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Investigation of guanidine hydrochloride induced unfolding of apolipoprotein A-I_{Milano}

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Abstract. A guanidine hydrochloride (GuHCl) induced unfolding study of apolipoprotein A-I_{Milano} (apo A-I_M) has been performed. The unfolding was followed by circular dichroism (CD) measurements at 222 nm and by isothermal titration (ITC) calorimetry at 25°C. In the ITC experiments enthalpies of transfer were determined for apo A-I_M from aqueous sodium phosphate buffer solution into solutions of different concentrations of GuHCl. The CD data and the ITC data give complementary and consistent results of the complex unfolding process. Analytical ultracentrifugation experiments were made on apo A-I_M in sodium phosphate buffer and in 0.6 M GuHCl, respectively. Analyses of the obtained sedimentation velocity data show that apo A-I_M is highly aggregated in sodium phosphate buffer. The aggregates are almost completely dissociated in 0.6 M GuHCl. Aggregation of the protein in sodium phosphate buffer solution induces an increase in α-helical content. The loss of α-helical secondary structural element of the protein upon dissociation of the aggregates destabilises the protein resulting in a low GuHCl concentration of unfolding, $[\text{GuHCl}]_m = 1.1$ M. The unfolded protein has a significant α-helical content at the unfolded state. From ITC- and CD data we suggest that increased binding of GuHCl to the unfolded protein results in a disruption of the residual secondary structure.

1. Introduction

Apolipoprotein A-I (apo A-I) is the major protein of high density lipoproteins (HDL). The main function of HDL is to transport cholesterol from peripheral tissue to the liver for reuse or excretion a process called reverse cholesterol transport (RCT) [1]. In this process apo A-I is a potent activator of the enzyme lecithin cholesterol acyltransferase (LCAT), which is a key enzyme in the cholesterol metabolism [2]. HDL levels in plasma are found to be inversely correlated by the occurrence of arterosclerosis [3].

Apo A-I is a 28 kDa protein with 243 amino acids [4] arranged in repeating units of 11 or 22 residues, predicted to form amphiphatic α-helices [5]. Apo A-I exists in both free and lipid-bound form, the free apo A-I is an asymmetric elongated protein [6], having about 45% α-helical content. When the protein binds to lipids the α-helical content increases to 75% [7]. The protein is in its free form highly self-associated and aggregated protein has a higher α-helicity than the non-aggregated protein [7].

A molecular variant of apo A-I named apolipoprotein A-I_{Milano} (apo A-I_M) was found in a family in Italy. The carries show low prevalence of developing atherosclerosis and longevity [8]. The variant protein has a single amino acid substitution at position 173, where an arginine is replaced by a cysteine. The resulting protein exists mainly as a disulphide-linked homodimer [9]. The substitution, situated in one of the α-helices, facilitates the interactions between α-helices. Consequently this affects the secondary as well as the tertiary structure and thereby the lipid binding properties of the protein [7]. The lipoprotein particles formed by phospholipids and apo A-I are more heterogeneous than the particles formed by apo

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A-I_M [10,11]. The restriction and heterogeneity in particle size for apo A-I_M has been suggested to be due to restrictions in the possible range of protein conformations of the disulphide linked dimer [10].

We have earlier reported studies on chemical and thermal induced unfolding of apo A-I and apo A-I_M [12,13]. The thermal induced unfolding showed intermediate states of both proteins. For apo A-I_M the intermediate state is well separated from the main transition, while for apo A-I the intermediate state is partly overlapping with the main transition [13]. Apo A-I_M is susceptible to low concentrations of GuHCl. The earlier data suggest that the changes in ellipticity involve changes in the state of oligomerization, as well as formation of intermediate states before unfolding [12].

Our aim in this study was to in more detail characterise qualitatively and quantitatively the unfolding of apo A-I_M and dissect the different processes involved in the unfolding process. The strategy was to study the isothermal GuHCl induced unfolding by isothermal titration calorimetry, ITC, and circular dichroism, CD, at 25°C.

The use of isothermal calorimetry to characterise and quantify the unfolding behaviour of proteins in the presence of denaturants has not gained much attention and use. Pfeil and Privalov used isothermal calorimetry for the study of GuHCl induced unfolding of lysozyme in 1976 [14]. More recently urea and GuHCl induced unfolding of lysozyme, cytochrome c and ribonuclease A was studied by isothermal calorimetry by Makhatadze and Privalov [15]. In these studies the denaturation of the proteins and the preferential binding of denaturants to the proteins were thermodynamically evaluated. In the earliest study it was demonstrated that the states of thermal induced unfolded and GuHCl induced unfolded lysozyme is thermodynamically indistinguishable [14].

