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Conformation and stability properties of B17: II. Analytical investigations using differential scanning calorimetry

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Abstract

Thermal and stability properties of B17, the 17 % N-terminal domain of apo B, were carried out using differential scanning calorimetry spectroscopy, where the thermal characteristics of the polypeptide were studied and analyzed. The heat capacity data of B17 showed that the protein undergoes two transitions between 50 and 90 °C, with T_m 's at 65.9 and 74.8 °C. While the first transition showed immediate reversibility, the second one—with the higher T_m —necessitated a longer cooling (several days) period for its reversibility to be observed and both transitions could be seen in the heat capacity profile of B17. Moreover, the van't Hoff enthalpies determined via calorimetric measurements agreed with the values calculated from the CD analysis reported previously.

Keywords

Apolipoprotein (Apo B); Atherosclerosis; Differential scanning calorimetry (DSC); Low-density lipoprotein (LDL)

Introduction

Apo B₁₀₀ is a large protein that plays an extremely important role in the pathology of several potentially fatal diseases, e.g., atherosclerosis, arteriosclerosis, coronary artery diseases (CAD), etc. (Baynes and Dominiczak 2009). The entire protein is a single peptide chain composed of 4,536 amino acid residues, and has—when glycosylated—a molecular mass of ~550 kDa (Chen et al. 1986; Cladaras et al. 1986; Knott et al. 1986; Law et al. 1986; Yang et al. 1986).

The size and insolubility of apo B necessitated that only indirect probing be done on this nonexchangeable protein, and; hence, biochemical and biophysical techniques, as well as computer algorithms have been used to study the structure of the protein in pieces, perhaps corresponding to structural or functional domains (Nolte 1994; Poulos 2001; Khachfe 2002; Al-Ali and Khachfe 2007; Khachfe and Atkinson 2011; Khachfe and Atkinson 2012). In this report, the N-terminal portion of the protein, a portion necessary for the secretion and interaction of the protein with other molecules, is studied in terms of its thermal stability using calorimetric probes. The calorimetric behavior of macromolecules has been elaborately and eloquently analyzed in several reviews (Privalov and Potekhin 1986; Privalov and Makhataadze 1990; Sturtevant 1974). Calorimetry measures the heat capacity (cal/K), which is the amount of heat absorbed by the system to raise its temperature by 1 K under constant pressure (C_p) or constant volume (C_v). A differential scanning calorimeter measures the excess heat capacity (C_p) of a protein as a temperature range (5–100 °C) is scanned at a specified constant rate (°C/h). Two cells, one for the reference buffer and one for the protein sample in that buffer, are enclosed in an adiabatic chamber. These cells are heated by two main heaters fed with the same input current (series); therefore, the same amount of energy will be delivered to both the sample cell and the reference cell. However, since the sample cell contains the protein moiety besides the reference solution, the energy delivered to this cell will be used to both raise the temperature of the solution and thermally unfold the protein, and the two cells will tend to have different temperatures. The difference in temperature between the two cells is maintained at zero by the presence of a cell feedback heater (CFH) that provides extra power to the sample cell. This extra power is proportional to the excess heat capacity, which is recorded versus temperature. As a protein unfolds, hydrophobic residues become exposed to the solvent, a factor that is a major contributor to the observed heat capacity effect of unfolding. The heat capacities of the pre-transition and post-transition regions are those corresponding to the folded and unfolded states in the solvent, respectively. Since the energy of the unfolded state is higher than that of the folded state, the heat capacity of the former is greater than that of the latter. Because the heat capacity of non-polar compounds in an aqueous environment is directly proportional to the surface area of the number of solvated water molecules, the heat capacity change defines the hydrophobic effect related to the exposure of non-polar groups to the aqueous solvent. The difference between the two, C_p , is—therefore—roughly proportional to the size of the hydrophobic core of the protein (Murphy et al. 1990). The value of C_p is determined by the vertical distance between the pre- and post-transition baselines extrapolated to the midpoint temperature of the transition, T_m , where the excess C_p is maximal. The initial and final temperatures of the transition, T_i and T_f , give the temperature range over which protein unfolding occurs. The enthalpy change, H , accompanying the transfer of a protein from the folded state to the unfolded state is the energy difference in the specific interactions—hydrogen bonding, Van der Waals contacts, and ionic forces—associated with the folded and unfolded states. The calorimetric enthalpy H_{cal} of unfolding one protein molecule is the area under the curve of the excess heat capacity. In the two-state mechanism of protein unfolding, the temperature dependence of the equilibrium constant yields the van't Hoff enthalpy H_{vH} , such that,

