Global Journal of Researches in Engineering: C
Chemical Engineering
Volume 15 Issue 3 Version 1.0 Year 2015
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN: 2249-4596 & Print ISSN: 0975-5861

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

By Maryam Ghadamgahi & Davood Ajloo
Damghan University, Iran

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 T_m, 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 (T_m) varied in the range 353-438 K and was higher for higher scan rates systems and lower for acidic condition.

Keywords: carbonic anhydrase, melting temperature, molecular dynamics simulation, scan rate, simulated annealing.

GJRE-C Classification: FOR Code: 030599, 030505

Strictly as per the compliance and regulations of:

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

Maryam Ghadamgahi [α] & Davood Ajloo [σ]

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 T_m, 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 (T_m) 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 T_m value.

Keywords: carbonic anhydrase, melting temperature, molecular dynamics simulation, scan rate, simulated annealing.

I. Introduction

Carbonic 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.[1-3]

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:

$$\frac{scan\ rate}{T_m^2} = \frac{AR}{E_a} e^{-E_a/RT_m} \qquad (1)$$

Such that a plot of ln(scan rate/T_m^2) against $1/T_m$ 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.[9-12] All above results are confirmed by a MD simulation study and a good agreement was found for the DSC data with experimental values.

Author α σ : School of Chemistry, Damghan University, Damghan, Iran. e-mail: ghadamgahi63_2010@yahoo.com

Finally, according to the two-state irreversible model, the scanning rate effect on the DSC transitions is given by:[13]

$$ln\left(v/T_m^2\right) = const - E/RT_m \quad (2)$$

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 .[14-20]

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.[21-24]

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.

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.

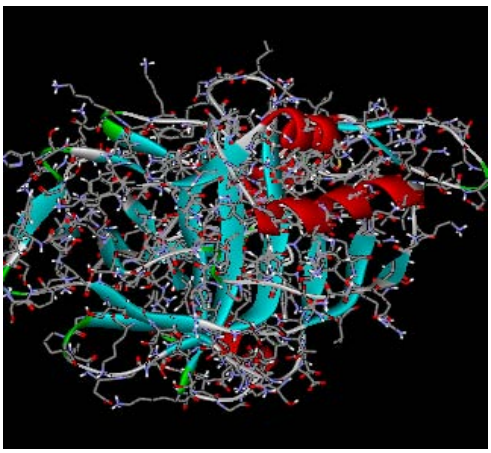

Fig. 1 : Protein structure (1CA2) taken from protein data bank www.RCSB.org

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 local-minimum 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.

b) Analyses

The conformational changes of the protein during MD simulations were monitored by the root-mean-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

$$RMSD = \sqrt{\frac{\sum_{i=1}^{N} m_i (r_i - r_i^0)}{\sum_{i=1}^{N} m_i}} \quad (3)$$

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:

$$H = E + PV \quad (4)$$

$$C_P = \frac{\langle H^2 \rangle - \langle H \rangle^2}{RT^2} \quad (5)$$

$$\langle H \rangle = \frac{\sum_V \sum_j H_i e^{-E_{Vj}/kT} e^{-pV/kT}}{\sum_V \sum_j e^{-E_{Vj}/kT} e^{-pV/kT}} \quad (6)$$

$\langle H \rangle$ is the average value of enthalpy, H_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.[26-30]

III. Results and Discussion

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.

