Differential Scanning Calorimetry and Protein Stability

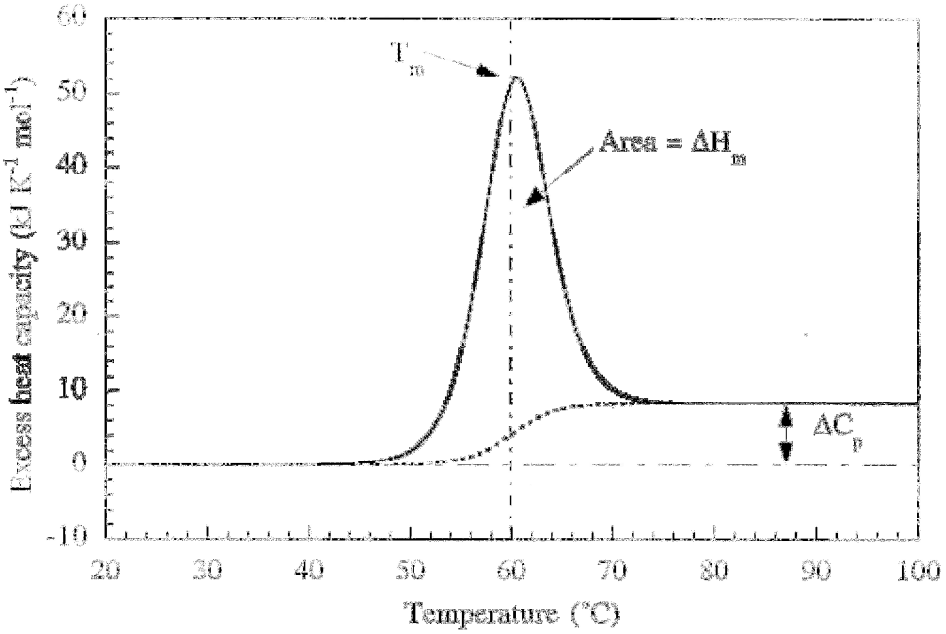

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Table of Contents

Picture at title page: Adapted from Robertson 1997

Abbreviations

CD	-	circular dichroism
DSC	-	differential scanning calorimetry
ΔG	-	free energy of denaturation
ΔH	-	enthalpy of denaturation
HSA	-	Human Serum Albumin
HTH	-	helix-turn-helix
RT	-	room temperature
Tc	-	tetracycline
TetR	-	tetracycline repressor
Tm	-	transition midpoint

Abstract

Differential scanning calorimetry (DSC) is a technique applicable to the study of protein stability. It measures the excess heat capacity of a protein relative to a reference sample. Deconvolution of a thermogram can provide insight in the unfolding process and about the presence of stable intermediate states. From the denaturation curve of a protein all the important thermodynamical parameters can be obtained. Protein fluorescence and circular dichroism (CD) on the other hand are spectral techniques, which can monitor the changes in the tertiary and secondary structure. The studies reviewed in this article demonstrate that DSC is a useful technique for studying protein denaturation and protein-ligand interactions. Especially the combination of DSC with a spectral technique appeared to be a very powerful in protein research.

Introduction

The way a polypeptide chain folds to a protein with a secondary, tertiary and quaternary structure is at the present moment the subject of many studies. One way to learn more about the folding of proteins is to study their unfolding. The denaturation process of a protein holds much information about the protein and can be used for a variety of purposes. Information regarding the stability of a protein is for example of great interest in the development of more stable proteins for use in industrial processes (Roberge 2003).

A variety of techniques are nowadays available for the study of proteins, among which circular dichroism (CD) spectroscopy, fluorescence spectroscopy and differential scanning calorimetry (DSC). CD is the difference in absorption of right-handed circulary polarized light and left-handed polarized light. With the CD of a protein it is possible to measure the amount of secondary and tertiary structures present in a protein, because not only the molecular structure of a protein influences the CD, but also the supramolecular structure does. The excitation and emission spectra of fluorescent aminoacids (tryptophan, tyrosine and phenylalanine) depend on the environment of the aminoacids. The fluorescence of a tryptophan residue differs when it is buried in the hydrophobic core of a protein or when it is exposed to a polar solvent. Fluorescence can thus be used to track changes in the tertiary structure of a protein, as the spectra of the fluorescent aminoacids changes upon unfolding.

DSC records the thermal events occurring in a protein upon heating, by measuring the excess heat capacity of a protein as a function of temperature. This is done by measuring the difference in energy needed to increase the temperature of a protein and a reference with the same amount. DSC can thus monitor the unfolding of proteins by measuring the energy released or absorbed by the thermal events which accompany protein denaturation. Of the aforementioned techniques, DSC is the only one by which the enthalpy of denaturation can be directly determined from the experimental data.

In this article the technique of DSC will be described, demonstrated and compared to CD and fluorescence in order to give an introduction on the technique and to present it's advantages and disadvantages. This review starts with a short introduction on each technique, followed by a chapter about the functioning of a calorimeter and an presentation of the basal thermodynamics used in studying thermal denaturation, after this three studies are reviewed which all used DSC in a different way.

Fluorescence

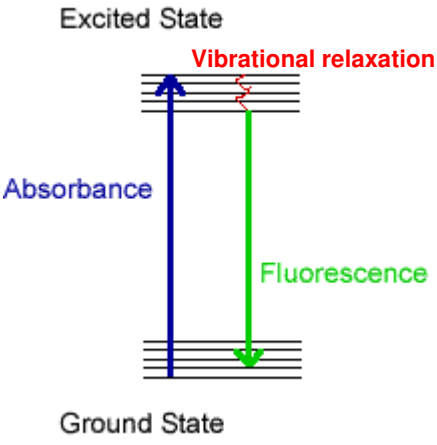

Figure 1: A Jablonski diagram showing the different events leading to fluorescence. (Adapted from Kutztown University 2008)

Fluorescence is a phenomenon in which an excited molecule returns to it´s ground state by radiative decay. The absorption of a photon takes a molecule to an excited electronic state, see figure 1. The excited molecule loses it's energy by radiationless vibrational relaxation through collisions with other molecules till it is in the ground state of the upper electronic state. The surrounding molecules, however, might be unable to absorb the energy which is needed for the transition from the upper electronic state to the ground electronic state. The molecule can therefore prolong long enough in it's excited state to generate a photon out of the remaining energy and return to it's ground state by the emission of it. The wavelength of the emitted photon is longer than the wavelength of the excitation photon for two reasons. At first because a part of the excitation energy is discarded as vibrational energy to surrounding molecules. Secondly, because the solvent may interact different with the solute in the excited state than in the ground state. (Atkins 2006)

The fluorescent properties of the aminoacids phenylalanine, tyrosine and tryptophan can be used to assess the stability of the tertiary structure of proteins, as their fluorescence depends on the molecular environment they are in. Tryptophan is the most dominant aminoacid in the fluorescence spectra, while phenylalanine is the least fluorescent aminoacid.

The absorption maxima of phenylalanine, tyrosine and tryptophan are between 250 and 300 nm, but this depends on the environment of the respective chromophore. In a more polar environment, there is a shift in the absorption spectra to shorter wavelength, while upon a decrease in polarity there is a shift to longer wavelengths. In folded native proteins the aromatic aminoacids are in general buried in the hydrophobic core of the molecule, but upon denaturation the tertiary structure changes and the respective aminoacids are exposed to an aqueous polar solvent, resulting in a decrease in the absorbance in the 285 – 295 nm region. (Yanari 1960, Righetti 2001)

The emission of the three aminoacids is also effected by the molecular environment. When a protein denatures, there are changes in both the wavelength and in intensity of the emission. The tryptophan emission in a native protein can be more or less intense than in the denatured protein. Consequently there can be an increase or decrease of the fluorescence during the unfolding of a protein. The emission maximum shifts in general from shorter wavelengths to 350 nm, the emission maximum in water. (Teale 1960, Righetti 2001)

The variations in the fluorescence of proteins reflect changes in the environment of the fluorescent aminoacids. Protein fluorescence can therefore be used to monitor the amount of tertiary structure present in proteins, as the unfolding of the tertiary structure results in changes in the environment of the aminoacids.

