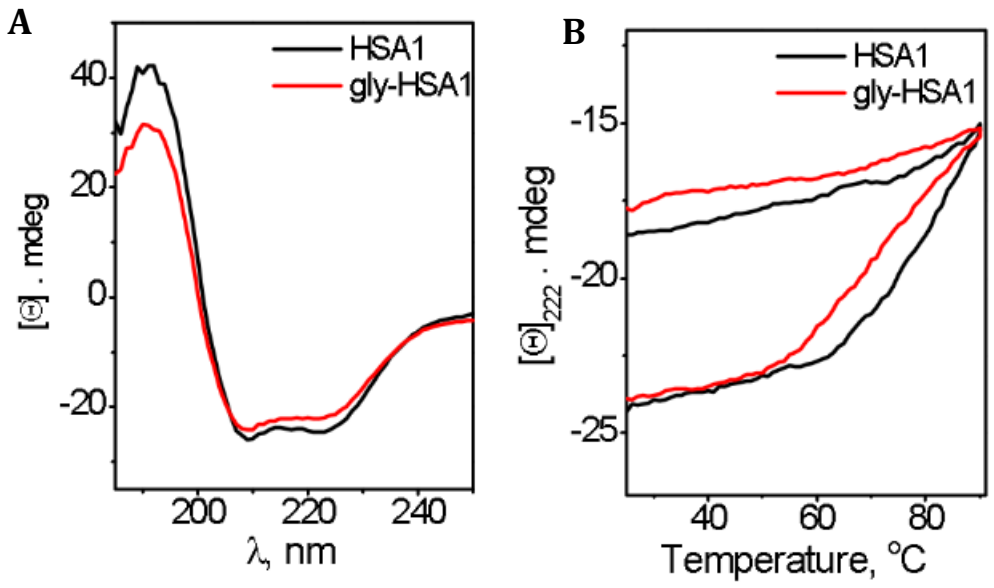

Figure 4.7: The effect of glycation on the secondary structure and stability of HSA

(A) Far-UV CD spectra were recorded with 0.2 mg/mL gly-HSA-1 (red) dissolved in PB. These spectra were compared with those for HSA-1 (black).
(B): 0.2 mg/mL of gly-HSA-1 was heated and then cooled at a rate of 2 °C/min while the CD signal at 222 nm (red) was measured. This melting curve was compared with that for HSA-1 (black).

4.3.2 The pro-oxidant effect of glycated albumin

LDL oxidation experiments were performed with the gly-HSA samples to compare the results to those found for the unmodified HSA samples. The results of these experiments are shown in Figure 4.8. Gly-HSA-1 had a pro-oxidant effect by decreasing the lag phase.

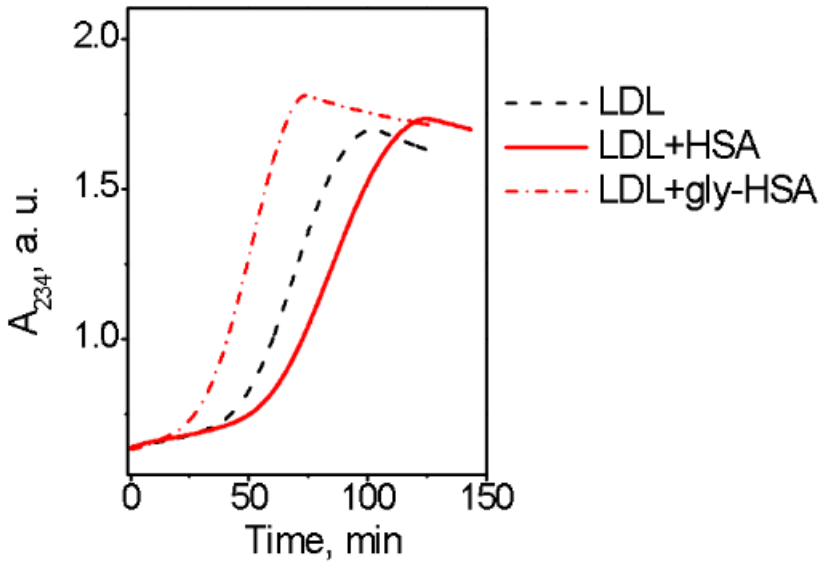

Figure 4.8: The effect of glycation on the anti-oxidant properties of HSA

LDL oxidation reactions were performed using the same procedure as described in 3.5: 0.1 mg/mL of apoB in LDL was dissolved in 10mm PB at pH 7.5, and 0.1 mg/mL of gly-HSA-1 was added. Then, 10 μm of $CuSO_4$ was added. The absorbance of 234 nm UV light was measured as a function of how much time had passed since the Copper Sulfate was added.