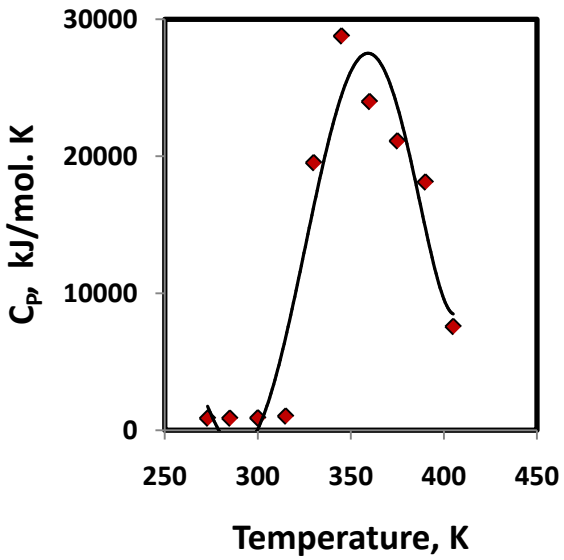

Fig 2 : DSC profile for the unfolding of BCA protein

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 show parameters of total SAS, hydrophobic SAS, radius of gyration and RMSD

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.

Fig. 3h shows averaged $CD_{222,nm}$ of CA by molecular dynamics vs. temperature this figure shows the helicity as $CD_{222,nm}$ which decreases in the high temperatures due to unfolding and denaturation of protein.

