Differential Scanning Calorimetry: A Powerful and Versatile Tool for Analyzing Proteins and Peptides

Abstract

Differential Scanning Calorimetry (DSC) is anefficient and versatile analytical technique widely used to study the thermal properties and stability of proteins. This review article provides a comprehensive overview of the principles and applications of DSC in protein research. DSC measures the heat flow associated with temperature-induced conformational changes in proteins, allowing for the direct determination of key thermodynamic parameters such as melting temperature (Tm), enthalpy change (ΔH), and heat capacity change (ΔCp). These parameters provide valuable insights into protein and peptide stability, folding mechanisms, and interactions with ligands. The review discusses the methodological aspects of DSC, including sample preparation, experimental setup, and data analysis techniques. It also highlights the application of DSC in various aspects, such as drug discovery, biopharmaceutical development, and the study of protein-ligand interactions. By elucidating the thermal behavior of proteins, DSC contributes significantly to our understanding of protein and peptide structure, function, and stability, making it an indispensable tool in biochemical and biophysical research.

Keywords: DSC for proteins, DSC interpretation, Thermal Stability, Biomarker disease, Protein Folding, Ligand Binding, Biopharmaceuticals, Thermal Analysis.

1 Introduction

In DSC studies, the sample under examination and an appropriate reference material (one that does not undergo a phase transition within the temperature range of interest) are simultaneously heated or cooled at the same time at predefined rates. If the sample undergoes a temperature-induced phase shift, heat will be absorbed (or released). As a result, a temperature difference (DT) arises between the sample and reference cells [1]. The instrumental control system minimizes the temperature discrepancy by supplying more (or less) heat.DSC profiles present thermal stability information and, to some extent, act as a structural "fingerprint" for assessing structural conformation. A differential scanning calorimeter is used to detect the melting temperature (Tm) and energy necessary to disrupt protein connections that stabilize the tertiary structure (enthalpy; ΔH) [2]. DSC is now extensively utilized in pharmaceutical research and development to examine the heat stability of biomolecules, particularly peptides and proteins [2]. This allows for the detection of endothermic or exothermic transitions, which can reveal important insights into the thermodynamic properties of proteins and peptides [3,29].The results expressed in terms of Molar heat capacity as a function of temperature and it is used to estimate the following thermodynamic parameters (change in heat capacity (ΔCp), enthalpy (ΔH), entropy (ΔS), and Gibbs free energy (ΔG)) [2].

2 Method Development

Effective method development in DSC analysis requires careful consideration of sample preparation, instrument setup, experimental parameters, and data analysis strategies. Iterative optimization and validation are often necessary to obtain reliable and reproducible results for complex protein and peptide systems.

2.1 DSC Instrumentation

Sample and Reference Cells/Pans These are small enclosures where the sample and an inert reference material are placed. Common pan materials include aluminum, platinum, and high-purity metals or ceramics. The pans are designed to provide good thermal conductivity and minimize sample contamination. Temperature Sensors thermocouples or platinum resistance thermometers are used to precisely measure the temperature difference between the sample and reference cells/pans. The number and arrangement of these sensors determine the sensitivity and resolution of the instrument. Furnace: A furnace or heating block surrounds the sample and reference cells/pans. It provides controlled heating or cooling over a wide temperature range, typically from sub-ambient to 600-700°C or higher for specialized instruments. Purge Gas System An inert purge gas like nitrogen, argon, or helium is used to create an inert atmosphere around the sample and prevent oxidation or other undesired reactions during heating. Cooling System Depending on the instrument, various cooling systems like liquid nitrogen, compressed air, or refrigerants are used to cool the sample and furnace for sub-ambient measurements or rapid cooling between experiments. Data Acquisition and Control System This includes hardware and software for controlling the instrument parameters (temperature ramp rates, purge gas flow, etc.), acquiring data from the temperature sensors, and performing data analysis and calculations. Sample Encapsulation Station A separate station is often used for precisely weighing and hermetically sealing samples in pans or crucibles under an inert atmosphere to prevent oxidation or moisture uptake. Autosampler Some modern DSC instruments are equipped with autosamplers that can automatically load and analyze multiple samples in sequence, improving throughput and reproducibility. Factors like sensitivity, resolution, temperature range, and automation capabilities are key considerations when selecting a DSC instrument for specific applications in materials science, pharmaceuticals, polymers, or biological systems [4-6].