Circular dichroism (CD)

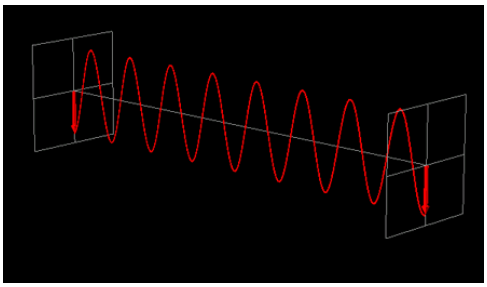

Figure 2: A wave of linearly polarized light. (Adapted from Applied Photophysics Ltd)

Linearly polarized light is light whose oscillations are confined to a single plane (figure 2). All polarized light states can be described by the summation of two waves of linearly polarized light oscillating perpendicular to each other (figure 3). If the two waves are out of phase by a quarter-wave, the

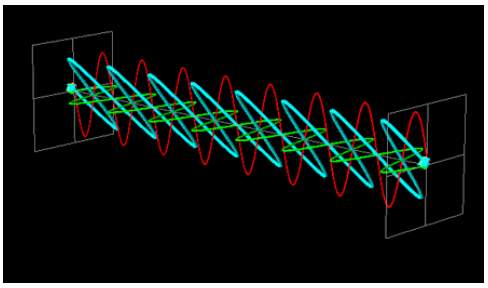

Figure 3: Two waves of linearly polarized light and the resultant wave. (Adapted from Applied Photophysics Ltd)

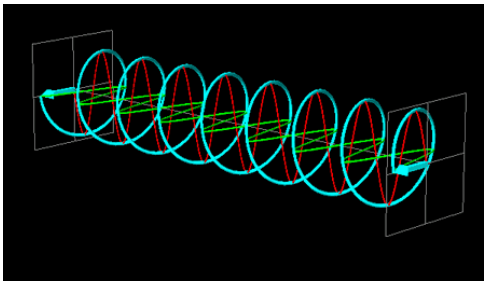

Figure 4: Two waves of linearly polarized light a quarter-wave out of light and the resultant right handed circularly polarized light wave. (Adapted from Applied Photophysics Ltd)

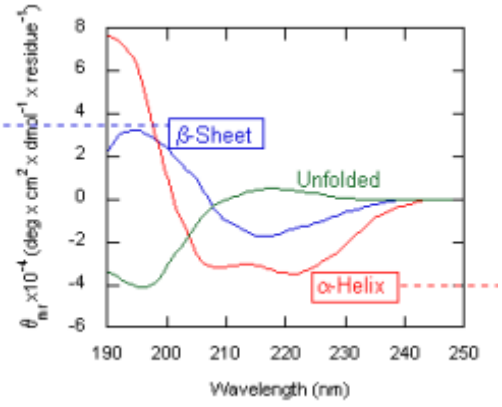

Figure 5: The CD spectra of an α-helix and a β-sheet. (Adapted from Applied Photophysics Ltd)

resultant wave is not linearly polarized anymore but describes a helix and

is known as known as circularly polarized light (CPL) (figure 4). The helix can be both right-handed or left-handed, rendering right handed and left-handed circularly polarized light (R-CPL and L-CPL).

Chiral molecules, like biomolecules, absorb left-handed circularly polarized light and right-handed circularly polarized light to a different extent. This is the basis of circular dichroism, which is defined as the difference in the absorption of L-CPL and R-CPL.

In a protein are not only the individual aminoacids chiral, but also it's secondary and tertiary structure. Consequently the CD spectra of proteins contain information about the secondary and tertiary structure a protein. In the far UV (170-250 nm) CD spectra provide information about the secondary structure of a protein, whereas CD bands in the near UV (250-300 nm) originate from aromatic aminoacids.

In the presence of ordered domains, aromatic aminoacids give rise to a CD signal in the near UV, but when no tertiary structure is present the ellipticity in the near UV is close to zero. The CD signal in the near UV is thus correlated with the amount of tertiary structure in the protein. The far UV is dominated by bands caused by the presence of α-helices and β-sheets (figure 5). α-helices give rise to a negative band near 222 nm, a negative band near 208 nm and a positive band near 192 nm in a CD spectrum. β-sheets have a negative band near 216 nm, a positive band between 195 and 200 nm and a negative band near 175 nm. Due to these specific bands in the far UV, the amount of β-sheets and α-helices can be monitored by CD.

CD spectra provide information about the amount of secondary and tertiary structure present in a protein. Therefore CD can be very useful in monitoring protein unfolding, because it is possible to really track the unfolding of the protein at a structural level.(Righetti 2001)

Differential Scanning Calorimetry

DSC is a powerful technique able to study thermal induced changes in proteins. The name of the technique, differential scanning calorimetry, stands for the method in which the heat release (calor) of a sample is differentially compared to the heat release of a reference as the protein is 'scanned' over a temperature range. The reference sample normally contains the buffer in which the protein is dissolved. The rate with which the sample is heated is called the scanrate , which is the increase in temperature per time unit.

DSC measures the excess heat capacity at constant pressure ($C_{p,ex}$) of a protein relative to a reference sample as a function of temperature, therefore the pressure in the cells is kept constant. Normally the heat capacity of a protein or solution changes slowly with temperature, but during a thermal event, like denaturation, it changes discontinuously (Friedli 1996). The

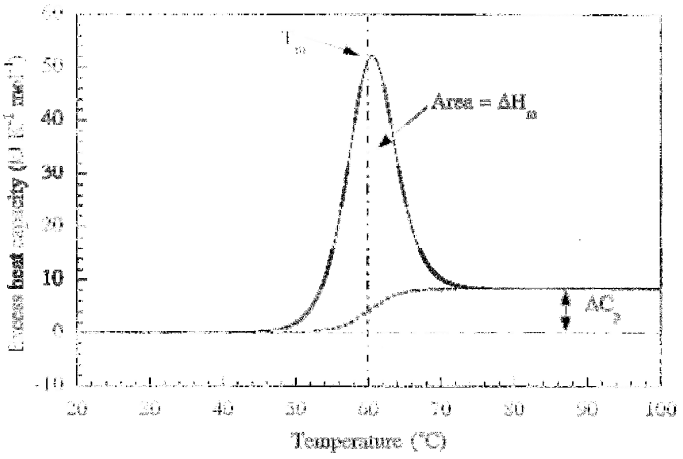

Figure 6: Simulated differential scanning calorimetry experiment for the two-state unfolding of a globular protein. (Robertson 1997)

unfolding of a protein is an endothermic event, due to the hydration of side chains that are in the native protein buried in it's hydrophobic core, but become exposed to the hydrophilic solvent upon denaturation (Bruylants 2005). During the unfolding of a protein more energy is thus required to raise the temperature of the sample, this extra energy needed to raise the temperature can be seen as an 'excess' heat capacity in addition to the 'normal heat capacity' of the protein. (Atkins 2006).