We have also conducted sedimentation velocity experiments on apo A-I_M in sodium phosphate buffer and in 0.6 M GuHCl solution for comparing the aggregation state of the protein in the two solutions.

2. Materials and methods

Apolipoprotein A-1_M was expressed in *E. coli* and purified as described elsewhere [7]. Protein was received in water for injection at a concentration of 22.5 mg/ml according to a SDS-gelfiltration method. 93% of the protein exists as dimer, 3.5% as monomer and 3.5% as multimers. Protein was diluted in 50 mM sodium-phosphate buffer at pH 7.4 to an approximate concentration of 5 mg/ml. Protein solutions were extensively dialysed. Protein concentrations were determined with the Lowry method (Protein Assay kit P5656, Sigma Diagnostics, St. Louis, MO, USA). The apo A-I_M solutions were stored in freezer (−80°C) before use as well as the buffer against which it was dialysed.

A stock guanidine hydrochloride (GuHCl) solution was prepared by weighting the appropriate amount of GuHCl and adding 50 mM sodium-phosphate buffer to a concentration of 6 M GuHCl. The stock solution was then diluted to the desired concentration with sodium phosphate buffer, and pH was adjusted to 7.40 with 0.5 M sodium hydroxide.

The microcalorimetric titrations were performed in the Thermal Activity Monitor (Thermometric AB, Järfälla, Sweden), equipped with nanowatt-amplifiers and 4 ml titration vessels. Experiment control and data collection was done using the Digitam Software (Scitech Software, Järfälla, Sweden). The calorimeters were calibrated electrically using both static and dynamic calibrations. 2.7 ml of a GuHCl solution (0–4 M) was placed in the titration cell, which was stirred with a turbine stirrer at a rate of 60 rpm. After equilibration of the system, five additions of 10 μl buffer were made in time intervals of 20 min between each addition. Thereafter five protein injections were made with the same volume and time intervals, the protein concentration in the syringe being 2.5 mg/ml. Standard deviation of each five injections was less

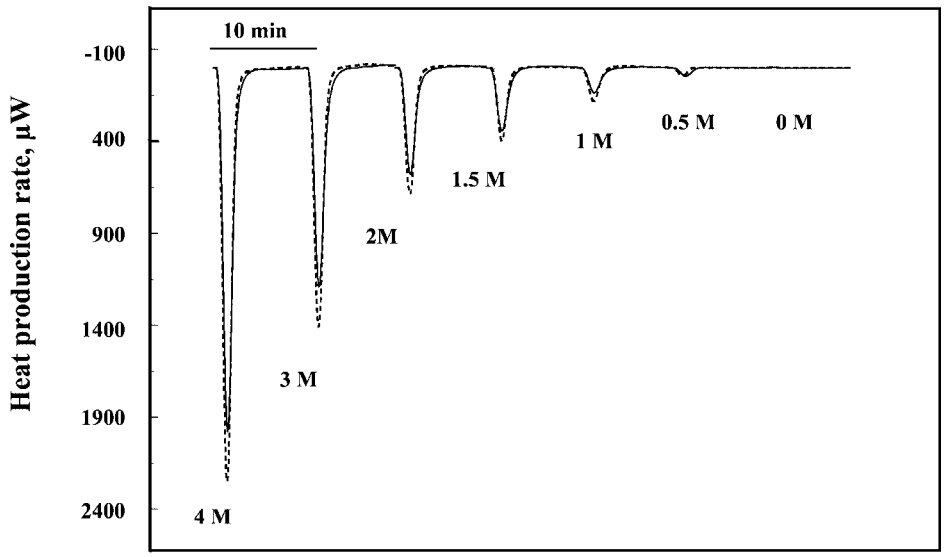

Fig. 1. This figure shows calorimetric injection peaks from the investigated GuHCl concentration range. The peaks are from different titration experiments, but put together in one figure to visualise the heat effects at different GuHCl concentrations. — buffer injections and - - - protein injections.

than 2%. In order to obtain the enthalpy of transfer of the protein the data was corrected for the contribution of adding aqueous phosphate buffer to the GuHCl solution. In Fig. 1 the calorimetric power-time curves of the protein transfer experiments and the phosphate dilution experiments are illustrated.

