Influence of Scan Rate on Simulation of Differential Scanning Calorimetry Profiles of Protein Denaturationt

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Abstract

The heat capacity has played a major role in proteins. Its calculation by atomistic simulation methods remains a significant challenge due to the complex and dynamic nature of protein structures and this work compares the denaturation effect of bovine carbonic anhydrase (BCA) by heat, pH and scan rate dependence of protein denaturation by molecular dynamics (MD) simulation. To better understand this factor on calculating a protein heat capacity and Tm, we have provided a comparative analysis of simulation models that differ in their scan rate and pH description. Our model protein system is the carbonic anhydrase, and a series of 20 ns simulated DSC with different scan rate (v= 0.10, 0.0125, 0.015 and 0.02 K/ps) and pH have been reported by simulated annealing performed at temperatures ranging from 250 to 575 K, starting from the carbonic anhydrase native structure. It was observed that, our systems were quite sensitive to the description and the calculated melting temperature (Tm) varied in the range 353-438 K and was higher for higher scan rates systems and lower for acidic condition. It was also demonstrated that increasing scan rate causes a slight shift to right and acidic pH cause a shift to left in Tm value.

Index terms— carbonic anhydrase, melting temperature, molecular dynamics simulation, scan rate, simulated annealing.

1 I. Introduction

arbonic anhydrase (CA) is a clinically relevant and biochemically well-characterized protein. It catalyzes hydration of carbon dioxide to carbonic acid and is involved in vital physiological processes such as pH and CO 2 homeostasis, transport of bicarbonate and CO 2 , biosynthetic reactions, bone resorption, calcification, tumorigenicity, and other physiological or pathological processes. Therefore, this enzyme is an important target for inhibitors with clinical applications, primarily for use as antiglaucoma agents but also for the therapy of various pathologies such as epilepsy and Parkinson's disease. Many groups have used carbonic anhydrase-both bovine and human-as a model protein for studies of folding and unfolding. [1][2][3] Differential scanning calorimetry (DSC) is a technique able to study thermally induced transitions and particularly, the conformational transitions of biological macromolecules (for example between the folded and the unfolded structure of a protein).

It measures the excess heat capacity of a solution (C p) of the molecule of interest as a function of temperature and has been extensively used to study protein thermal denaturation. 4 A variety of techniques have evolved which can be used to gain structural information on protein stability. DSC has become one of the key physicochemical methods to study the stability of protein biopharmaceuticals. 5,6 In experimental study of carbonic anhydrase unfolding by DSC the enthalpy of unfolding in the temperature range of 39 to 72 °C by carrying out DSC experiments at various pH was determined. 7 T m (effectively the transition peak) is defined as the temperature at which 50% of the protein molecules are unfolded or as a midpoint in a thermal ramp and represents a temperature where the free energy of the natives and nonnative forms are equivalent. Protein melting temperatures can

be determined by numerous methods, including differential scanning calorimetry and optical methods (circular dichroism, fluorescence or absorbance spectroscopy). These techniques have low throughput, are time consuming, and require significant amounts of protein and, thus, are not generally utilized when testing the large numbers of compounds generated during drug development.

Scan rate dependence was determined using the methods described by Sanchez-Ruiz et al. 8 The scan-rate-dependent shift in T m for denaturation was fitted to the equation:???????? ???????? ?? ?? 2 = ???? ?? ?? ?? ??? ?? /???? ?? (1)

Such that a plot of ln(scan rate/?? ?? 2) against 1/ ?? ?? yields a slope ? E a /R, where E a is the activation energy for denaturation, R the gas constant and A the pre-exponential factor in the Arrhenius equation. The effects of scan rate on the DSC profiles are given. These results are expressed in the form of DSC profiles (excess C p , vs. T).