The denaturation of a protein appears as a peak in a DSC curve, the top of the peak, where the excess heat capacity is maximal, is the transition midpoint (T_m) (figure 6). The integral of the

area under the denaturation peak is the enthalpy of denaturation (ΔH_m), which reflects the amount of secondary structure present in a protein. (Friedli 1996, Koshiyama 1981, Spink 2008). The shift in the baseline between the pre- and post transition area is the change in heat capacity (ΔC_p) between the native and the denatured protein. In general the heat capacity of a denatured protein is higher than of a native protein, due to a restructuring of solvent (Edsall 1935, Madan 1996, Robertson 1997). With ΔH_m and ΔC_p, it is possible to determine the change in the Gibbs free energy ($\Delta G(T)$) and the entropy ($\Delta S(T)$) of denaturation at a temperature (T) of interest, which means that in principle all the important thermodynamical parameters can be obtained by DSC. The stability of a protein is in general quantified by the ΔG of denaturation, a stable protein has a high and positive ΔG. Next to the calorimetric determined enthalpy (ΔH_m), there is also the van 't Hoff enthalpy (ΔH_{vH}), which is calculated from the DSC curve assuming a two state transition. The ratio between $\Delta H_{vH}/\Delta H_m$ is an indication of the cooperativity of the unfolding and the presence of stable intermediate states. If $\Delta H_{vH}/\Delta H_m$ is less than one, there are probably stable intermediate states during the unfolding, while a ratio greater than one is an indication that the transition is cooperative. If $\Delta H_{vH} = \Delta H_m$ the unfolding follows a two state model.

In a DSC scan it is very important that the denaturation is reversible. In most studies this is tested by scanning the protein two times and check that the second scan gives the (almost) same curve. However, this is not a test for reversibility, but for repeatability. Thermodynamic reversibility requires that the system is at equilibrium during the reaction. Scanning the protein perturbs the equilibrium, thermodynamic reversibility is thus better demonstrated by demonstrating that the T_m does not change with the scanrate. Unfortunately such a test is rarely preformed. (Robertson 1997)

A negative aspect of DSC is that it requires protein concentrations of at least 1 mg/mL, which is relatively high compared to the concentrations needed for fluorescence or CD measurements, which can be as low as 0,01 mg/mL. Apart from the need of a lot of protein to perform a scan, as a typical DSC instrument has cells of 0,3-2 mL, these high protein concentrations also favor aggregation.

The calorimeter

A DSC instrument or calorimeter measures the energy needed to maintain a zero temperature difference between a sample and a reference, as the two samples are both heated

or cooled according to a temperature protocol. A general layout of the basic components of a calorimeter is shown in figure 7. A DSC instrument has a sample and a reference cell, to each of them a thermometer and are heater are attached. The two cells with their temperature sensors and heaters are surrounded by a shield to which shieldheaters are attached. All the sensors and heaters are connected to a computer.

The function of the shield varies with the type of calorimeter. In the instruments of the *Calorimetric Sciences Corporation*, the shield controls the temperature of scanning. The cells are in thermal contact with the shield and the temperature of the cells increases when the shield is heated by the shieldheaters. The cell temperature is monitored by the cell thermometers, in this way the computer can determine if there is any temperature difference between the two cells. If there is a temperature difference, compensating power is supplied through the individual cell heaters in order to keep the cells at the same temperature. The output of the calorimeter is the difference in energy supplied to the two heaters plotted as a function of temperature. In the instruments of *MicroCal, LCC*, the cells are not primarily heated by the shield, but by the cellheaters. The shieldheaters keep the shield temperature at the same level as the temperature of the cells. This means that there is no temperature difference between the shield and the cells and that the experiment is done under adiabatic conditions, with no heat transfer form the cells to the surroundings. Down scanning is not adiabatic, because it requires a temperature gradient to cool the cells. Also in these calorimeters the temperature of the two cells is monitored and power compensating is applied through the individual cellheaters to keep the cells at the same temperature. The output of the instrument is again the difference in energy consumption of the cellheaters. The performances of the two types of calorimeters are very similar. (Spink 2008)

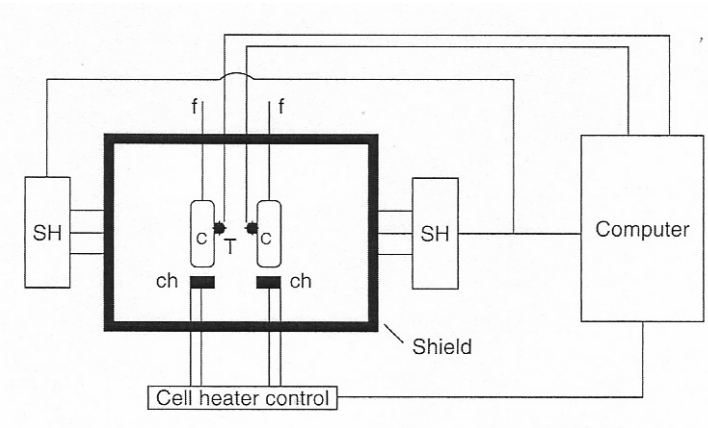

Figure 7: Schematic diagram of a differential scanning calorimeter. Cells (c) are located within a shield, which is in contact with shield heaters (SH). Individual cell heaters (ch) control the temperature of the sample and reference cells. Temperature sensors (T) are located on the cell surfaces, which determine if there is a temperature difference between the two cells, and through computer control apply appropriate compensating heat to the cells to keep temperature difference near zero. The compensating energy per unit time is recorded as the calorimetric signal. (adapted from Spink 2008)

Thermodynamics for DSC

The temperature , T, at time, t, during a linear scan can be calculated with:

$$T = T_0 + \alpha t \quad (1)$$

where T_0 is the initial temperature and α the scanrate in (Kelvin s^{-1}). In case there is no thermal event during the scan, the supplied heat at constant pressure (q_p) can be calculated by:

$$q_p = C_p \Delta T \quad (2)$$

with C_p being the specific heat at constant pressure and where $\Delta T = T - T_0 = \alpha t$, assumed that C_p does not change with temperature. Although the C_p changes slightly with temperature, the assumption that C_p stays constant does not lead to significant errors in the other parameters (Robertson 1997, Privalov 1988). On the occurrence of a thermal event more or less heat has to be supplied to the sample to achieve the same change in temperature as the reference. In the case of an endothermic event there is thus additional 'excess' heat, $q_{p,ex}$ supplied to the sample and this can be expressed as an additional contribution to the heat capacity, $C_{p,ex}$ (the excess heat capacity) by $q_{p,ex} = C_{p,ex} \Delta T$. From this equation the following can be derived:

$$C_{p,ex} = \frac{q_{p,ex}}{\Delta T} = \frac{q_{p,ex}}{\alpha t} = \frac{P_{ex}}{\alpha} \quad (3)$$

where $P_{ex} = q_{p,ex} / t$ is the excess, or in an exothermic event the decrease, electrical power supplied to the heaters to keep the reference and the sample at the same temperature. (Atkins 2000)

The stability of proteins is quantified by the standard free energy ΔG, which is calculated by:

$$\Delta G(T) = \Delta H(T) - T\Delta S(T) \quad (4)$$

where ΔH is the change in enthalpy and ΔS the change in entropy at the temperature (T). ΔG is related to the equilibrium constant (K) by the following formula (Bruylants 2005):

$$\Delta G(T) = -RT \ln K(T) \quad (5)$$

where R is the gas constant.