CD measurements were carried out on a Jasco J-720 Spectropolarimeter (Jasco Corporation, Tokyo, Japan) equipped with peltier element (PTC-348 WI) for temperature control. Instrument control and data collection was done with the J-700 software. Ellipticity was measured at 222 nm, at a protein concentration of 0.1 mg/ml in a 0.1 cm quartz cuvette. Three scans of the same solution were averaged. Protein was incubated for two hours in the GuHCl solution at 25°C before measurement. All measurements were performed at 25°C. Ellipticity of the pure GuHCl solution at every concentration was subtracted from the ellipticity of the corresponding protein solution. The CD-signal was converted to molar mean residue ellipticity, $[\theta]$, expressed in degrees $\mathrm{cm}^2\,\mathrm{dmol}^{-1}$ and calculated as

$$[\theta] = (\theta)_{\mathrm{obs}} \times 115/(10 \times l \times c), \tag{1}$$

where $(\theta)_{\mathrm{obs}}$ is the observed ellipticity in degrees, 115 is the mean residue molecular weight of the apo A-I_M, l is the optical path-length in centimetres and c is the protein concentration in g/ml.

For the sedimentation velocity experiments, a Beckman Optima XL-I analytical ultracentrifugation with interference optical detection system was used. Epon double-sector centrepieces were filled with 400 μl of sample solution. The samples were 1 mg/ml apo A-I_M in 50 mM sodium phosphate at pH 7.4, and 1 mg/ml apo A-I_M in 50 mM sodium phosphate and 0.6 M GuHCl at pH 7.4. The experiments were performed at a rotor speed of 50,000 rpm at 25°C. At each experiment 200 scans of interference data were acquired in time intervals of 40 s.

The data were analysed applying the Lamm equation on sedimentation using the program SEDFIT [16]. The specific volumes of the protein, density of the solvents and viscosity of the solvents needed for

the data analysis were calculated using the program SEDNERP by John Philo, which can be downloaded from the RASMB file archive.

3. Results and discussion

From the sedimentation velocity experiments of apo A-I_M in 50 mM sodium phosphate it is obvious that the protein has a high prevalence to aggregate, Fig. 2A. According to the results from the analysis more than 80% of the protein is aggregated at the concentration at which the analytical ultracentrifugation experiments were performed. The size distribution shows that there are non-aggregated protein and oligomers of containing two, thee and four protein molecules present.

Calorimetric dilution experiments of apo A-I_M in sodium phosphate buffer resulted in small and negligible enthalpies and did not show any concentration dependency (data not shown). The results from the dilution experiments indicate that the aggregation equilibrium is not shifted at the concentration range of the study, when the protein is in sodium phosphate buffer. The enthalpies of transfer, $\Delta_{trans}H$, of apo A-I_M from aqueous phosphate buffer solution into aqueous GuHCl solution has a complex variation in magnitude as a function of GuHCl concentration, as shown in Fig. 3A. Strikingly, the magnitudes of $\Delta_{trans}H$ to the different GuHCl solutions seems unlikely large, -9 to 1 MJ/mol apo A-I_M. However, as will be seen below in the text, the analysis of the data agrees with earlier published data. Expressing the enthalpies in terms per mol residues the enthalpies are in the range of -18 to 1 kJ/mol residue, which is