2.2 Sample preparation

Sample preparation is a critical step for accurate and reliable DSC analysis of proteins and peptides in solution. Proper sample preparation is essential to obtain reliable DSC data for proteins and peptides, as improper handling can lead to denaturation, aggregation, or artifacts that complicate data interpretation.Here are some common sample preparation techniques used for DSC analysis of protein/peptide solutions [7-9, 24].

3 Sample Concentration

DSC has limited sensitivity at low concentrations, so samples often need to be concentrated to 1-5 mg/mL or higher for reliable measurements (The required concentration range may vary depending on the model of the instrument).To increase the sample concentration common techniques, include ultrafiltration (centrifugal filters or stirred cells) and lyophilization followed by reconstitution.Care must be taken to avoid protein denaturation or aggregation during concentration [26].

3.1.1 Buffer Exchange/Dialysis

Buffers including phosphate, formate, and acetate are ideal since they have minimal ionization heats and their pH stays constant with temperature fluctuations [1].

Proteins are typically buffer-exchanged into a volatile buffer (e.g., sodium phosphate) compatible with DSC analysis to avoid interference from non-volatile salts or buffers.
Dialysis or desalting columns can be used for efficient buffer exchange [27].

3.1.2 Degassing

Dissolved gases can cause bubbles which may lead to baseline distortions in DSC thermograms.
Both reference and samples are typically degassed under vacuum or by gentle stirring before loading into DSC pans.

3.1.3 Hermetic Sealing

Samples are sealed in DSC pans or crucibles under an inert atmosphere (nitrogen or argon) to prevent oxidation and evaporation during heating.
Specialized sample encapsulation stations are often used for this purpose.
Sample Homogeneity: Ensuring sample homogeneity is crucial for accurate DSC measurements.Techniques like vortexing, gentle pipetting, or sonication may be used to achieve homogeneous solutions before loading into pans.
Sample Cutting/Sizing (for solids):For solid proteins or peptides, samples may need to be cut or sized to fit DSC pans using specialized cutters or mills.
Automated Sample Preparation:Modern DSC instruments often integrate with automated sample preparation workstations for improved throughput, reproducibility, and minimized handling errors.

3.2 Experimental Parameters

Depending on the equipment, samples can be loaded into the cell manually with a syringe or automatically using an autosampler. Set the initial temperature of the instrument at 20°c and final temperature at 120°C. Temperature can vary depending on prior knowledge of the sample. Set the scan rate for the experiment, *e.g.*60 °C/h, which is the most commonly used scan rate. Unknown samples can be scan at variable temperature (20°C-80°C) to assess the kinetics of unfolding.Slower rates improve resolution but may promote kinetic effects. Set the post-experiment thermostat to 10 °C to protect the calorimeter's cells. Before running the experiment, ensure that the setup parameters are valid. If everything is in place, begin the experiment.

3.3 Data Analysis

3.3.1 Determining thermodynamic parameters

Integrating the area under the endothermic unfolding peak yields the enthalpy of unfolding (ΔHcal). The temperature at the peak maximum is the melting temperature (Tm). These parameters provide a measure of protein thermal stability.

3.3.2 Assessing reversibility

Rescanning the sample after the initial DSC scan can reveal if the unfolding is reversible. If an identical endotherm is observed, the native and denatured states are in equilibrium. Irreversible unfolding can occur due to processes like aggregation.

3.3.3 Analyzing scan rate dependence

Performing DSC at different scan rates can provide insights into the kinetics and mechanism of unfolding. Faster scan rates shift the transition to higher temperatures. Analyzing the scan rate dependence can distinguish between two-state and non-two-state unfolding.

3.3.4 Studying concentration dependence

Varying the protein concentration in the DSC experiment can reveal if the unfolding is concentration-dependent. Aggregation is often concentration-dependent, so monitoring the Tm and ΔH as a function of concentration can detect aggregation.