The calorimetric transitions for carbonic anhydrase denaturation are highly scanning-rate dependent, which indicates that the thermal denaturation is under kinetic control. [9][10][11][12]

2 C

Finally, according to the two-state irreversible model, the scanning rate effect on the DSC transitions is given by: 13???? ? ?? ?? ?? 2 ? ? = ?????????? ? ?? ???? ?? ?**(2)**

Scan rate and pH dependence of carbonic anhydrase stability were determined by measuring the T m values at various scan rates and pH to generate a DSC curve. Models relating the scan rate-dependent increase in protein thermal stability to association constants require an accurate knowledge of the thermodynamics of protein stability. Thus, carbonic anhydrase stability was studied by MD simulation of differential scanning calorimetry (DSC), giving a complete thermodynamic description of the Gibbs free energy, calorimetric enthalpy, and heat capacity of unfolding . [14][15][16][17][18][19][20] We chose to conduct the study on carbonic anhydrase-a protein commonly used as a model for biophysical and physical-organic studies by us and others. [21][22][23][24] Thermal denaturations of carbonic anhydrase have been examined using simulation of differential scanning calorimetry by molecular dynamic simulation. Thermal denaturation has never been directly examined previously theoretically. Carbonic anhydrase have been examined as a function of scan rate. In this work we have found denaturation of protein. Finally, it is noteworthy that CA could become important biotechnological materials. Therefore investigation of thermal stability of the CA has not only academic, but also applied, interest. This model correctly predicts scan rate and pH-dependent changes in T m of BCA. These T m values are then compared to those obtained by experimental methods that are in good agreement.

3 II. Experimental a) Molecular dynamics simulations

All MD simulations were carried out using the GROMACS 4.5.0 package together with the GROMOS96 force field in parallel by the BirgHPC. The starting structure of bovine carbonic anhydrase was constructed based on the X-ray crystal structure of BCA (PDB ID: 1CA2, Fig. 1). The simple point charge (SPC) model was used to describe water. A different time step was used to integrate the equations of motion with the Verlet algorithm. A non bond pair list cutoff of 0.9 nm was used. Temperatures and pressures were controlled by a Nose-Hoover thermostat and Parrinello-Rahman barostate with coupling constants of 0.1 and 0.5, respectively. For all simulations, the atomic coordinates were saved every 50 ps for analysis. A cubic simulation box of the volume 321 nm 3 was made and then water molecules were randomly added into the simulation box and initial configurations were minimized using steepest descent algorithm with 5000 integration step. BirgHPC (Bioinformatics Research Group High Performance Computing) which is a free Linux Live CD distribution based on Pelican HPC and Debian Live including 56 processors was also used for our simulations. BirgHPC has been developed to create high-performance clusters for bioinformatics and molecular dynamics studies using any Local Area Network (LAN)-networked computers. The latest versions of GROMACS 4.5.0 was run in parallel by the birgHPC.

Variations of temperature were adjusted in mdp file. In order to study thermal denaturation, temperatures varied in the range of 273 to 405 K to calculate the stability of the protein. Simulated annealing is a special case of MD or MC simulation, in which the temperature is gradually reduced during the simulation. Often, the system is first heated and then cooled. Thus, the system is given the opportunity to surmount energetic barriers in a search for conformations with energies lower than the localminimum energy found by energy minimization. One of the applications of MD is involved in utilization of MD, often with simulated annealing protocols, to determine or refine structures with data obtained from experiments. 25 Creation of different scan rates by variation of temperature in each step was adjusted in mdp file by simulated annealing. DSC calculations were performed, keeping a constant pressure of 1 atm over the simulation. Different scanning rates within the range 0.010-0.02 K/ps were employed. In order to simulate scan rate of 0.01 K/ps, temperature increased from 250 to 450 K during 20 ns. It means that in each 500 step temperature increased 5 K. For the scan rate of 0.0125, initial and final temperature was 225 to 475 and in each 2000 ps step temperature increased 25 K, for scan rate of 0.015, initial and final temperature was 270 to 570 and in each 1000 ps step temperature increased 15 K and for scan rate of 0.02, initial and final temperature was 150 to 545 K and in each 250Ps step, temperature increased 5 K. In order to simulate lower pH (acidic form), all carboxyl groups (COO-) were protonated and converted to

COOH by definite tools implemented in GROMACS. In addition, at neutral pH, we used the crystal structure (pdb code; 1CA2) downloaded from the protein data bank. All MD simulations for comparison of pH effect were carried out for three scan rates of 0.01, 0.0125 and 0.015 K/ps during 20 ns. Variations of RMSD, CD 222,nm , hydrogen bond (HB), solvent accessible surface (sas) area, radius of gyration and Hamiltonian energy were also calculated.