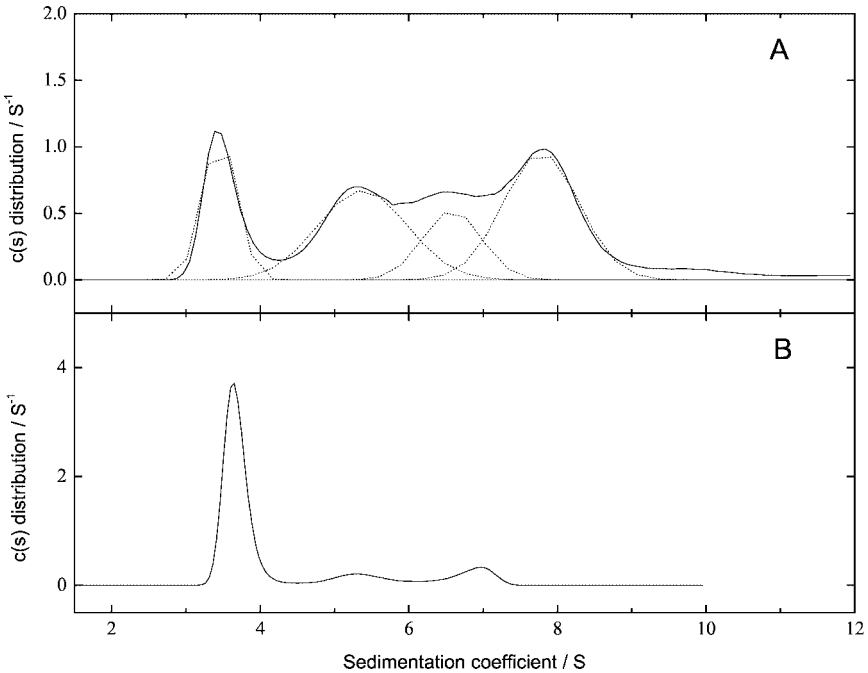

Fig. 2. Size distribution plots, $c(s)$ versus sedimentation coefficient S, calculated from sedimentation velocity data with the program SEDFIT [16]. Each plot is based upon 200 interference data scans acquired at 40 s intervals at a rotor speed of 50,000 rpm. (A) 1 mg/ml apo A-I_M in 50 mM sodium phosphate at pH 7.4. The analysed data was deconvoluted into four species by Gaussian distribution shown as dotted lines. The calculated frictional ratio, f/f_0, from the size distribution analysis frictional ratio was 1.37. (B) 1 mg/ml apo A-I_M in 50 mM sodium phosphate and 0.6 M GuHCl at pH 7.4. The calculated frictional ratio, f/f_0, from the size distribution analysis frictional ratio was 1.08.

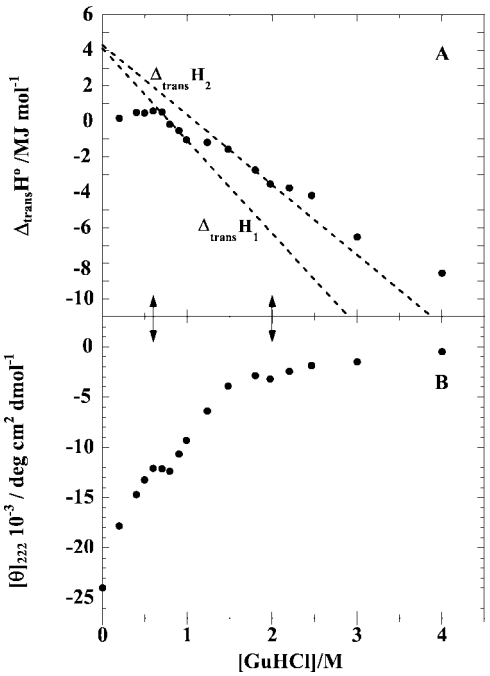

Fig. 3. The double graph shows experimental results from ITC and CD. The double arrows at 0.6 M and 2 M GuHCl mark the concentration ranges discussed in the text. (A) Enthalpy of transfer, Δ_{trans}H, for 43 μM apo A-I$_{\text{M}}$ aqueous buffer solution into different concentrations GuHCl solutions. The final concentrations of apo A-I$_{\text{M}}$ for all experiments were 0.16 μM. The dotted lines represent the estimated Δ_{trans}H of the stable states, denoted $\Delta_{\text{trans}}H_1$ and $\Delta_{\text{trans}}H_2$, described in the text. (B) The ellipticity, $[\theta]_{222}$, of apo A-I$_{\text{M}}$ in different GuHCl concentrations.

in a range that is perhaps more easy to conceive. Looking at the plot of Δ_{trans}H against the GuHCl concentration to which the protein has been transferred one can identify three concentration ranges at which different processes occur. Interestingly, the same division in concentration ranges can be identified from the results of ellipticity of apo A-I$_{\text{M}}$, Fig. 3B. When there is a change in tendency of the enthalpy there is also a change in tendency of the ellipticity at 222 nm. Having data from two different techniques sharing the same features clearly indicates that we can separate three different concentration ranges that describe different processes.

The comments and the discussions about the GuHCl concentration dependence of the Δ_{trans}H refer to Fig. 3A and the ellipticity data of apo A-I$_{\text{M}}$ at different GuHCl concentrations refer to Fig. 3B.