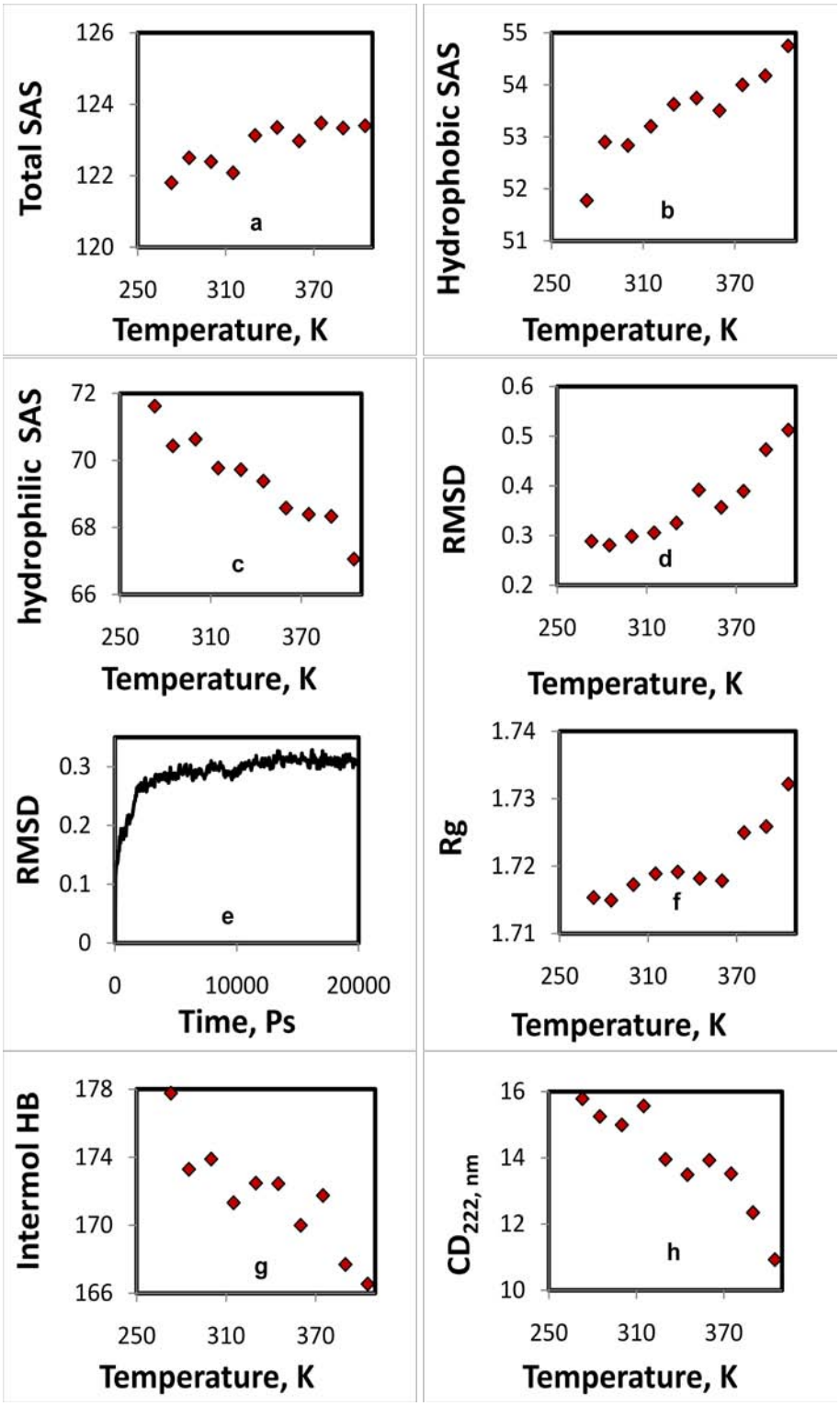

Fig. 3 : a) averaged total solvent accessible surface area of CA by molecular dynamics b) and c) averaged solvent accessible surface area of hydrophobic and hydrophilic part of CA by molecular dynamics, respectively d) averaged RMSD by molecular dynamics for CA e) Calculated RMSD by molecular dynamics for CA in the 273 K f) averaged radius gyration of CA by molecular dynamics g) averaged inter molecular hydrogen bond of CA by molecular dynamics h) averaged CD222,nm of CA by molecular dynamics vs.temperature

b) Scan rate dependence

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 temperature-dependence of excess heat capacity for CA at several scan rates.