4 b) Analyses

The conformational changes of the protein during MD simulations were monitored by the rootmean-square derivations (RMSD) with its X-ray structure as a reference. The RMSD value, a measure of molecular mobility, is calculated by translating and rotating the coordinates of the instantaneous structure to superimpose the reference structure with a maximum overlap. The RMSD is defined as???????? = ? ? ?? ?? (?? ?? ??? ?? 0) ?? ??=1 ? ?? ?? ?? ??=1**(3)**

Where m i is the mass of atom i. r i and r i o are the coordinates of atom i at a certain instance during MD simulations and at its reference state, respectively. RMSDs were calculated, for the trajectories, from the starting structures of carbonic anhydrase as a function of time. In the all systems, RMSDs reach a stable value within the first nanosecond of all the analyses.

The simulation trajectories were analyzed using several auxiliary programs provided with the GROMACS package. The programs include g_energy that calculate all energies such as Hamiltonian, total pressure, box volume etc. and displays averages. Calculation of the heat capacity at constant pressure (C P) can be used to directly compare with experimental DSC results. In general, a straightforward but difficult method to accomplish this is to use the trajectory energy fluctuations to determine the C P , directly. From a trajectory, one can determine the trajectory average energy and the enthalpy of each step i H i . to determine the heat capacity: ???? is the average value of enthalpy, ?? i is enthalpy of ith state and k is bultzman constant. It is important to note that in all calculations of DSC profiles, heat capacity has been subtracted from solvent heat capacity and it has been done for all scan rates calculation. **??**26][27][28][29][30] ?? = ?? + ????**(4)**C P = ?H 2 ???H? 2 RT 2**(5**

5 III. Results and Discussion

6 a) Thermal denaturation (Tm of carbonic anhydrase)

We used the MD simulation of differential scanning calorimetry (DSC) to monitor the thermal unfolding of BCA. The melting temperature of BCA in temperature range from 273 to 405 K was about 345 K which is near the experimental value. 7,[31][32] Fig. 2 shows DSC profile or representative thermo gram in several temperatures for unfolding of CA protein. Thermodynamic of CA unfolding was studied by DSC using various temperature conditions previously demonstrated to give T m using MD simulation. This observation was consistent with experiments, where the T m for BCA was estimated to be 343 K. Here, we describe simulations of CA at various temperatures, focusing on the unfolding process. Increasing temperature accelerates protein unfolding without changing the pathway of unfolding. Temperature is believed to alter the structure of hydrogen bonds network of protein in water and increase the SAS and protein size and decrease the intermolecular hydrogen bond, electrostatic and hyrophobic interactions of proteins. Structure parameters were obtained from MD simulation for each temperature and results were averaged. The structure information such as solvent accessible surface, inter molecular hydrogen bonding (HB) between CA and solvent molecules, gyrate radii (Rg), CD 222,nm and RMSD were obtained and averaged at each temperature. Fig. 3 that increase by temperature due to unfolding of protein while intermolecular hydrogen bond, hydrophilic SAS and CD 222,nm decrease with temperature. The variation of surface area during 20 ns time evolution was significant and obtained. Fig. 3a shows the averaged value of total solvent accessible surface area of CA in 20 ns time interval in the temperature ranged from 273 to 405. These figure shows increase of surface by increasing temperature. This proves that the CA structure has been unfolded and it is obvious that surface area of CA in system with 273 K is less than higher temperatures. Fig. 3b and 3c show average solvent accessible surface area of hydrophobic and hydrophilic part for CA in 20 ns time interval vs. temperature respectively. Solvent accessible surface area of hydrophobic part and total surface area of CA is more in the presence of higher temperatures. This proves that the CA structure has been unfolded more in the presence of higher temperature so the surface areas of protein increase due to unfolding process. Totally temperature cause more interaction and structural change in CA and this result is in good agreement with experiment data.