In a thermogram the difference in the energy (P_{ex} or $C_{p,ex}$) supplied to the sample and the reference is plotted against the temperature and thermal events appear as a peak in the curve. The transition midpoint is the temperature at which the excess heat capacity ($C_{p,ex}$) is

maximal. From the area under a peak the enthalpy of the reaction (ΔH_m) can be calculated by the integral of the excess heat capacity (Spink 2008):

$$\Delta H = \int_{T_1}^{T_2} C_{p,ex} dT \quad (6)$$

with T_1 and T_2 being the start and end temperature of the thermal reaction. This is unique feature of DSC, as it is the only technique where the enthalpy can be directly obtained from the results (Bruylants 2005). The change in entropy can be calculated by the following formula (Spink 2008):

$$\Delta S = \int_{T_1}^{T_2} \frac{C_{p,ex}}{T} dT \quad (7)$$

The heat capacity (C_p) of a native protein is lower than of a denatured protein, as more energy is required to heat an unfolded protein. This is due to the restructuring of solvent molecules around the non polar sidechains, which become exposed during the unfolding process. (Bruylants 2005)

The van't Hoff (ΔH_{vH}) is the calculated enthalpy of denaturation assuming a two state transition. It can differ from the experimental determined enthalpy (ΔH_m) as it does not take all processes into account, but assumes a simple two state model. It can be calculated from a DSC trace using the following equation:

$$\Delta H_{vH} = 4RT_m^2 \frac{C_{p,m}}{\Delta H_m} \quad (8)$$

with $C_{p,m}$ being the excess heat capacity at T_m and ΔH_m the experimentally determined calorimetric enthalpy. If the ratio $\Delta H_{vH}/\Delta H_m$ is less than one there are probably intermediate states during the melting, while if the ratio is greater than one, it indicates cooperative melting and if the ratio is one and if the ratio is one the approximates a two state model. (Bruylants 2005, Spink 2008)

To determine the ΔG of a protein by thermal analysis requires it's heating and the obtained parameters are only representative for that temperature. With the 'modified Gibbs-Helmholtz' equation the ΔG can be calculated for any temperature T:

$$\Delta G(T) = \Delta H_m (1 - \frac{T}{Tm}) + \Delta C_p \left[T - T_m - T \ln(\frac{T}{T_m}) \right] \quad (9)$$

where ΔH_m is the enthalpy at the T_m and ΔC_p is the heat capacity change upon denaturation (Bruylants 2005, Pace 2000).

Kirchoff's law can be used to determine the enthalpy and entropy at a temperature T:

$$\Delta H(T) = \Delta H_m + \Delta C_p (T - T_m) \quad (10)$$

$$\Delta S(T) = \Delta S_m + \Delta C_p \ln \frac{T}{T_m} \quad (11)$$

Where ΔS_m is the entropy at T_m (Bruylants 2005)

The denaturation of L-PGDS

1 - Protein denaturation

Differential scanning calorimetry is a technique capable of studying the unfolding of proteins. As the instrument measures the excess heat capacity ($C_{p,ex}$), the denaturation of a protein is visible as an endothermic peak in a thermogram, which reaches it's maximum at the transition midpoint (T_m). The T_m is used as an indicator of the thermostability and in general proteins with a high T_m are more stable. The sharpness of the transition peak can be measured as the width at half-peak weight and is an indicator of the cooperativity of the unfolding. A small peak indicates a cooperative transition, while a broad peak is an indicator of a multi-state denaturation (Bruylants 2005). Comparison of the experimentally obtained enthalpy and the calculated van't Hoff enthalpy also yields information about the cooperativeness of the unfolding and the presence of stable intermediate states during the denaturation process. A necessity for obtaining relevant parameters is that the scanning is thermodynamically reversible, which means that the system is at equilibrium during the scan and that the protein does not aggregate during or after the denaturation. If aggregation occurs there are also other processes happening besides the unfolding which influence the data.

2 - DSC measurements

In a study from Iida *et al.* the stability and the unfolding of L-PGDS was assessed with DSC and CD measurements. Lipocalin-type prostaglandin (PG) D synthase (L-PGDS) is an enzyme responsible for the production of PGD_2. PGD_2 is a neuromodulator with various

functions in both the central nervous system and the peripheral nervous system. Besides it's function as enzyme, L-PGDS is also a member of the lipocalin superfamiliy, which compromises various lipid transporters.

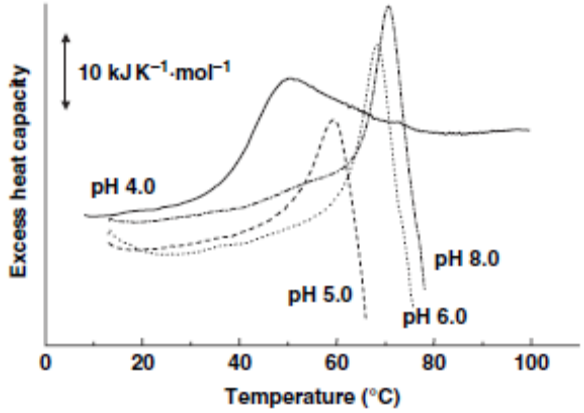

Fig. 8: Thermogram profiles of L-PGDS. The DSC traces for the unfolding of L-PGDS were obtained at pH 4.0 (solid line), pH 5.0 (dashed line), pH 6.0 (dotted line) and pH 8.0 (dot-dashed line). (Adapted from Ida 2008)

The reversibility of the protein was tested by performing DSC scans at various pH's (figure 8)The scans of L-PGDS at pH's of 5,0, 6,0 and 8,0 ended in an exothermal event, indicating aggregation and rescanning of the samples did not result in any calorimetric transition, indicating that the denaturation was irreversible. Scanning at pH 4,0 resulted in a single endothermic event with a T_m at 50 °C, this time the denaturation appeared to be reversible. The van't Hoff enthalpy for the transition was calculated and the ratio between the experimental determined enthalpy and the van't Hoff enthalpy appeared to be 0,5, indicating

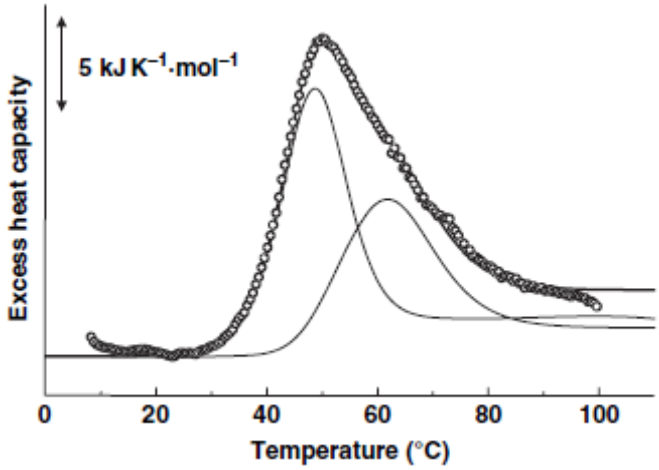

Fig. 9 **DSC curve deconvolution for thermal unfolding of L-PGDS.** A sequential three-state transition model, N ↔ I ↔ U. Open circle, observed DSC data; solid line, component curves; and dotted line, theoretical curve. (adapted from Iida 2008)

the unfolding does not follow a two state model, but has stable intermediate states. The data could be fitted very well to the three state equilibrium unfolding model which describes the transition from a native state (N) via an intermediate state (I) to a unfolded state (U), figure 9. The T_m and the enthalpy of the first transition are 48,0 °C and 206 kJ mol^{-1}, for the transition from the intermediate to the unfolded state these parameters are 60,8 °C and 163 kJ mol^{-1}. The dependency of the Gibbs free energy on temperature was calculated with use of Kirchoff's

law for both transitions and is plotted in figure 10. As can be seen in the graph, both the native and the intermediate state of L-PGDS are more stable at temperatures below 0 °C.