3.3.5 Fitting models to the data

The DSC profile can be fit to thermodynamic models to extract equilibrium parameters like the van't Hoff enthalpy (ΔHvH). The ratio of ΔHcal to ΔHvH provides a measure of the cooperativity of unfolding. Models can also account for linked protonation reactions and oligomeric dissociation.

3.3.6 Data Interpretation

Data interpretation is a crucial aspect of using Differential Scanning Calorimetry (DSC) to study proteins [11].

In differential scanning calorimetry (DSC), an endotherm is a peak in the DSC curve that indicates an endothermic process, where the sample absorbs heat relative to the reference. This typically occurs during processes like melting, boiling, sublimation, and some chemical reactions.An exotherm, on the other hand, is a peak in the DSC curve that indicates an exothermic process, where the sample releases heat relative to the reference. Exothermic processes include crystallization, curing reactions, and some chemical reactions.The direction of the peak (up or down) depends on the DSC instrument design. In a heat flux DSC, an endotherm appears as a downward peak, while an exotherm appears as an upward peak. In a power compensation DSC, the directions are reversed.The area under an endothermic or exothermic peak is proportional to the enthalpy change (ΔH) of the transition. Integrating the peak area allows quantifying the heat absorbed or released during the process.In summary, endotherms and exotherms are the characteristic peaks in a DSC curve that indicate heat absorption and heat release processes in the sample, respectively, relative to an inert reference material. Their direction and area provide information about the nature and enthalpy of the thermal transitions occurring in the sample.

3.3.7 Endotherms

Endothermic peaks in a protein DSC curve correspond to heat absorption processes, typically protein unfolding or denaturation. When a protein is heated, its higher-order structure undergoes an irreversible change, causing activity loss due to degeneration. This thermal denaturation results in an endothermic peak in the DSC analysis. The area under the endothermic peak is proportional to the enthalpy change (ΔH) of protein unfolding. Analyzing the endotherm provides the melting temperature (Tm) and enthalpy of unfolding of the protein. This information is crucial for understanding protein stability and folding.

3.3.8 Exotherms

Exothermic peaks in a protein DSC curve are less common, but can occur due to processes like:

Ligand binding Binding of a ligand to the native protein can stabilize the folded state, shifting the unfolding endotherm to higher temperatures. At high enough ligand concentrations, an exothermic ligand binding peak may precede the endothermic unfolding.
Aggregation Unfolded proteins can aggregate exothermically at high temperatures. This competes with the endothermic unfolding.
Refolding If the protein refolds upon cooling, an exothermic refolding peak may be observed.