Root mean square deviation (RMSD) of the CA for all temperatures was obtained. Fig. 3d shows the average of CA RMSD in the 20 ns time interval in all temperatures. The figure shows that CA has more structural changes (RMSD) in the higher temperature. Fig. 3e shows the RMSD of CA in the 20 ns time interval for 273 K which has been selected randomly. It shows that the system reaches a stable state after about 5 ns. Fig. 3f shows the average values of radius gyration of CA in 20 ns time interval vs. temperature. This figure shows increase of radius gyration of CA in the presence of higher temperature. This result is in good accordance with increase of hydrophobic and total surface area of CA. This proves that the CA structure has been unfolded and it is obvious that surface area and therefore radius gyration of CA in system with 405 K temperature is increased more due to more increase of CA surface area. This proves that the CA structure has been unfolded more in the higher temperatures.

Fig. 3g shows averaged value of intermolecular hydrogen bond of CA in 20 ns time interval vs. temperature. Reduction of this parameter proves that the CA structure has been unfolded in high temperatures and this result is in good agreement with above results. The number of intermolecular hydrogen bonds decreased in the process of increasing temperatures and protein structure is unfolded and denatured. The number of hydrogen bonds between protein-solvent is also increased due to unfolding of protein in high temperatures and is not shown here. In some of the temperatures these trends are reverse due to formation of helix and beta sheets. Decrease of SAS may be due to formation of beta sheet and helix structures. In the case of the thermal denaturation of CA, however, the DSC transitions are strongly scanning-rate dependent. This indicates that the state of the transitions at any given temperature (within the denaturation range) depends on the time required to reach that temperature; therefore, the thermal denaturation of CA is under kinetic control and the DSC transitions are distorted by changing scan rates. The thermal denaturation of several soluble proteins such ac CA has been found to conform to this model. Fig. 4 shows temperaturedependence of excess heat capacity for CA at several scan rates. As it may be seen in this figure the traces were scan rate-dependent. It may be concluded, therefore, that the thermal denaturation of CA is kinetically controlled under the conditions employed. The denaturation shapes of the transitions agree in general with the results of Brouillette et al. 19 The results displayed in this figure show, however, that the DSC transitions are highly scanning rate-dependent. The curves of C P , vs T are shifted to higher temperature with increasing scan rate (Fig. 3). This illustrates the importance of varying the scan rate or the rate of unfolding on the profiles. The temperature of maximum C P , (T max) obeys the equation (2). As scan rate increases further, the profile shifts to higher temperature which is obvious in our results. Fig. **??** shows scan rate dependence of CA denaturation. The area under a DSC curve normally yields the calorimetric enthalpy of denaturation of the protein, Î?"H. The position of the peak yields the T m for denaturation. CA thermally denature as a single peak in DSC. The fitted values for the calorimetrically determined apparent thermodynamic parameters for the denaturation of the CA in each scan rate are shown in Table 1.

7 C

Where Î?"S Tm is the entropy of unfolding at the melting temperature obtained by: ??? ?? ?? = ??? ?? ?? (7) Since the Gibbs free energy of unfolding at T m is equal to zero: ??? ?? ?? = 0

The enthalpy of melting Î?"H was determined by integrating the area under the peak. It is common practice to determine the enthalpy of protein unfolding by differential scanning calorimetry (DSC). This approach limits the available data to higher temperatures where most proteins denature, ranging from about 273 to 405 K.

Proteins have to be artificially destabilized to reduce their melting temperature. One of the most common means to destabilize a protein is to reduce the pH. It was shown using CA as a model protein that the enthalpy of unfolding determined by simulated DSC at various pHs is equal to the enthalpy of unfolding determined by isothermal calorimetric titration of the protein with acid. 9 The free energy of denaturation at a reference temperature T was calculated.) Versus 1/T m , where each data point refers to one of the four scan rates used Fig. 7 shows Arrhenius plots of the scan-rate dependent changes in T m . The slopes of these lines provide the apparent activation energies of denaturation. 31 Since Sanchez-Ruiz and co-workers and other authors 33,34 reported more or less equivalent activation energies with each of the different Arrhenius-based analysis methods, we have demonstrated only one of these methods here (in Figure 7). T m values for CA were found to be scan-rate-dependent. Kinetic activation energies for irreversible denaturation were derived from the scan rate dependence of the DSC transitions using Arrhenius plots as described by Sanchez-Ruiz et al., 18 and these plots of ln(scan rate/T m 2) against 1/T m are also given in Fig. 7. The slopes of these plots provide the activation energies for irreversible denaturation, which are 338.7/R kJ/mol for CA which R is gas constant.