4.3.3 The glycoxidation of albumin

In order to determine the structural and functional properties of glycoxidized HSA, 1 mg/mL HSA in 10mM PB, pH 7.5, was incubated with 0.5 mM methylglyoxal purchased from Sigma-Aldrich (catalog number M0252) at 37 ℃ for 3 hours. Then the reaction mixture was dialyzed against standard phosphate buffer to remove excess methylglyoxal, and the glycoxidized albumin was stored at 4 ℃.

The structure of glycoxidized HSA-1 (methylglyoxyl-HSA-1) and glycoxidized HSA-2 (methylglyoxyl-HSA-2) was studied utilizing CD spectroscopy using the same method as that used to study the unmodified HSA-1 and HSA-2 samples; results of these experiments are shown in Figure 4.9. A steeper slope in the melting data observed for glycoxidized HSA-1 and glycoxidized HSA-2, as compared to unmodified HSA-1 and HSA-2, suggests that glycoxidation slightly increases the unfolding cooperativity of HSA regardless of the presence of bound FA. The significance of this effect and its underlying /mechanism requires further investigation.

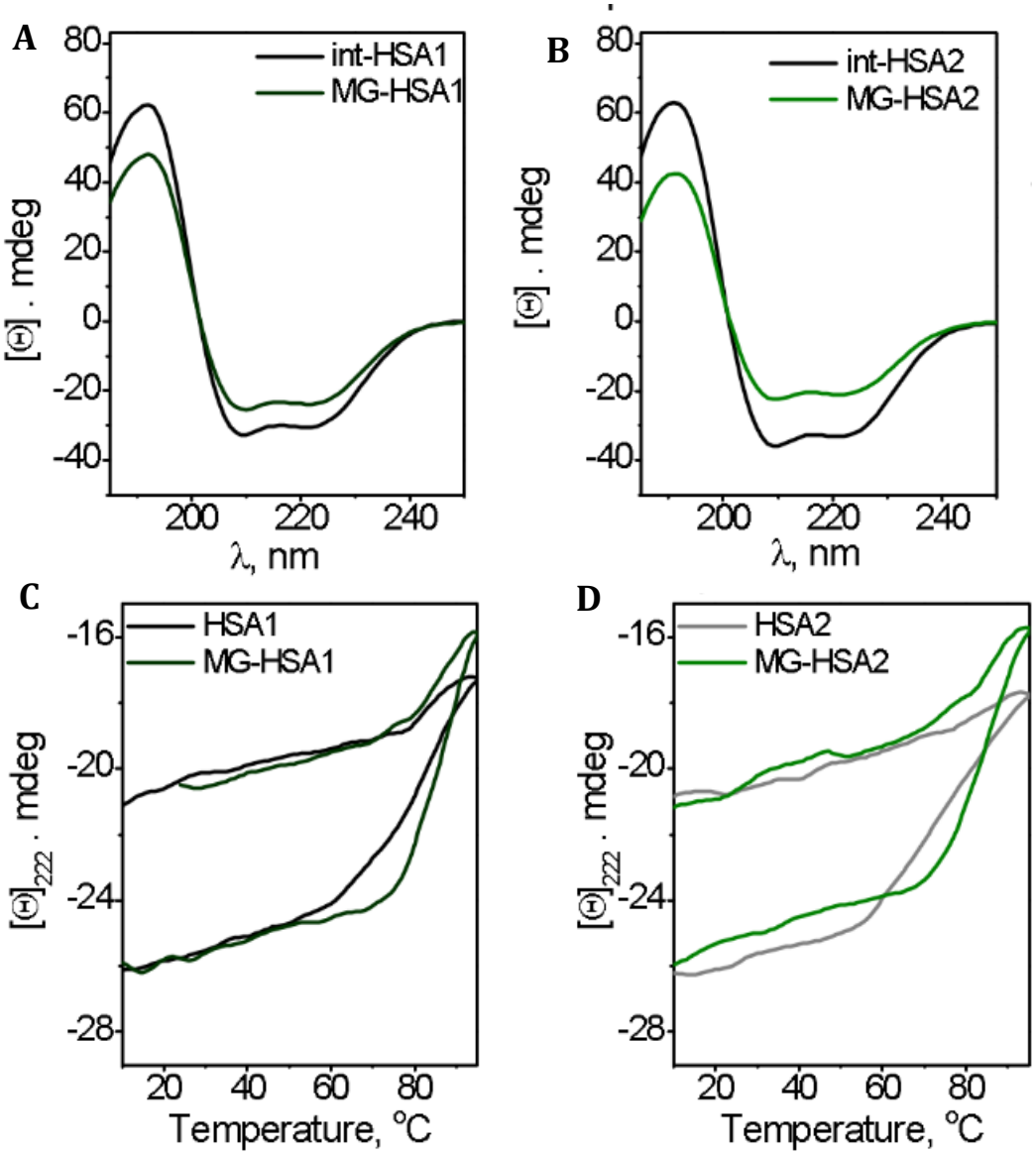

Figure 4.9: The effect of glycoxidation on the secondary structure and stability of HSA

(A,B) Far-UV CD spectra were recorded with 0.2 mg/mL methylglyoxyl-HSA-1 (dark green) and methylglyoxyl-HSA-2 (green) dissolved in PB. These spectra were compared with those for HSA-1 (black) and HSA-2 (black).
(C,D): 0.2 mg/mL of methylglyoxyl-HSA-1 was heated and then cooled at a rate of 2 °C/min while the CD signal at 222 nm (dark green) was measured. This melting curve was compared with that for HSA-1 (black). A similar experiment was performed to compare the thermal stability of methylglyoxyl-HSA-2 (green) with HSA-2 (black).