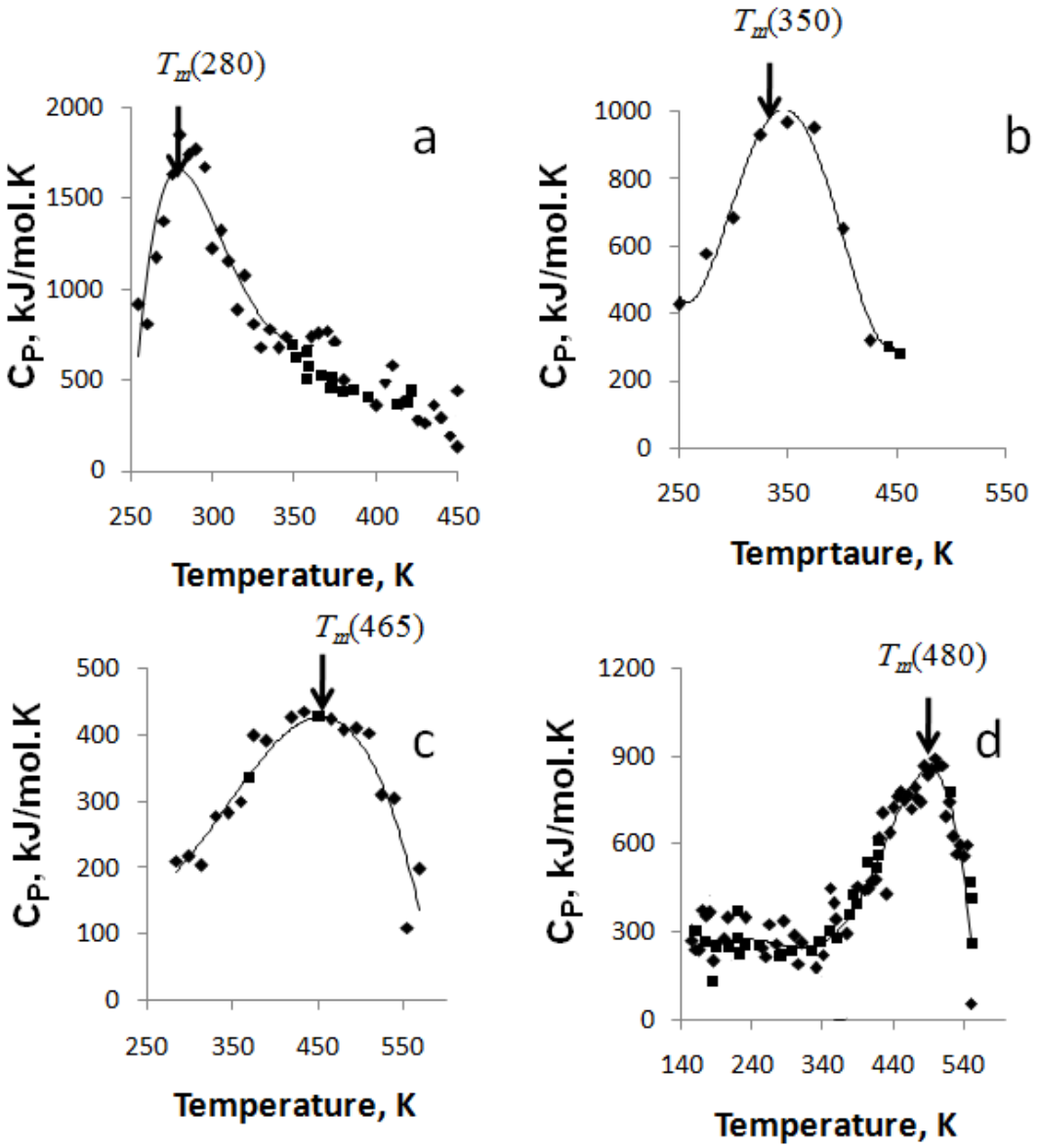

Fig. 4 : Characteristic heat-capacity curves for CA, with four scan rates a) 0.01, b) 0.0125, c) 0.015 and d) 0.02 K/ps

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. 5 shows scan rate dependence of CA denaturation.

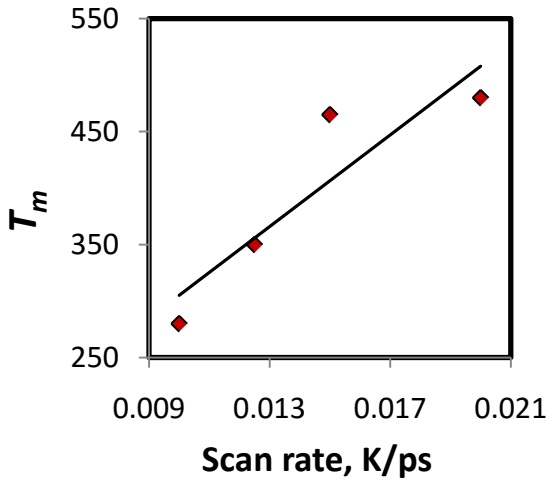

Fig. 5 : Scan rate dependence of thermal denaturations of CA

Calculated RMSD by molecular dynamics for CA for studied scan rates is depicted in Fig. 6. It shows that all of the systems reach a stable state.