8 c) pH Effect

Further exploration of the denaturation of CA was also performed as a function of scan rate in acidic pH. Protein stability could be altered dramatically by changing pH or by changing pH in a scan rate mode. For example, lowering the pH to acidic value lowered the T m by 8, 16, or 25 °C experimentally. 9 Here we used the MD simulation and showed thermal denaturation of the sample was pH and scan-rate dependent. We have made DSC studies into the thermal stability of CA within different pH at neutral and acidic pH values and different scan rates and compared these calculation results to those of the native protein in experimental condition. Effects of the pH on T m of both scan rate and thermal denaturation can be taken as an interesting result. The scan rates used were 0.01, 0.0125 and 0.015 K/ps in a lower acidic media which all carboxyl groups (COO -) were protonated. Fig. 8 shows a typical DSC profiles for a protein in three different scan rates and acidic media. Melting temperature as a function of pH for different scan rates was obtained. T m values in each scan rate for acidic condition are presented in Table 1. Thermal stability was essentially pH dependent. In lower pH, the T m gradually decreased as the pH became more acidic, as expected for a protein that binds protons more tightly in the non-native state. 15

9 IV. Conclusion

BCA is a protein that is commonly used as a model for biophysical studies, and this work provides additional information on the effect of different denaturants of scan rate, pH and temperature on the Increasing the scan rate in neutral media improves the stability of BCA. Calculations were performed using molecular dynamics simulation with four scan rates. The calorimetric traces were found to be scan-ratedependent under the conditions employed.

10 V. Acknowledgments

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Figure 1: Fig. 1 :