$$\Delta H_{\text{vH}} = RT^2 \frac{d(\ln K_{\text{eq}})}{dT}, \quad (1)$$

Where,

$$K_{\text{eq}} = \frac{[\text{Unfolded}, U]}{[\text{Native}, N]} \quad (2)$$

and R is the gas constant. Therefore, the van't Hoff enthalpy measures the enthalpy change associated with unfolding of one cooperative unit of the protein. It can be approximated from the width of the transition peak at half of its height,

$$\Delta H_{\text{vH}} = \frac{4RT_m^2}{\Delta T_{1/2}}. \quad (3)$$

The process of unfolding can be described by the ratio of calorimetric enthalpy to van't Hoff enthalpy, $H_{\text{cal}}/H_{\text{vH}}$. This ratio can be calculated from the following equation:

$$\frac{\Delta H_{\text{cal}}}{\Delta H_{\text{vH}}} = \frac{\Delta H_{\text{cal}}^2}{4RC_{\text{pmax}}T_m^2}, \quad (4)$$

where C_{pmax} is the maximum heat capacity, R the gas constant, and T_m the midpoint temperature. A value equal to one means a two-state transition; greater than one indicates that intermediates are involved, and less than one suggests the presence of an irreversible process (Privalov and Potekhin 1986; Privalov and Makhatadze 1990; Sturtevant 1974).

Materials and methods

Pure B17 was obtained as previously described (Khachfe and Atkinson 2011). Samples were dialyzed to the appropriate buffer conditions using Slide-A-Lyzer dialysis cassettes (10,000 MWCO) obtained from Pierce, USA.

High resolution calorimetry experiments were done on a computerized MC-2 calorimeter (Microcal, Inc., Northampton, MA), containing two identical tantalum cells for sample and reference (volume 1.2314 ml). Samples were dialyzed against buffer, and dialysate was used as the reference. Protein concentration of the four samples used was 1.5–2 mg/ml. The sample and the reference were degassed under room temperature vacuum prior to introduction—at room temperature—into the calorimeter cells, which were subsequently cooled. The scans were recorded from 5 to 100 °C at a heating rate of 90 °C/h under nitrogen pressure. To baseline-correct for buffer, dialysate versus dialysate scans were

recorded and then subtracted from the following protein versus dialysate scans. Protein scans were then normalized for protein concentration.

The resulting heat capacity curves of the protein were subjected to deconvolution or curve-fitting analysis (Freire and Biltonen 1978; Privalov 1979, 1982) using the ORIGIN software from Microcal, Inc. (Northampton, MA).

Results and discussion

Using differential scanning calorimetry (DSC), the packing of B17 was investigated vis-à-vis its thermal properties. Calorimetric analysis was performed on four samples of B17. The samples were at 0.5, 1.2, 1.4, and 1.5 mg/ml, and in 5 mM K-phosphate at pH 7.4. The sample at 0.5 mg/ml B17 did not produce measurable change in the heat capacity with respect to the buffer baseline. The unfolding of B17 at 1.2 mg/ml of protein in 5 mM K-phosphate, pH 7.4, indicated the presence of at least two transitions in the region from ~60 to ~80 °C, as shown by the large peak at ~75 °C and its shoulder at ~65 °C (Fig. 1a). When the heat capacity measurements are immediately repeated on the same samples, the characteristic peaks are lost, and no transition information is obtained (Fig. 1b), indicating a loss in structural content.