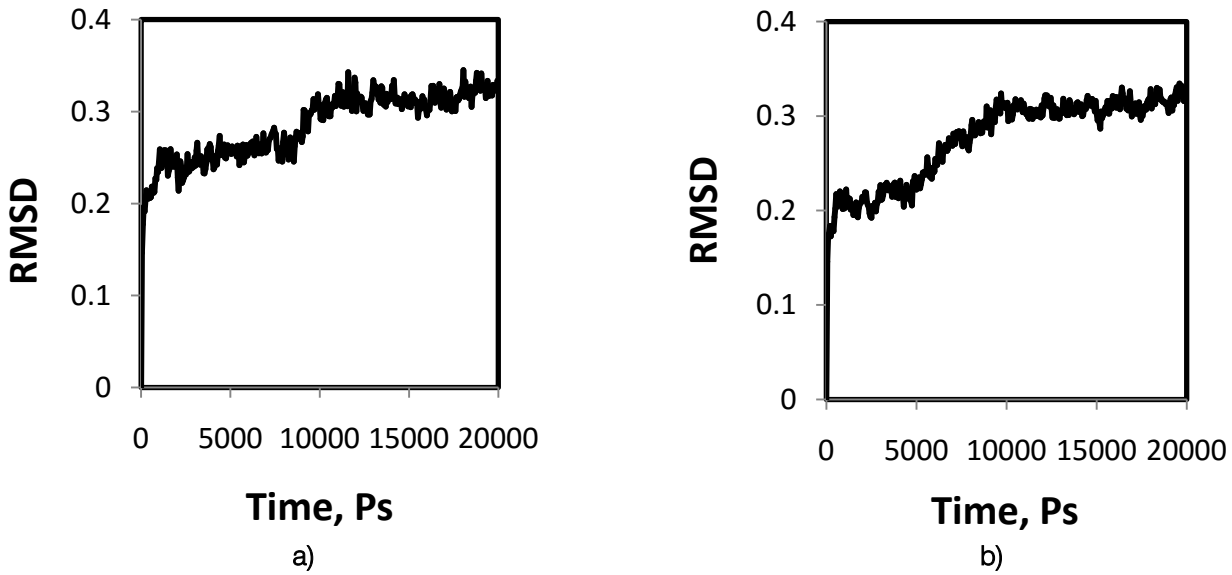

Fig. 6 : Calculated RMSD by molecular dynamics for CA for scan rates of a) 0.01 and b) 0.0125K/ps

The area under a DSC curve normally yields the calorimetric enthalpy of denaturation of the protein, ΔH. 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.

Table 1 : T_m and thermodynamic parameters of CA denaturation in different scan rates.

Scan rate	ΔH(neutral)(kJ/mol)	ΔS(kJ/mol)	T_m(neutral)	T_m(acidic)
0.01	62207	222.17	280	278
0.0125	76986	219.96	350	341
0.015	78914	169.71	465	415
0.02	79638	165.91	480	Not done

Where ΔS_{Tm} is the entropy of unfolding at the melting temperature obtained by:

$$\Delta S_{T_m} = \frac{\Delta H}{T_m} \quad (7)$$

Since the Gibbs free energy of unfolding at T_m is equal to zero: $\Delta G_{T_m} = 0$

The enthalpy of melting ΔH 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.

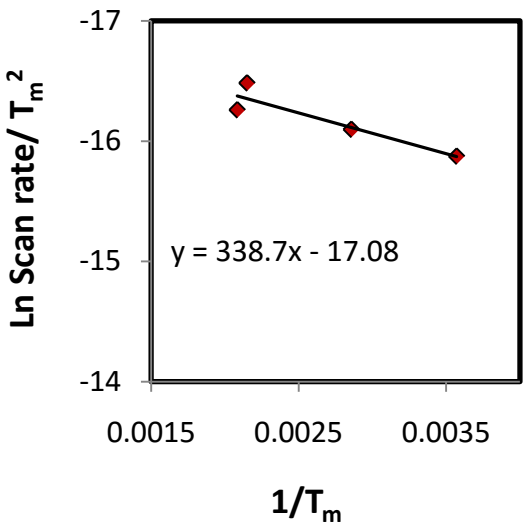

Fig. 7 : Plot of Ln(V/T_m^2) *Versus* 1/T_m, where each data point refers to one of the four scan rates used

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.

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.