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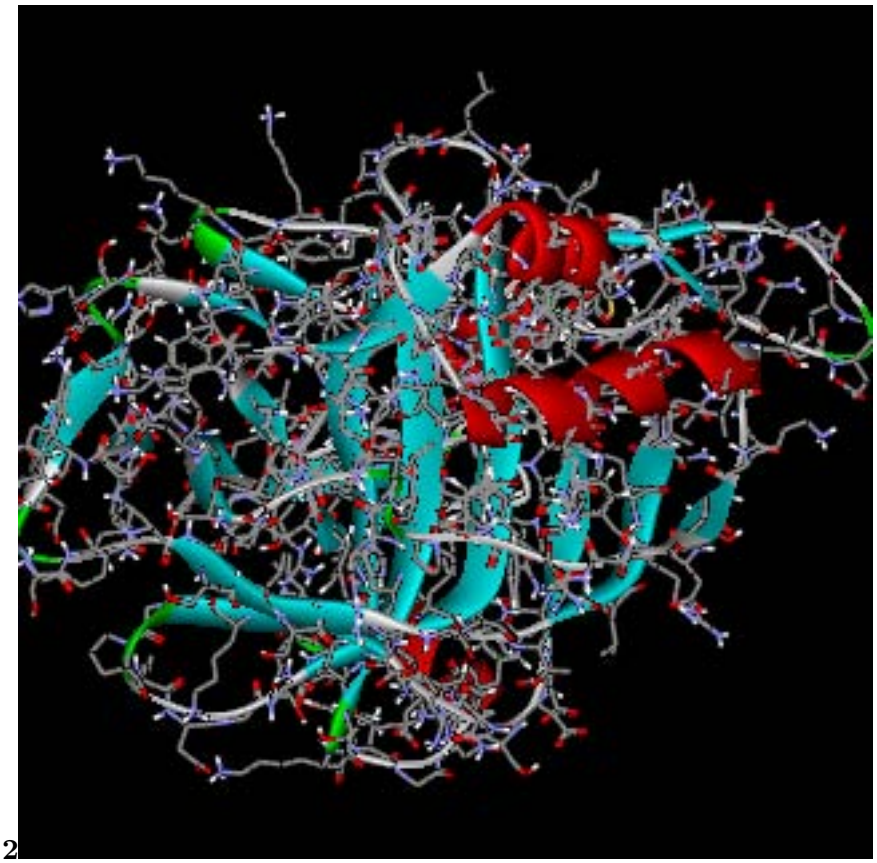
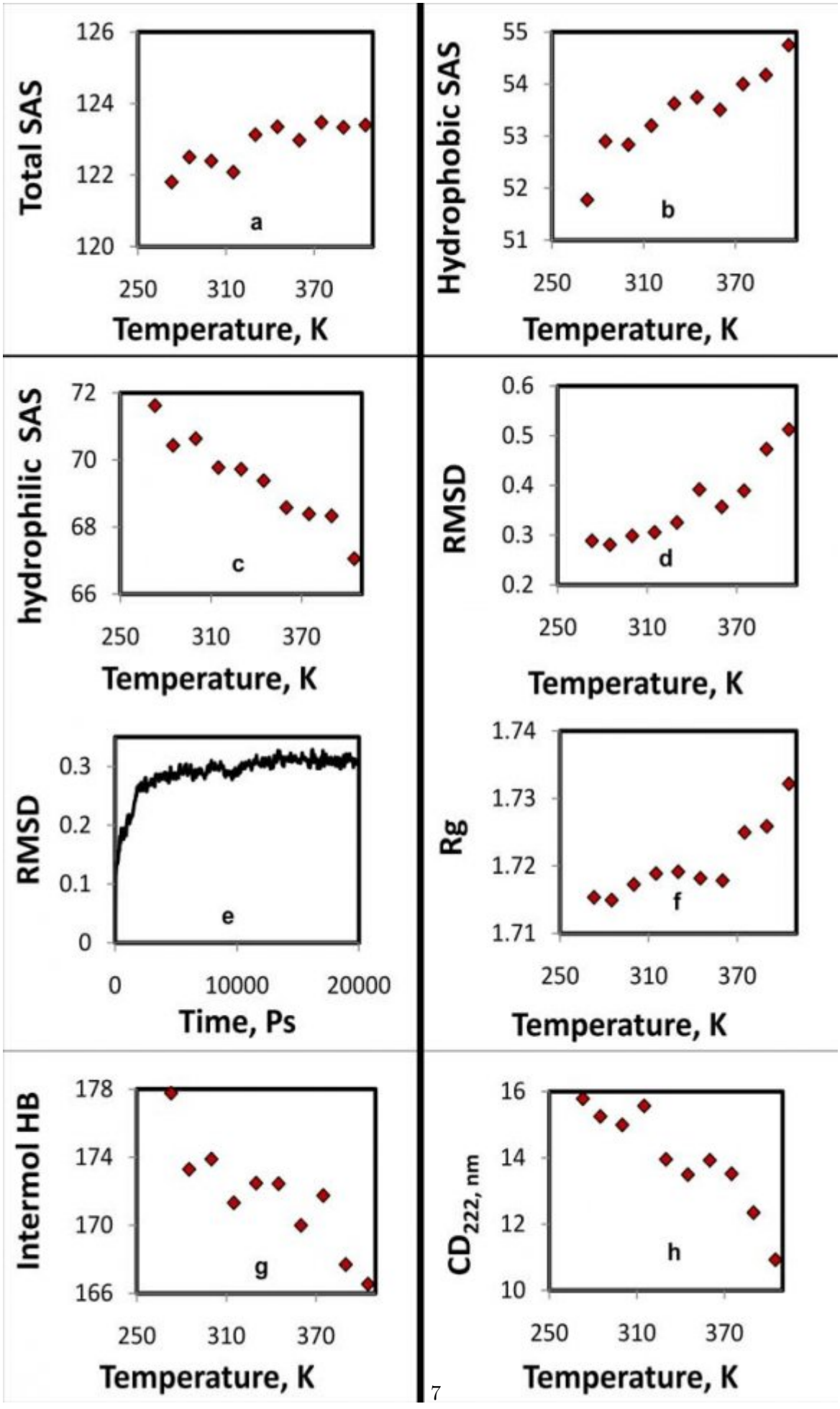


Figure 2: Fig 2 :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124
122
120
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250
310
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Temperature, K
Hydrophobic SAS
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54
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b
250
310
370
Temperature, K
hydrophilic SAS
72
70
68
66
c
250
310
370
Temperature, K
RMSD
0.6
0.5
0.4
0.3
0.2
d
250
310
370
Temperature, K
RMSD
0.3
0.2
0.1
0
e
0
10000
20000
Time, Ps
Rg
1.74
1.73
1.72
1.71
f
250
310
370
Temperature, K
Intermol HB
178
174
170
166
g
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Temperature, K
CD222, nm
16
14
12
10
h
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Temperature, K