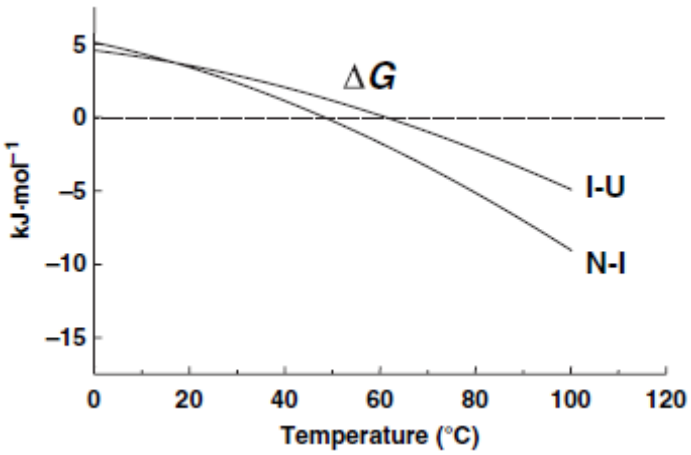

Figure 10: **The temperature dependency of the Gibbs free energy.** For the N↔I and I↔U transition of L-PGDS (Adapted from Iida 2008)

3 - CD measurements

To obtain insight in the structural changes accompanying the transitions, the thermal unfolding of L-PGDS at pH 4,0 was also monitored by CD. Near and far UV spectra were recorded at different temperatures (data not shown). The far UV spectra showed that in the native protein a lot of β-sheets are present. The structural reversibility of the transition was tested by remeasuring the spectrum of the protein at 20 °C after heating it to 90 °C, the CD spectrum remained the same, indicating that the unfolding of the protein at pH 4,0 is also structural reversible. Equilibrium transition curves were recorded at 200, 235 and 290 nm, the wavelengths at which the change in ellipticity was larger (figure 11). The transition curves show, depending on the wavelength a decrease or an increase in ellipticity upon unfolding. The T_m's for the transition at the three wavelengths were calculated, at 200 nm the T_m was at 54,6 °C, at 235 nm 47,3 °C and at 290 nm 46,9 °C.

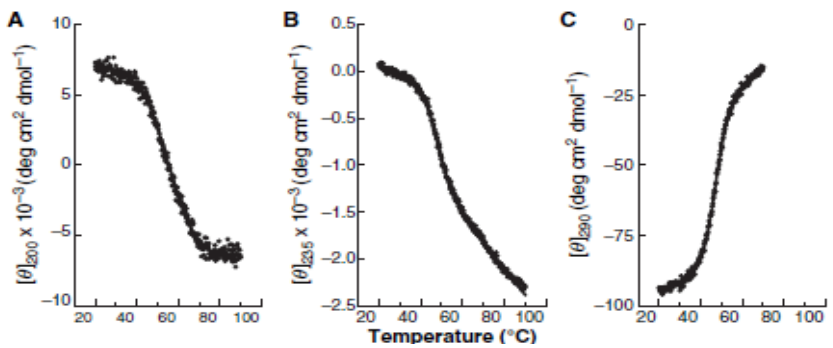

Figure 11: Thermal unfolding curves of L-PGDS at pH 4,0. Unfolding transitions were monitored by measurement of CD at 200 (A), 235 (B), and 290 nm (C).

4 - Conclusion

Both the DSC and CD data showed that the denaturation of L-PGDS is reversible at pH 4,0. Deconvolution of the DSC measurements showed that the denaturation of L-PGDS follows a three state model, with two transitions and an intermediate state. The T_m values obtained from the DSC and CD data do not completely correspond, but the CD data can provide insight in the structural changes during the denaturation. The changes in ellipticity at 235 and 290 nm reflect probably the transition from the native state to the transition state, because the T_m values correspond to the temperature at which the first transition occurs. As the near UV is dominated by the CD of the aromatic aminoacids, the transition from the native state to the intermediate state is probably accompanied by a change in the tertiary structure of the protein. The ellipticity at 200 nm on the other hand represents the amount of β-sheets present in the protein. The change in the ellipticity at 200 nm is assumed to correspond to the transition from the intermediate state to the unfolded state, reflecting a change in the secondary structure.

From these results it is possible to conclude that although the transition state has lost it's tertiary structure, it still has a substantial amount of it's secondary structure. For the structure of the intermediate state is therefore a molten-globule like state suggested, as it still contains a lot of secondary structure, but not a strong tertiary structure. (Iida 2008)

Iida *et al.* demonstrated that DSC and CD are together a powerful combination. The DSC measurements provided the researchers with the important parameters of the two transitions, while the CD experiments visualized the structural changes in the protein during the transitions

DSC and the blood plasma proteome

1 - Protein mixtures and DSC

The thermogram of a protein mixture is the summation of the thermograms of the individual proteins, provided that the proteins do not interact. A thermogram is an extensive property, which means that each protein is represented according to their total mass in the thermogram. This makes it possible to deconvolute a DSC curve into the thermograms of the different proteins according to their relative concentrations. A powerful capability of DSC is

the ability to discern between the ligand bound state and unbound state of a protein. Depending on which ligand is bound a protein may be stabilized or destabilized. If a ligand binds the native state of a protein, it will stabilize this conformation which will result in a higher T_m of the protein, but when a ligand preferentially binds the unfolded protein, it will destabilize it which results in a lower T_m (Le Chatelier-Braun principle). DSC is thus very sensitive to both protein composition and interactions. It does not detect a ligand directly, but by the interaction with the protein it binds. In a recent study Garbett *et al*. showed that making use of these capabilities, DSC could be used as a diagnostic tool in the detection of disease markers which bind more abundant proteins in the bloodplasma.

2 - The blood plasma and biomarkers

Detection of diseases in an early stage saves lives, as treatment can then be started before the disease turns malignant. Many diseases alter the composition of the blood plasma and a lot of effort has been put in the identification of disease biomarkers in the blood plasma. The blood plasma proteome is made up for 99 % out of 22 proteins, in the remaining 1 % up to 1000 different proteins have been identified (Down 2005) and this fraction contains the biomarkers. DSC is able to detect changes in the plasma proteome composition and might be used in the future as a tool clinical for the diagnosis of diseases, as research of Garbett *et al.* showed.

3 - Thermograms of the blood plasma

In the research conducted by Garbett *et al.* thermograms of the bloodplasma of healthy individuals were recoreded and averaged (figure 12). To understand the structure of this thermogram, the DSC curves of the sixteen most abundant proteins in the blood plasma were recorded and weighted according to their normal concentration in the blood plasma, as shown in figure 13. The summation of these weighted thermograms is very similar to the thermogram obtained from the blood plasma (figure 12). This indicates that DSC is very sensitve to protein concentrations and that the proteins in the blood plasma do not interact. (Garbett 2009)

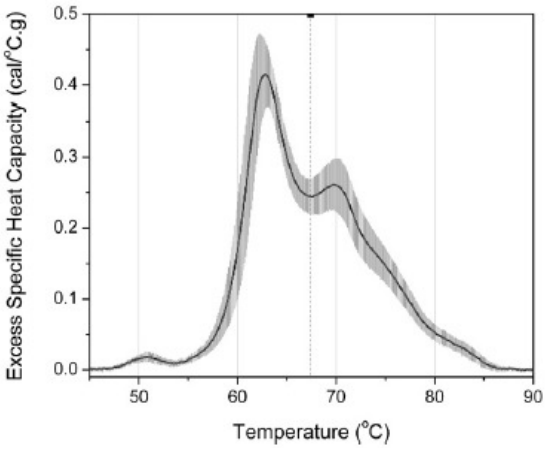

Figure 12: DSC thermogram of plasma from healthy individuals. The solid line is the average of 15 thermograms of plasma samples from 15 healthy individuals (9 male, 6 female; ages 22–50). The shaded area is the standard deviation at each temperature. The vertical dashed line is the first moment of the thermogram at 67.4 °C. (Adapted from Garbett 2009)