3.1. GuHCl concentration 0–0.6 M

Sedimentation velocity data analysed by applying the Lamm equation by the SEDFIT program [16] shows clearly that the degree of aggregation of the protein changes drastically when going from sodium phosphate buffer to 0.6 M GuHCl, Fig. 2B. At this GuHCl concentration the protein is essentially all in non-aggregated form. Noticeable from the analysis was the difference in fractional ratio, f/f_0, for the protein in 50 mM sodium phosphate buffer and in the 0.6 M GuHCl buffer. The frictional ratio was 1.37 in sodium phosphate buffer, while the values dropped down to 1.08 in 0.6 M GuHCl buffer. This would reflect that the aggregated particles are more elongated than the non-aggregated form.

At this GuHCl concentration range there is a significant change in ellipticity. The ellipticity increases from $-24\,000$ deg cm^2 dmol^{-1} to $-12\,000$ deg cm^2 dmol^{-1}. The rapid increase in ellipticity can be ex-

plained by that the aggregation of the protein induces an increase in α-helical secondary structure elements. This is analogous to the increase in α-helical structure when the protein is bound to lipids. Its α-helical content is then higher than its free monomeric form [7]. Thus, lacking interactions with hydrophobic surfaces such as other apo A-I_M molecules or lipids the secondary structural elements in the protein is destabilised.

The enthalpy of transfer is essentially constant at this concentration range. A constant enthalpy change is obtained if the degree of aggregation is considerably different in the two solutions. The magnitude of the enthalpy, $\Delta_{trans}H \approx 550$ kJ/mol, implies that the aggregation number is relatively large and/or that there are additional processes occurring in the transfer process. A plausible additional process is a transition to an intermediate protein state, stabilised by low concentrations of GuHCl. Earlier studies in the near-UV region suggest an intermediate state at GuHCl concentrations above 0.25 M and also in buffer solutions above 35°C [12]. A significant ratio of apo A-I_M occupies the intermediate state at 25°C in phosphate buffer as has been shown by scanning calorimetry [12].

3.2. GuHCl concentration 0.6–2 M

The ellipticity is essentially constant at [GuHCl] = 0.6–0.8 M, followed by a sigmoidal increase in the ellipticity. The ellipticity reaches a new plateau at [GuHCl] = 1.5–2 M.

The enthalpy of transfer decreases linearly with GuHCl concentration, [GuHCl] = 0.6–1 M,

$$\Delta_{trans}H_1([GuHCl]) = 4112 - 5211 \cdot [GuHCl], \tag{2}$$

and with another linearity in the concentration range [GuHCl] = 1.5–2 M,

$$\Delta_{trans}H_2([GuHCl]) = 4303 - 3941 \cdot [GuHCl]. \tag{3}$$

The enthalpical linear regions coincide with the ellipticity stable regions. The midpoint of the transition between the two stable states is 1.1 M GuHCl.

The linear dependencies of enthalpy of transfer of the two states, $\Delta_{trans}H_1$ and $\Delta_{trans}H_2$, describe the difference in hydration when transferring the protein from phosphate buffer solution to the intermediate state and the unfolded state, respectively. The difference between the two enthalpies extrapolated to [GuHCl] = 0 M is the enthalpy of transition between the two states in phosphate buffer solution. The difference in enthalpy is equal to the change in enthalpy due to transition of the protein from the intermediate state to the unfolded state, $\Delta_{unfold}H$.

$$\Delta_{unfold}H = \Delta_{trans}H_2(0) - \Delta_{trans}H_1(0). \tag{4}$$

The value obtained, $\Delta_{unfold}H = 191$ kJ/mol, from the extrapolation of the ITC data is the same as earlier obtained from DSC, $\Delta_{unfold}H(DSC) = 190$ kJ/mol at 55°C [12]. The DSC studies could not detect any significant change in heat capacity for the unfolding process. This means that the enthalpy is temperature independent.

The stability of apo A-I_M is lower in GuHCl solution than would be anticipated from the thermal stability of the protein in phosphate buffer, $T_m = 55$°C [12]. One explanation for this that in phosphate buffer the protein is highly aggregated and the aggregates are dissociated in GuHCl. The aggregation process of apo A-I_M has a stabilising effect on the protein [12]. The small heat capacity change of

unfolding indicates incomplete unfolding or aggregation of unfolded protein. The small heat capacity difference might also be due to that the intermediate state of the protein already exposes non-polar groups to water.