Then, LDL oxidation experiments were performed with the glycoxidized HSA samples to compare the results to those found for the unmodified HSA samples. The results of these experiments are shown in Figure 4.10. Like oxidized HSA-1 and HSA-2, glycoxidized HSA-1 and HSA-2 had a clear pro-oxidant effect and shortened the lag phase of LDL oxidation. The exact cause of this effect will be explored in future studies.

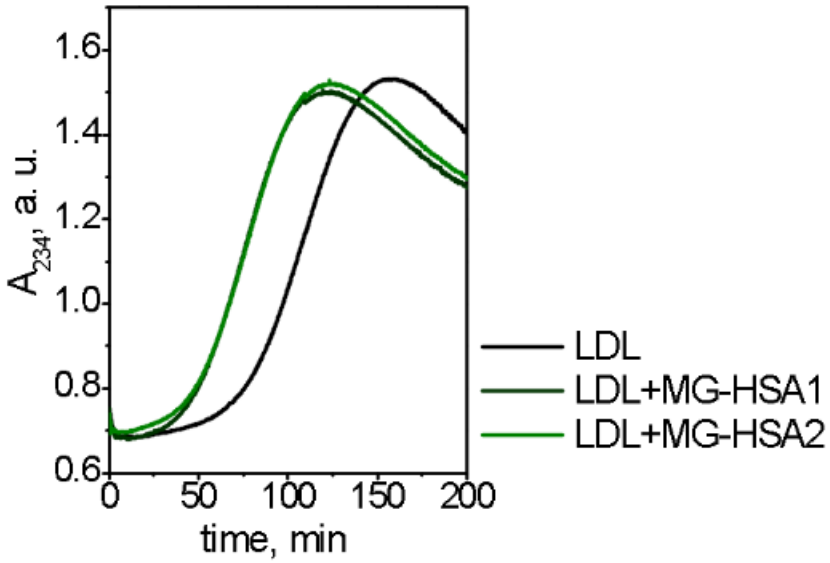

Figure 4.10: The effect of glycoxidation on the anti-oxidant properties of HSA

LDL oxidation reactions were performed using the same procedure as described in 3.5: 0.1 mg/mL of apoB in LDL was dissolved in 10mm PB at pH 7.5, and 0.1 mg/mL of glycox-HSA-1 (light green) and glycox-HSA-2 (dark green) were added. Then, 10 μm of $CuSO_4$ was added. The absorbance of 234 nm UV light /was measured as a function of how much time had passed since the Copper Sulfate was added. The reaction kinetics for glycox-HSA-1 (dark green) was then compared with those for HSA-1 (black), and the same was done for glycox-HSA-2 (green) and HSA-2 (black).

DISCUSSION

The results reported in this thesis show that fatty acid binding did not significantly alter the structural, stability or anti-oxidant properties of HSA. However, all post-translational modifications of HSA, including cystenylation of Cys-34, glycation, oxidation, and glycoxidation, reversed the effect of HSA on LDL oxidation by copper: unmodified HSA was clearly anti-oxidant, while modified HSA acted as a pro-oxidant by decreasing the lag phase of LDL oxidation. To our knowledge, this effect has not been reported previously. The biochemical and structural basis underlying this general effect and its relevance to various oxidation processes *in vivo* remain to be determined in the future.

One important aspect of future studies is to consider the structural and functional effects of the post-translational modifications themselves, without the artefacts resulting from the experimental protocols involved. For example, the HSA glycation performed by Sigma-Aldrich, which did not involve protein heating, had very little effect on secondary structure observed by CD spectroscopy. This suggests that HSA heating used in the other methods to induce the other post-translational modifications caused significant changes to the protein structure. To eliminate these artefacts, in the future alternative methods will be used to introduce HSA modifications without heating. The results will help establish the molecular basis for the observed functional changes in modified HSA, such as its effects on LDL oxidation.

Another important aspect for future studies is to consider the effects of these post-translational modifications on drug binding and other properties of albumin besides its anti-oxidant function. Various post-translational modifications of HSA are known to exist in higher concentrations in various diseases states; for example, high levels of glycated HSA have been found in the serum of diabetic patients,[21] and high levels of oxidized HSA are thought to potentially correlate with the development of advanced cardiovascular disease.[41-3] Further *in vitro* experiments, building on the work done here, could elucidate potential mechanisms for altered function of such modified forms of albumin.

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