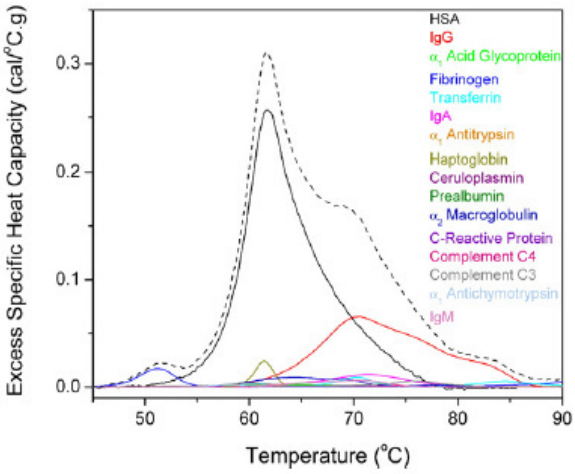

Figure 13: Basis of the plasma thermogram. Calculated thermogram (dashed line) obtained from the sum of the weighted contributions of the 16 most abundant plasma proteins. (Adapted from Garbett 2009)

To test if there are differences between the thermograms of healthy and diseased blood plasma, DSC runs were preformed on the blood plasma of individuals with various cancers and other diseases (figure 14). The DSC curves of the diseased blood plasma show some major changes compared to the thermograms of healthy individuals. By some of the diseases the peaks of the plasma are shifted to the right, indicating that the proteins in the blood plasma are more stable. There is a decrease in the amplitude of the peak at ~ 63 °C while the amplitude of the peak at ~70 °C shows an increase. A remarkable feature is that each disease has it's unique thermogram, which would make it possible to use DSC as a diagnostic tool. (Garbett 2009)

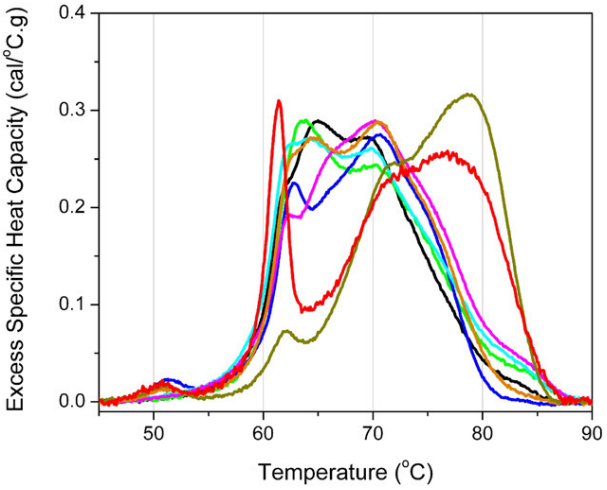

Figure 14: Average thermograms for individuals diagnosed with various cancers and diseases. Endometrial cancer (black line; duplicate DSC runs on samples from 8 individuals); amyotrophic lateral sclerosis (green line; duplicate DSC runs on samples from 12 individuals); lung cancer (blue line; duplicate DSC runs on samples from 30 individuals); ovarian cancer (cyan line; duplicate DSC runs on samples from 12 individuals); Lyme disease (dark yellow line; duplicate DSC runs on samples from 4 individuals); systemic lupus erythematosus (red line; duplicate DSC runs on samples from 2 individuals); rheumatoid arthritis (orange line; duplicate DSC runs on samples from 5 individuals); melanoma (magenta line; duplicate DSC runs on samples from 5 individuals). These data suggest that each type of cancer or disease may have a characteristic signature thermogram. (adapted from Garbett 2009)

4 - Protein interaction and thermograms

Previous research (Garbett et al., 2008) showed that the changes in the diseased thermograms are not due to changes in the concentration of the major plasma proteins. A probable explanation for the observed changes is that they are the result of an interaction event, because when a ligand binds to a folded protein, the folded conformation is stabilized, which results in a higher melting temperature of the protein. To test if the shifts in the thermograms could indeed be the result of the binding of a ligand to a protein, thermograms of human serum albumin (HSA) with bromocresol green and healthy blood plasma with bromocresol green were recorded. Bromocresol green binds HSA to site I with an affinity constant of $7\times10^{5}M^{-1}$ (Peters 1996) and is likely to stabilize HSA by it's binding. HSA dominates the thermogram of the blood plasma (figure 13) so it is likely that if there is a change in the thermogram of HSA upon bromocresol green binding, a similar change will occur in the thermogram of the blood plasma. In figure 15 the DSC curves of HSA with bromocrenol green and healthy blood plasma with various concentrations of bromocrenol green are shown. In both thermograms there is a shift of the thermograms to higher temperatures upon the addition of bromocresol green and the thermogram of the plasma starts to resemble the thermograms of some of the diseased thermograms, shown in figure 14. This shows that the addition of a small molecule to the blood plasma can change it's thermogram

significantly and that similar processes can lead to the observed changes in the thermograms of diseased blood plasma. (Garbett 2009)

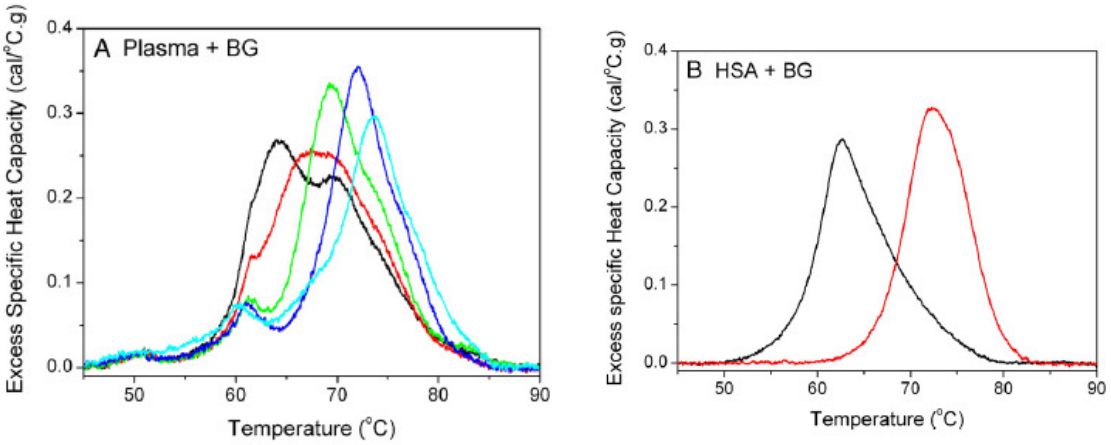

Figure 15: Effect of bromocresol green on the plasma thermogram from healthy individuals (panel A) or the HSA thermogram (panel B). (A). Bromocresol green was added to 25- fold diluted plasma (black) to final concentrations of 30 µM (red), 148 µM (green), 290 µM (blue) or 686 µM (cyan). (B) Bromocresol green was added to 46 µM HSA (black) to a final concentration of 460 µM(red). (Adapted from Garbett 2009)

5 - Conclusion

Garbett *et al.* demonstrated DSC is a valuable method which is very sensitive to the protein composition of a sample and to protein-ligand interactions. The various diseases yielded thermograms which were clearly distinct from the DSC curves of healthy plasma. Furthermore each disease had it's own unique thermogram, although some more clear than others, making it possible to identify a disease on the basis of a thermogram of the blood plasma. Although DSC does not detect and identify biomarkers in the blood plasma directly, it is very sensitive to the interactions which biomarkers make with more abundant proteins in the blood plasma. More research should be realized on the variations between thermograms of individuals, but the study by Garbett *et al.* demonstrates that DSC has potential to be used as a tool in clinical diagnosis.