3.3. III GuHCl above 2M

At GuHCl concentrations above 2 M the ellipticity slowly increases towards zero. Enthalpy of transfer at 25°C changes non-linearly as a function of GuHCl concentration, reaching a saturation enthalpy at higher concentrations.

Applying a simple model for α-helix content calculations [17], which is based upon that there are solely random coil and α-helix structural elements present, the α-helical content of the protein at the unfolded state is small, but still significant. The model is defined as,

$$\%\ \alpha\text{-helix} = (R - [\theta]_{222\,\mathrm{nm}})/(R - A) \times 100, \tag{5}$$

where R is the ellipticity contribution from random coil at 222 nm (3900 deg cm^2 dmol^{-1}) and A from α-helix at 222 nm ($-38\,000$ deg cm^2 dmol^{-1}).

A tentative interpretation from the CD data and calorimetric titration data would be that GuHCl bind to the incompletely unfolded protein. The binding of GuHCl would then induce dissolution of the residual secondary structure of the incompletely unfolded protein. The binding of GuHCl to the protein is likely to be weak and heterogeneous in nature. This will make it difficult to apply any simple model to calorimetric data or any other equilibrium data. The most simple model assumes that each site has the same binding constant and enthalpy of binding, indifferent whether the protein is folded or not [18]. The enthalpy of transfer at initial unfolded state is in the order of -4 MJ/mol protein, while the asymptotically end value for the enthalpy is in the order of -9 MJ/mol protein. The difference in enthalpy that would be due to binding of GuHCl to the unfolded protein is in the order of -5 MJ/mol protein. Privalov and Makhatadze have utilised a simple equilibrium model for the unfolding of lysosyme, ribonuclease A and cytochrome c from isothermal calorimetry data [15]. The parameters they obtained were stoichiometries for the native and denatured proteins, equilibrium constants and enthalpies of binding. They obtained the average binding enthalpy per residue, Δh, of -11 ± 2 kJ/mol GuHCl for the three proteins. If we use the Δh value from Privalov and Makhatadze on our system and take the ratio between the excess enthalpy after unfolding and Δh we obtain an approximation of how many GuHCl binding site there are on the protein. The value we obtain is approximately 450 GuHCl per apo A-I_M, or 0.9 GuHCl/residuals. Compared to the three model systems chosen by Privalov and Makhatadze the number of binding sites varies between 0.5–0.7 GuHCl /residuals of the unfolded proteins. Although the gross approximation in the calculation, we obtain a value that is in good agreement with Privalov and Makhatadze [15].

3.4. Comparison with normal apo A-I

In this work we have solely focused on the mutated variant (173 Arg $\rightarrow$ 173 Cys) of apo A-I, which forms a covalent dimer by a S–S bond from oxidation of the cysteins from two monomeric protein molecules. There are, however, data published on the non-mutated variant that makes it possible to make a comparison of properties the two proteins that have implication on the physiological effects seen [8].

Although the difference between normal apo A-I and apo A-I_M is solely one amino acid substitution (173 Arg $\rightarrow$ 173 Cys) they differ significantly in lipid binding properties and physiochemical properties

[10]. The formation of the covalent disulphide linked dimer of apo A-I_M change dramatically the thermal stability of the protein [12]. The GuHCl induced unfolding of normal apo A-I and the non-covalent linked form of apo A-I_M, assuming a two-state unfolding model, have both transition midpoints at 1.2 M GuHCl at 25°C [13]. A slight increase in ellipticity was also observed for apo A-I at low GuHCl concentrations before the unfolding occurred. Edelstein and Scanu have reported that GuHCl induced unfolding of apo A-I is protein concentration dependent and this is due to self-aggregation [19]. GuHCl induces dissociation of aggregates of all forms of normal apo A-I and destabilise the proteins. It is, thus, the non-aggregated proteins that unfold in GuHCl. The difference in thermal stability of the proteins and the formation of and the stability of the intermediate states for the different forms of apo A-I can explain the lipid binding properties [11,12]. At physiological temperature the intermediate state of apo A-I_M is the most stable state, while normal apo A-I is only partially in its intermediate state [12].