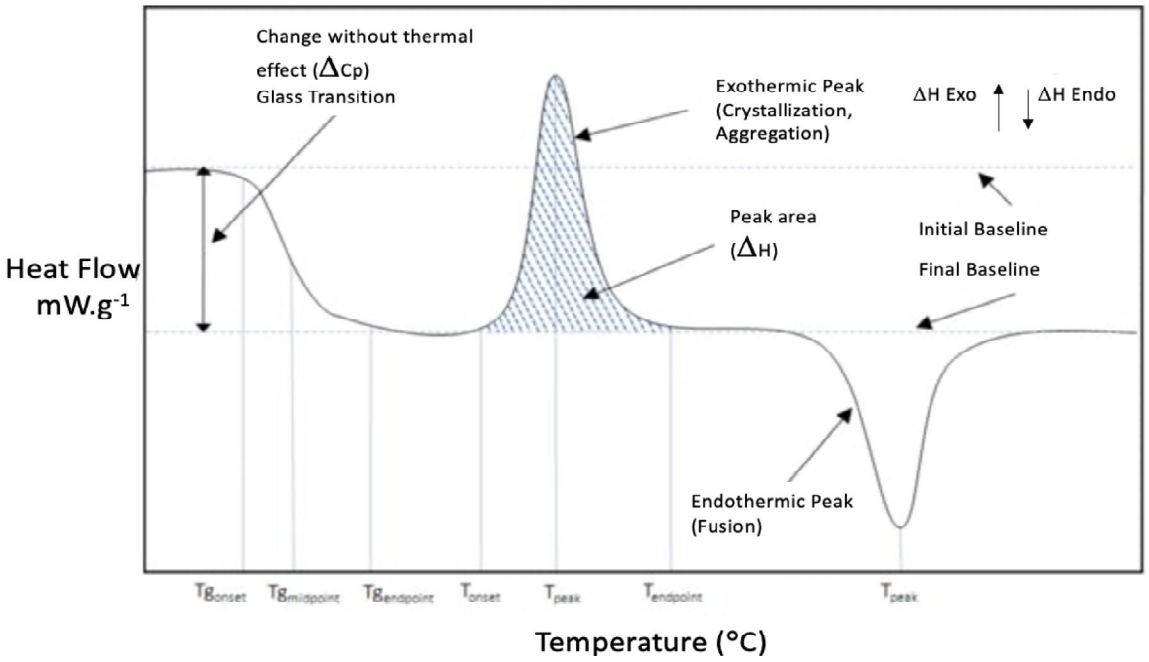

Figure 1 DSC Thermogram (heat flow against temperature):exothermic peak, endothermic peak [22]

3.4 Protein Stability:

The melting/denaturation temperature (Tm or Td) is a measure of the protein's thermal stability.
Shifts in Tm upon changes in solution conditions (pH, ionic strength) or ligand binding indicate changes in protein stability.

3.5 Reversibility and Kinetics:

Reversible unfolding transitions indicate a two-state folding mechanism without intermediates or aggregation.Irreversible transitions may suggest aggregation, degradation, or kinetically-trapped states.Scan rates can influence the observed transition temperatures and shapes, providing insights into unfolding kinetics.

3.6 Ligand Binding and Interactions:

Shifts in Tm or appearance of new transitions upon ligand binding can indicate binding interactions and allow quantification of binding affinities.
Changes in transition shapes or enthalpies can reveal the nature of the binding interaction (e.g., coupled folding/binding).

4 Application of DSC in Biomolecules study

DSC is an invaluable tool for studying the thermodynamics of various biomolecular interactions and conformational changes in biochemistry and drug development. It provides direct, non-destructive measurements of thermodynamic parameters that are crucial for understanding and engineering the stability and function of proteins, nucleic acids, lipids, and their interactions with drugs and other ligands.

4.1 Peptide Screening

DSC identified the membranotropic areas of hepatitis C virus proteins E1, E2, core, and p7 by evaluating the influence of protein-derived peptide libraries on model membrane integrity. In this work, they investigated the capacity of specific sequences of these proteins to alter the Lb-La and La-HII phospholipid phase transitions, as well as the viability of screening a protein-derived peptide library using both DSC and SAXD [12].

4.2 Protein Stability and Folding

DSC can directly measure the thermodynamic parameters that characterize protein stability, such as the enthalpy change (ΔH), transition midpoint (Tm), heat capacity change (ΔCp), Gibbs free energy (ΔG), and entropy change (ΔS) during thermal denaturation. This allows studying protein stability, domain structure, conformation, and the stabilizing effects of ligand binding[13]. It allows quantitative characterization of how factors like pH, ionic strength, ligand binding, and mutations affect protein stability and folding cooperativity.Understanding protein stability is crucial for developing stable biotherapeutics, enzymes for industrial applications, and elucidating the principles of protein folding.Unlike other methods like circular dichroism (CD) spectroscopy, the accuracy of DSC is not dependent on the secondary structure of the protein. DSC can be used to study proteins with a wide range of secondary structures [2].

4.3 Disease Biomarker Detection

DSC is an emerging technique that can provide a unique thermodynamic signature of disease by analyzing the thermal stability of proteins in biological samples. It has shown promise for detecting a variety of cancers and other diseases, and may be applicable to saliva as well as blood. Whilst more studies are completed, a reference library of normal and disease DSC profiles could aid in future clinical diagnosis and monitoring.

Studies have successfully used DSC to identify unique thermodynamic signatures correlated with various diseases in body fluids and tissues, including:Glioblastoma multiforme brain cancer, Melanoma with lymph node or distal metastases, Breast cancer, Colorectal cancer, Cervical cancer, Chronic pulmonary disease and Type 1 diabetes with early renal function decline [25].