Tetracycline repressor

1 - The protein concentration and scanrate

In a study of Kędracja-Krok *et al.* the influence of Tetracycline (Tc) binding on the stability of the Tetracycline repressor protein (TetR) was studied by DSC and CD. DSC scans

were realized on both the liganded protein and the protein in it's free state. The effects that increasing protein concentrations have on the thermograms of TetR and Tc bound TetR was studied and the influence of the scanrate on the DSC curves. Variations in the thermogram with increasing protein concentrations are an indication of changes in the molecularity of the protein during the unfolding process (Sanchez-Ruiz 1992, Grasso 1995). A homodimer can for example first dissociate into monomers and then unfold or the monomers of the dimer can unfold without prior dissociation. The effect of the protein concentration on the T_m provides thus information on the denaturation process. The relationship between T_m and the scanrate is a measure for the reversibility of a transition, as mentioned before (Robertson 1997). When the transition appears to be irreversible, the denaturation is under kinetic control and can not be studied using equilibrium themodynamics, instead of this a kinetic analysis can be performed (Sanchez-Ruiz 1992, Grasso 1995). The thermal denaturation was also monitored with CD.

2 - Tetracycline resistance

Resistance to Tc is mediated in most Gram-negative bacteria by the TetA antiporter, which exports the drug out of the plasma membrane. Expression of the *tetA* gene is under strict control of TetR. TetR is a homodimer with two identical helix-turn-helix (HTH) motifs which bind in the absence of $[Mg\text{-}Tc]^+$ to two adjacent major grooves in the DNA helix encoding *tetR* and *tetA* genes, hereby suppressing the transcription of these genes. When Tc enters a bacterial cell, it will bind TetR with high affinity, hereby inducing conformational changes in the protein. Upon these conformational changes the specific interactions with the DNA are abolished leading to the release of the TetR-$[Mg\text{-}Tc]^+$ complex and enabling transcription of the *tetA* and *tetC* genes. (Kędracja-Krok 2003)

3 - The DSC curves of TetR

The denaturation of TetR in the absence and in the presence of Tc appeared to be fully irreversible, even when the scans were stopped directly after the transition peak (figure 16). After the scans aggregations was evident from the samples. The T_m of the unbound TetR is 60,4 and the Tm of the TetR-$[Mg\text{-}Tc]^+$ complex is 70,4 °C, the binding of Tc raises the T_m of TetR thus with 10 °C. The denaturation enthalpy of TetR more than doubles upon the binding of Tc, with the ΔH_m of TetR only being 397,84 $kJ \cdot mol^{-1}$ and of the ligand bound TetR

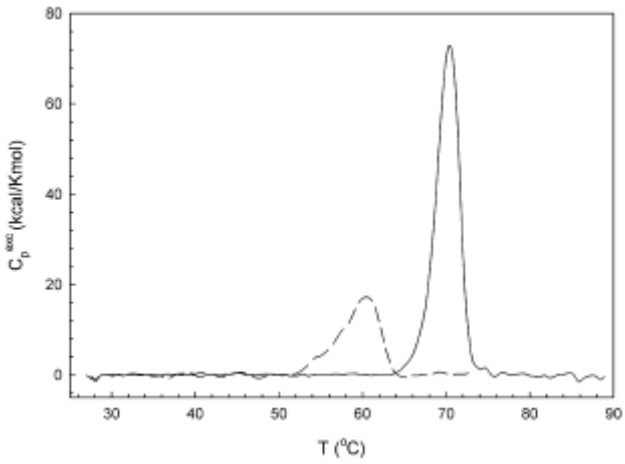

Figure 16: Typical thermograms of TetR (broken line) and complex of TetR with Tc (solid line). (Kędracja-Krok 2003)

1058,14 kJ·mol^{-1}, although the values are a bit inaccurate due to the irreversibility of the process. The increase in of the T_m and the doubling of the enthalpy of denaturation of TetR upon binding of Tc indicates that the complex of TetR-[Mg-Tc]$^+$ is much more stable than the unbound state of the protein. The ratio between the measured and the van 't Hoff enthalpy for the unbound protein is 0,59 which is an indication of oligomerization, this is no surprise because the denaturation peak of unbound TetR is very asymmetrical. The peak of the liganded TetR is on the other hand is more symmetrical and has a ratio between the van 't Hoff enthalpy and the calorimetric enthalpy of 0,92, showing that the denaturation follows a two-state process.

4 - The concentration effect

The T_m of TetR decreased at higher protein concentrations (figure 17). This indicates that the protein does not dissociate into monomers upon denaturation, as the T_m of multimeric proteins, which do unfold and dissociate at the same time, increases with the protein concentration (Takahashi 1981, Sanchez-Ruiz 1992). Although there is a slight increase of the T_m of the Tc bound TetR with protein concentration, there is a decrease at higher protein concentrations. This and the fact that the shape of the transitions did not change at higher protein concentrations indicates that also the Tc bound TetR does not unfold and dissociate simultaneously (Kędracja-Krok 2003)

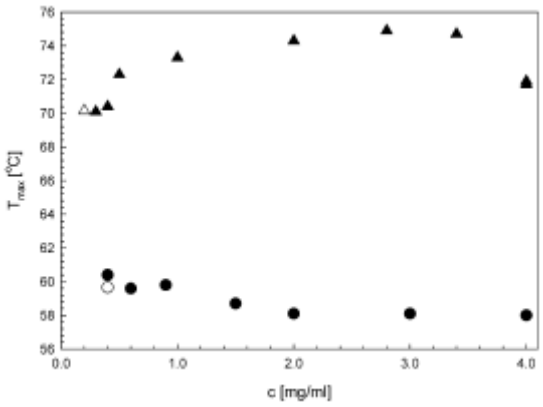

Figure 17: Effect of protein concentration on the transition midpoint (T_m).
The dark circles correspond to T_m for TetR obtained from DSC measurements and the white circle to T_m obtained from CD experiments. The dark triangles correspond to T_m for the complex of Tet repressor with Tc obtained from DSC experiments and the white triangle to the T_m obtained from CD measurements. (Kędracja-Krok 2003)

5 - The scanrate effect

The T_m of the repressor increases linearly with the scanrate, as shown in figure 18. This indicates that the denaturation of TetR is not reversible, but a kinetically controlled process and cannot be described by equilibrium thermodynamics (Robertson 1997, . Kinetic analysis of the thermograms showed that the denaturation of TetR can be described by the simple two-state model:

$$N_2 \xrightarrow{k} D_2$$

where *N* and *D* are the native and irreversible denatured form of the monomer and *k* is the first-order rate constant.