Figure 3:

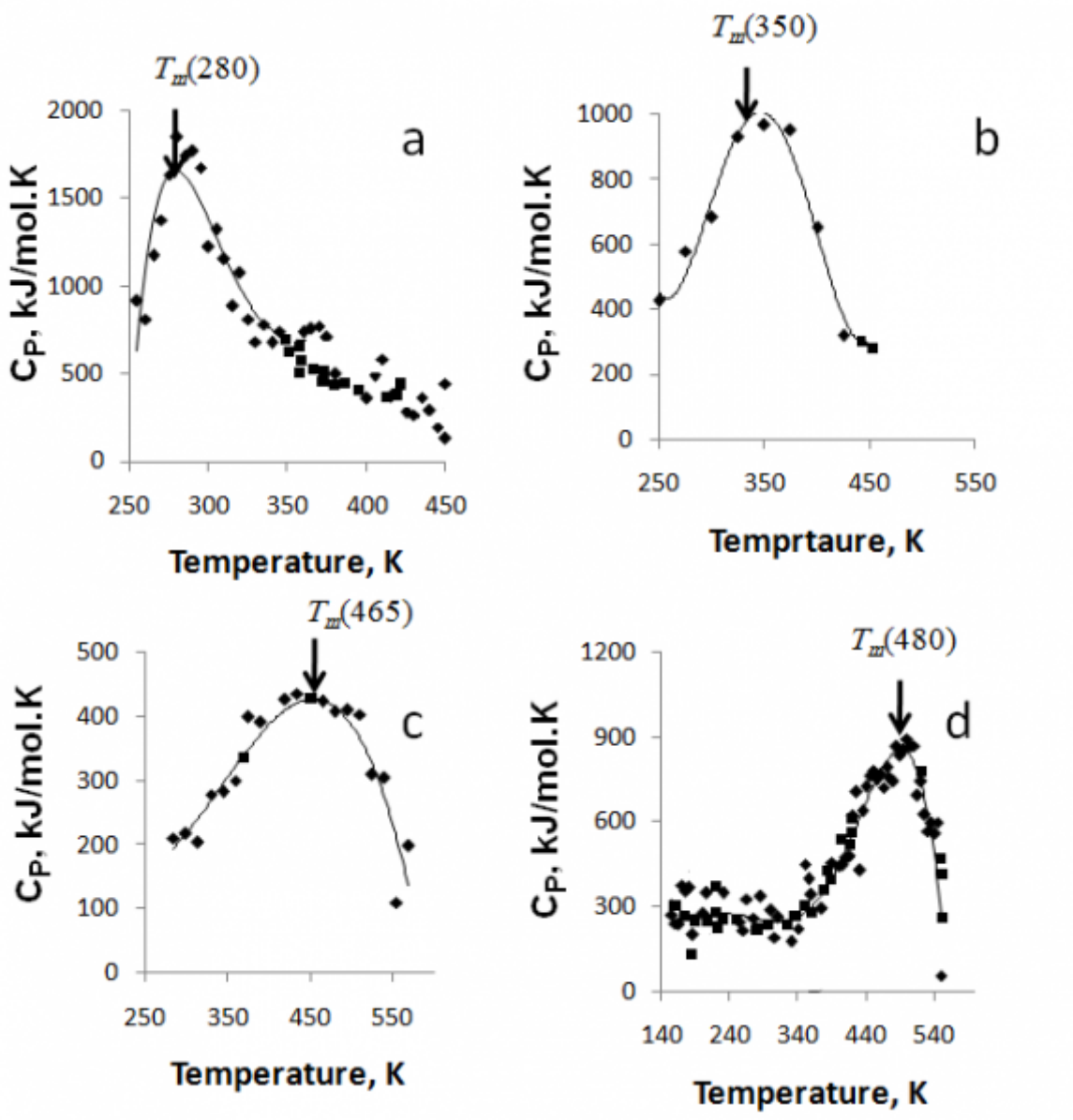

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Figure 4: Fig. 3h showsFig. 3 :