References

[1] J.A. Glomset, The plasma lecithin: Cholesterol acyltransferase reaction, *J. Lip. Res.* **9** (1968), 155–162.
[2] Q.-H. Meng, L. Calabresi, J.-C. Fruchart and Y.L. Marcel, Apolipoprotein AI domains involved in the activation of lecithin: cholesterol acyltransferase, *J. Biol. Chem.* **268** (1993), 16 966–16 973.
[3] A.R. Tall, Plasma high density lipoproteins, *J. Clin. Invest.* **86** (1990), 379–384.
[4] H.B. Brewer Jr., T. Fairwell, A. LaRue, R. Ronan, A. Houser and T.J. Bronzert, The amino acid sequence of human apoA-I, an apolipoprotein isolated from high density lipoproteins, *Biochem. Biophys. Res. Com.* **80** (1978), 623–630.
[5] J.P. Segrest, M.K. Jones, H. Deloof, C.G. Brouillette, Y.V. Venkatachalapathi and G.M. Anantharamaiah, The amphiphathic helix in the exchangeable apolipoproteins – A review of secondary structure and function, *J. Lip. Res.* **33** (1992), 141–166.
[6] D.L. Barbeau, A. Jonas, T.-I. Teng and A.M. Scanu, Asymmetry of apolipoprotein A-I in solution assessed from ultracentrifugal, viscometric, and fluorescence polarization studies, *Biochemistry* **18** (1979), 362–369.
[7] L. Calabresi, G. Vecchio, R. Longhi, E. Gianazza, G. Palm, H. Wadensten, A. Hammarström, A. Olsson, A. Karlström, C.R. Sirtori and G. Franceschini, Molecular characterization of native and recombinant apolipoprotein A-I_{Milano} dimer, *J. Biol. Chem.* **269** (1994), 32 168–32 174.
[8] G. Franceschini, P. Apebe, L. Calabresi, V. Gualandri and C.R. Sirtori, Apolipoprotein A-I, Milano variant: Genetic, clinical and biochemical features, *Aterosclerosis Rev.* **16** (1987), 149–159.
[9] S.C.R. Weisgraber Jr., T.P. Bersot, R.W. Mahley, G. Franceschini and C.R. Sirtori, Apolipoprotein A-IMilano, *J. Biol. Chem.* **258** (1983), 2508–2513.
[10] L. Calabresi, G. Vecchio, F. Frigerio, L. Vavassori, C.R. Sirtori and G. Franceschini, Reconstituted high-density lipoproteins with a disulfide-linked apolipoprotein A-I dimer: Evidence for restricted particle size heterogeneity, *Biochemistry* **36** (1997), 12 428–12 433.
[11] M. Suurkuusk and S.K. Singh, Formation of HDL-like complexes from apolipoprotein A-I-M and DMPC, *Int. J. Pharm.* **194** (2000), 21–38.
[12] M. Suurkuusk and D. Hallén, Apolipoprotein A-IMilano unfolds via an intermediate state as studied by differential scanning calorimetry and circular dichroism, *Eur. J. Biochem.* **264** (1999), 183–190.
[13] M. Suurkuusk and D. Hallén, Denaturation of apolipoprotein A-I and the monomer form of apolipoprotein, *Eur. J. Biochem.* **265** (1999), 346–352.
[14] W. Pfeil and P.L. Privalov, Thermodynamic investigations of proteins. II. Calorimetric study of lysozyme denaturation by guanidine hydrochloride, *Biophys. Chem.* **4** (1976), 33–40.
[15] G.I. Makhatadze and P.L. Privalov, Protein interactions with urea and guanidinium chloride: A calorimetric study, *J. Mol. Biol.* **226** (1992), 491–505.
[16] P. Schuck, Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling, *Biophys. J.* **78** (2000), 1606–1619.
[17] B.-M. Backlund, G. Wikander, T.L. Peeters and A. Gräslund, Induction of secondary structure in the peptide hormone motilin by interaction with phospholipid vesicles, *Biochem. Biophys. Acta* **1190** (1994), 337–344.
[18] C. Tanford, Theoretical models for the mechanism of denaturation, *Adv. Prot. Chem.* **24** (1970), 1–95.
[19] C. Edelstein and A.M. Scanu, Effect of guanidine hydrochloride on the hydrodynamic and thermodynamic properties of human apolipoprotein A-I in solution, *J. Biol. Chem.* **255** (1980), 5747–5754.