The same set of experiments was done after measuring the heat capacity up to 70 °C. Heating up to 70 °C produced a slight increase in the heat capacity profile over the baseline, corresponding to the shoulder (Fig. 1c). A second immediate scan over the range 20–100 °C on the same sample reproduced the full features, including the shoulder and a major peak (Fig. 1d). However, when the solution was cooled to 20 °C and then heated back up to 100 °C, both characteristic peaks were lost, and the curve was indistinguishable from the baseline (Fig. 1e). This showed that up to 70 °C, immediate reversible changes occur in the protein resulting in the initial increase in the heat capacity. Beyond 70 °C, other changes take place in such a way that the overall organization of the protein is disrupted. This might also suggest that the first changes occur in a subdomain that is somehow structurally independent. The second set of changes, however, completely destroys the protein fold, so that the protein does not refold upon immediate cooling, but rather needs a longer cooling time for refolding to occur, as was observed by storing the above samples at 4 °C for 7 days and then measuring the heat capacity again. A full scan over the range 20–100 °C on those samples reproduced the full features, including the shoulder and the major peak (Fig. 1f).

The reversibility upon prolonged cooling, observed when the heat capacity curve of a long-cooled sample looked almost identical to the original heat capacity curve, confirmed the reversibility of the refolding and the kinetic nature of the process, previously observed by the authors using circular dichroism spectroscopy (Khachfe and Atkinson 2012).

To analyze quantitatively the DSC profile of B17, a cubic baseline was subtracted from the original scan (A) of Fig. 1. This is a nonlinear approach whose performance depends on the data point determination accuracy (Jané et al. 1992), a characteristic that is readily available in our type of plots. The new graph is presented in Fig. 2, along with the mathematical deconvolution of its peaks. Table 1 summarizes the thermodynamic data of B17. The

midpoint (melting temperature), T_m , and the van't Hoff enthalpy, H_{vH} , were also determined from the van't Hoff plots of the CD data reported previously by the authors (Khachfe and Atkinson 2012) and the plots of the first derivative of the temperature curves. The analysis is shown in Figs. 3 and 4. The calculated values are in good agreement with the observed ones. This confirms the results of the DSC experiments in terms of the reversibility of the transitions and the high melting temperatures.

Walsh and Atkinson reported that full-length apo B solubilized in sodium deoxycholate, NaDC, undergoes multi-stage melting, suggesting the presence of multiple domains (Walsh and Atkinson 1990). Similar to what has been presented in our report, they reported that specific regions or domains of apo B may be melting or unfolding at specific temperatures, indicating that some domains of the large apo B molecule may be thermodynamically more stable than others (Walsh and Atkinson 1990). Indeed, our results indicate that the first transition takes place at $\sim 65^\circ\text{C}$ while the second one takes place at $\sim 75^\circ\text{C}$.

Jiang and McKnight studied the thermal unfolding of the N-terminal 5.9 % of apo B. Although their protein aggregates at high temperatures, their results also showed high temperature unfolding (Jiang and McKnight, personal communication). This difference in thermal behavior between B17 and the full-length apo B may be explained in terms of the degree and type of packing of B17. While the full structure of apo B contains eight disulfide bonds, six of them are found in B17. Therefore, the tight packing of the helices and sheets in the lipid-free B17, coupled with the presence of a high number of disulfide bonds between its structural elements may be the reason behind the high melting temperatures seen in both the CD (Khachfe and Atkinson 2012) and calorimetric data. Hence, the last transition seen in the NaDC model system might very well encompass the transitions of the N-terminal domain of apo B seen in our experiments.

Conclusions

The medical interest in studying apolipoprotein B₁₀₀ (apo B), the only protein found on human low density lipoprotein (LDL) particles, stems from the fact that LDL is the *inducing factor* for atherosclerosis and other coronary heart diseases. Being a very large (4,536 amino acids, 550 kDa) secretory glycoprotein, apo B has unique structural properties that might be studied in pieces corresponding to its structurally organized domains. In the present work, we studied the conformational and stability properties of B17, the 17 % N-terminal domain of apo B—a portion of the protein that is secreted predominantly lipid-free, and plays an important role in the initiation and assembly of the LDL particle (Herscovitz et al. 2001)—based on its calorimetric behavior. Previous reports indicated that the protein is composed of two structurally-independent domains (Al-Ali and Khachfe 2007) that exhibit distinct thermal behaviors (Khachfe and Atkinson 2012).