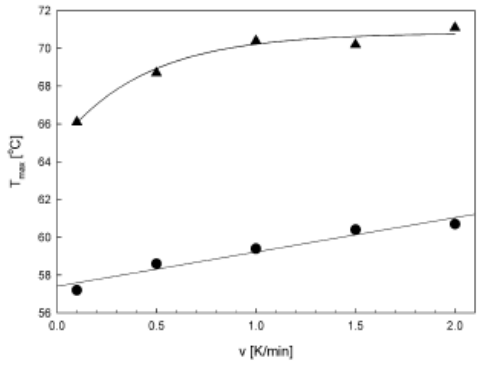

Figure 18: Effect of scan rate on transition temperature. Data obtained from DSC experiments. The circles are the T_m for TetR and the triangles the T_m for the complex of Tet repressor with Tc. The continuous lines have no theoretical meaning and are shown to guide the eye. (Kędracja-Krok 2003)

The T_m of the TetR-Tc complex initially rises rapidly with the scanrate, but stabilizes at higher scanrates, (figure 18). This means that although the transition is irreversible at lower scanrates, the system is in equilibrium at higher scanrates, because the T_m does not increase further. Equilibrium thermodynamics can therefore be used to analyze the transition

(Sanchez-Ruiz 1992). Furthermore the $\Delta H_{vH} = \Delta H_m$ at higher scanrates, indicating that the unfolding follows a two-state model at higher scanrates. The transition was then fitted to a two-state reversible model:

$$N_2Tc_2 \overset{K}{\leftrightarrow} 2TC + U_2 \overset{k}{\rightarrow} D$$

where *N* and *U* are respectively the native and unfolded monomers and *D* the denatured protein, *K* is the equilibrium constant and *k* the rate constant. According to this model the dimeric Tc bound TetR undergoes reversible denaturation with ligand loss, but without dissociation. The unfolded protein, U_2, then undergoes an irreversible alteration to a denatured state. From the fit the ΔH and ΔS values were calculated and respectively are 1067,1 kJ·mol^{-1} and 3,10 kJ·mol^{-1}. Unfortunately the C_p could not be determined because the of the low signal-to-noise ratio of the data.

6 - CD measurements

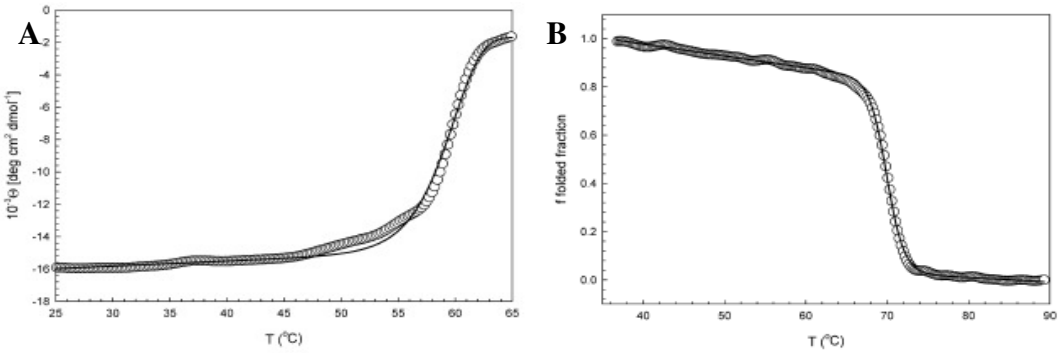

Figure 19: A: Temperature dependence of residue ellipticity at 222 nm for TetR fitted to a kinetic model. B: Temperature dependence of folded fraction (*f*) of the TetR complex with Tc fitted to a two-state model. (Kędracja-Krok 2003)

The thermal denaturation of TetR and TetR in complex with Tc was also monitored with CD (figure 19). In figure 19 is the ellipticity at 222 nm of TetR, which represents the amount of α-helix, plotted against the temperature, the data are fitted to a kinetic model. In figure 19B the fraction of folded TetR in complex with Tc is plotted against the temperature and fitted to a two-state model. The figures show that binding of Tc to TetR leads to a more symmetrical transition. Furthermore these data confirm that the liganded TetR denatures at an higher temperature.

7 - Conclusion

Kędracja-Krok *et al.* showed in this research that the binding of Tc by TetR leads to a stabilization of the protein. The T_m increases by 10 °C and the enthalpy of denaturation doubles when Tc binds the repressor. Although the denaturation was in all cases irreversible, by performing scans at different scanrates and protein concentrations there could still be a lot of information obtained about the protein. Scans with different protein concentrations made clear that both the liganded and unliganded protein unfold without prior dissociation into monomers. Analysis of the scanrate effect showed that the denaturation of unliganded TetR is a kinetically controlled process, but the TetR-$[Mg\text{-}Tc]^+$ complex unfolds according to a two state model. Although the unfolding of the complex is irreversible, the denaturation could be studied by equilibrium thermodynamics at higher scanrates , yielding a ΔH of 1067,1 $kJ \cdot mol^{-1}$ and a ΔS of 3,10 $kJ \cdot mol^{-1}$. Studying the thermally induced unfolding by CD gave similar results, supporting the results of the DSC. With this study Kędracja-Krok *et al.* showed with different techniques that the unfolding of a native protein can completely change when it binds a ligand.

Conclusion

CD, fluorescence and DSC all have their specialties. The amount of secondary and tertiary structure present in a protein can be assessed with CD and fluorescence is capable of monitoring changes in the tertiary structure of a protein. DSC on the other hand measures the excess heat capacity of a protein as a function of temperature.

The three discussed studies demonstrate that from a DSC scan a lot of information can be obtained. The research of Iida *et al.* showed that CD and DSC are a powerful combination. Deconvolution of a thermogram can yield information about the transitions occurring during the unfolding and allows calculation of the ΔS, ΔH_m and ΔC_p of the denaturation. With these parameters the ΔG of denaturation can be calculated at every temperature, which makes it possible to determine the conditions under which the protein is the most stable. CD measurements provided structural insight in the unfolding process and by taking the CD and DSC data together, a complete picture of the unfolding can be made.

The DSC curve of a protein and a ligand bound protein can differ a lot. Depending on whether the ligand preferentially binds the folded or unfolded state of a protein, binding results in a higher or lower T_m. Ligand binding can thus result in a higher or lower stability of the protein. This principle can be used to detect proteins which are in low abundance present in a sample, but through interactions with more abundant proteins leave their signature in the thermogram. Garbett *et al.* demonstrated that disease biomarkers in the bloodplasma can be detected by DSC through their interactions with other proteins and that DSC might serve as a diagnostic tool for the detections of diseases in an early stage.

Kędracja-Krok *et al.* studied the thermal denaturation of TetR and the TetR–$[Mg\text{-}Tc]^+$ complex. Although the denaturation of both was irreversible, still a lot of information could be obtained from the unfolding process. From the effect of the protein concentration on the T_m it could be concluded that the TetR unfolds without prior dissociation into monomers, both when bound to Tc and in it's unbound state. Analysis of the changes of the T_m with the scanrate showed that the the unfolding of the unliganded TetR is kinetically controlled, while the unfolding of the liganded TetR, although irreversible, can be described by a two-state model at higher scanrates.

It is clear that with DSC a lot of information can be obtained from a protein and it is the only technique with which it is possible to directly determine the enthalpy of denaturation. A downside of the technique is that high concentrations of protein are necessary, at least 1 mg/mL and with sample volumes of 0,3-2 ml a DSC run requires a lot of protein. The high protein concentration may also lead to difficulties arising from aggregation of the denatured state. Spectral techniques like CD and fluorescence on the other hand require less protein, the sample concentration can be as low as 0,01 mg/mL. Drawbacks of these techniques it is impossibility to directly determine the ΔH and the lack of direct measures for intermediate states in the unfolding process, which is possible with DSC as shown by Iida *et al.*

In summary, DSC is an excellent method for studying protein thermodynamics, because it can provide direct information on the thermodynamic parameters of a reaction. Especially the combination of DSC and a spectral technique is very powerful, because together they can present a complete image of a denaturation.

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