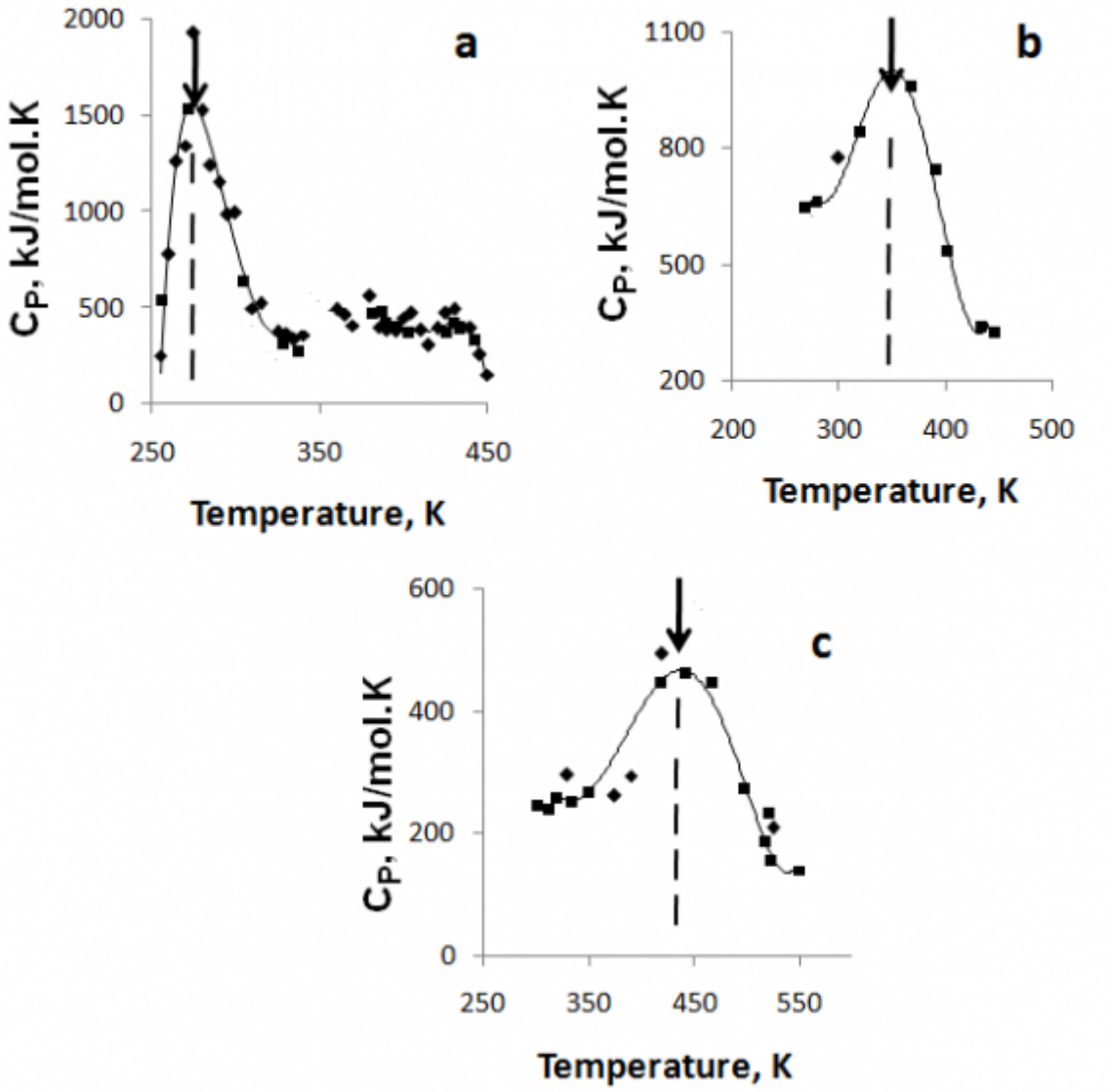

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Figure 5: Fig. 4 :

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Figure 6:

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Figure 7: Table 1 :

The calorimetric enthalpy of unfolding was calculated as the area of the unfolding peak, normalized to the molar protein concentration. The unfolding enthalpy was linearly proportional to T m , and the slope of H vs T m yielded C p .

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