The key advantages of DSC for biomarker detection are:

It is sensitive to changes in protein composition, interactions and abundance in complex samples like plasma, serum, urine and tissue homogenates. It does not require labeling or fractionation of the sample. Unique thermograms are obtained that correlate with disease type and progression [14].

4.4 Amyloid formation

DSC has provided insights into how binding of native protein partners can influence amyloid formation. For example, the binding of retinol binding protein (RBP) to the amyloidogenic protein transthyretin (TTR) was found to inhibit TTR fibril formation. The binding stoichiometry

of RBP, vitamin A, and thyroid hormone T4 to TTR was determined by DSC to be an important factor controlling TTR amyloidogenicity. DSC studies have shown that the heat capacity change (ΔCp) associated with amyloid fibril formation is similar to that of native protein folding, suggesting amyloid fibrils and globular proteins have comparable overall burial of polar and charged groups upon formation. However, the lower enthalpy of unfolding for amyloids indicates they have less internal packing and side chain interactions compared to native folds [15-17].

4.5 Protein-Ligand Interactions

DSC can detect and quantify binding interactions between proteins and small molecules or ligands by monitoring shifts in the protein's melting temperature upon ligand binding.This provides valuable information on binding affinities, stoichiometry, and the thermodynamics of protein-ligand complexes, aiding in drug discovery and understanding molecular recognition [18-19].

4.5.1 Determining binding affinity

Performing DSC at increasing ligand concentrations yields a series of curves that can be fit to obtain the protein-ligand dissociation constant (Kd). The enthalpy of unfolding directly measured by DSC helps improve the precision of the binding constant determination[11].

4.5.2 Measuring extremely tight binding constants

DSC is particularly useful for determining binding constants in the picomolar Kd range, which are difficult to measure by other methods. It can also separate binding steps and measure weak binding to secondary sites at high ligand concentrations[11,28].

4.5.3 Analyzing linked protonation reactions

If ligand binding is coupled to protonation reactions, DSC can be used at different pH values to determine the intrinsic, pH-independent binding constant. This provides a detailed thermodynamic picture of the linked ligand binding and protein unfolding process[11,28].

4.5.4 Studying the effect of ligands on protein stability

A shift of the protein denaturation peak to higher temperatures in the presence of a ligand is a sign of protein-ligand binding. DSC can be used to monitor how ligands affect the thermal stability of proteins[11].

4.6 Peptide-Lipid Interactions

DSC is extensively used to study the interactions between membrane-active peptides (e.g., antimicrobial, cell-penetrating) and lipid membranes, which are crucial for their biological functions. It reveals the mode of interaction, membrane perturbation, and thermodynamics of peptide-lipid binding, contributing to the development of peptide-based therapeutics and understanding membrane biology.

DSC can determine the binding affinity of peptides to lipid membranes and distinguish between surface adsorption and insertion into the hydrophobic core. The ratio of binding affinities to lipid monolayers vs bilayers indicates the contribution of the membrane core to binding.

The extent to which a peptide penetrates into the membrane core can be inferred from the ratio of binding affinities to lipid monolayers vs bilayers. A ratio above ~10 indicates the peptide has inserted into the bilayer.

5 Conclusion

Differential scanning calorimetry (DSC) is a powerful and versatile technique that provides a wealth of thermodynamic information about proteins, peptides, and their interactions. As reviewed in this article, DSC has numerous applications in protein science, drug development, disease diagnosis, and membrane biophysics.The key strengths of DSC are its ability to directly measure heat effects, obtain thermodynamic parameters like enthalpy and melting temperature, and analyze complex systems like protein mixtures and biological fluids. DSC yields crucial insights into protein stability, folding, and interactions that are difficult to obtain by other methods.In the field of protein engineering, DSC provides essential data on protein thermal stability and unfolding thermodynamics. This information guides the design of more stable proteins for industrial and therapeutic applications.Its ability to directly measure heat effects and binding interactions in complex systems makes it a valuable complement to spectroscopic techniques.

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