Calorimetric analysis of the heat capacity behavior of B17 showed that the protein undergoes two transitions between 50 and 90°C , with T_m 's at 65.9°C and 74.8°C . While the first transition showed immediate reversibility, the second one—with the higher T_m —necessitated a longer cooling (several days) period for its reversibility to be observed and both transitions could be seen in the heat capacity profile of B17. Compared with the model

structure of B17 (Al-Ali and Khachfe 2007) and the CD analysis of the protein (Khachfe and Atkinson 2012), it is evident that the first transition corresponds to the β -sheet domain (N-terminus) of the polypeptide, whereas the second transition is descriptive of the bigger, structurally mixed, α -helix-rich $\alpha\beta$ domain. In addition, not only does the presence of three disulfide bridges in each of these domains stabilize the fold of the protein, but it also contributes to the high melting temperatures observed in the current calorimetric study (Turunen et al. 2001).

The van't Hoff enthalpies determined via calorimetric measurements agreed with the values calculated (Eqs. 1 and 3, above) from the CD data that was previously partially reported (Khachfe and Atkinson 2012), and hence, confirmed the two-state thermal change associated with the unfolding of cooperative protein domains. In addition, the enthalpy ratio (Eq. 4, above) calculated using calorimetric data verified the two-state transitions, just in agreement with previous spectroscopy reports. Moreover, the mathematical manipulation of the CD data (Fig. 4) gives yet another verification that the first transition corresponds to the unfolding of the β -sheet N-terminal domain of the protein, whereas the second transition describes the denaturation of the α -helix-rich core, as witnessed by the peaks of the first derivative plots.

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Fig. 1.

High resolution calorimetry of B17. Differential scanning calorimetry (DSC) of BV B17 in 5 mM K-phosphate. Buffer baselines have been subtracted from each scan. *a* 20–100 °C; *b* 20–100 °C; *c* 20–70 °C; *d* 20–100 °C; *e* 20–100 °C immediate repeat; *f* 20–100 °C long-wait repeat

Fig. 2.
DSC analysis. Deconvolution of the heat capacity profile shown in figure. A cubic baseline was subtracted from the original data, which was normalized for B17 concentration

Fig. 3. van't Hoff enthalpies. The slopes of the above curves represent the van't Hoff enthalpies divided by the universal gas constant R. (1) $H_{vH} = 15.3$ kcal/mol; (2) $H_{vH} = 24.2$ kcal/mol. The original data was previously partially reported by the authors (Khachfe and Atkinson 2012)

Fig. 4.

Thermal data analysis. The first derivatives of the temperature CD plots recorded using the thermal unfolding of B17 monitored at constant wavelengths characteristic for the various secondary structure elements. The *upper panel* is the derivative of the thermal unfolding of B17 monitored at 217 nm, characteristic of β -sheet content; the *lower panel* is the derivative of the thermal unfolding of B17 monitored at 222 nm, characteristic of α -helical content. The original data was previously partially reported by the authors (Khachfe and Atkinson 2012)

Table 1

Summary of thermodynamic data for B17

Transition	$[T_i - T_f], T_m$ (°C)	H_{cal} (kcal/mol)	H_{vH} (kcal/mol)	H_{cal}/H_{vH}	C_{pmax} (kcal/mol K)
Overall	$[45.2 \pm 1.3 - 84.7 \pm 2.4], 75.6 \pm 0.9$	42.2 ± 0.8			4.4 ± 0.2
1st	$[47.7 \pm 2.4 - 82.9 \pm 1.5], 65.9 \pm 0.2$ (69.9) ^b	16.4 ± 1.0 (17.1) ^b	14.0 ± 0.6 (15.3) ^a	1.2 ± 0.4	2.0 ± 0.2
2nd	$[58.9 \pm 1.6 - 92.1 \pm 1.2], 74.8 \pm 0.1$ (73.5) ^b	25.8 ± 0.9 (25.3) ^b	23.8 ± 0.2 (24.2) ^a	1.1 ± 0.7	4.2 ± 0.3

All cited temperatures are in °C. $[T_i - T_f]$ represents the range of the transition between the initial temperature and the final temperature

^aValues from van't Hoff plots of the CD data

^bValues calculated from the first derivatives of the CD thermal unfolding curves