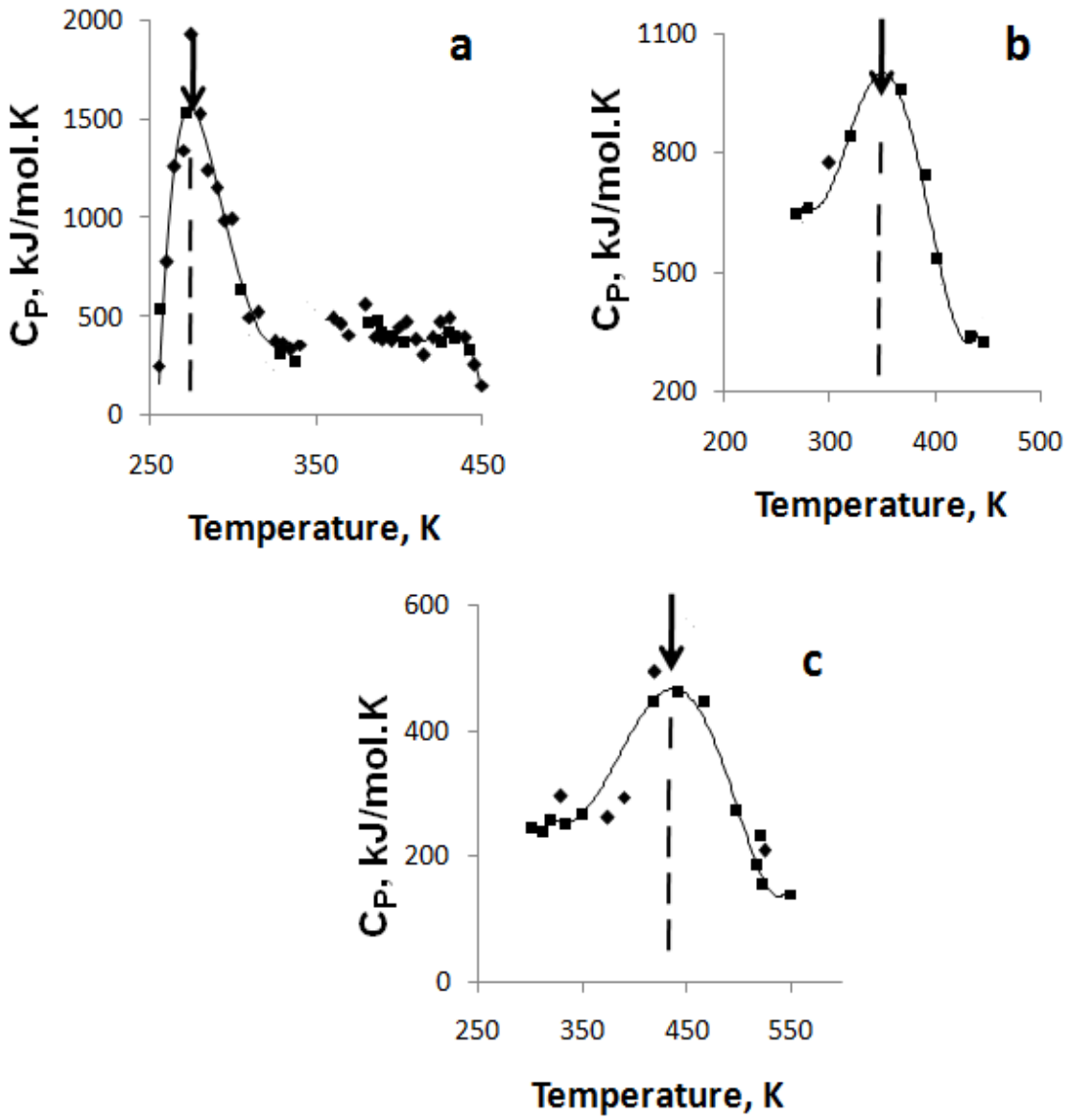

Fig. 8 : DSC profiles for CA in three different scan rates in created acidic media

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]

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.

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 stability of BCA. It demonstrates that increasing the scan rate in acidic media reduces protein stability. 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-rate-dependent under the conditions employed.

V. Acknowledgments

The financial support of Damghan University is acknowledged.

References Références Referencias

1. Sly WS H P 1995 *Annu Rev Biochem* 64 375-401
2. Lindskog S 1997 *Pharmacol Ther* 74 1-20
3. Thoms S 2002 *J Theor Biol* 215 9399-404
4. Bruylants G, Wouters J and Michaux C 2005 *Curr Med Chem* 1 2011-2020
5. Vermeer A W 2000 *Biophys J* 79 2150–54

6. Vermeer AW and Norde W 2000 *Biophys J* 78 394–404
7. Baranauskiene L, Matuliene J and Matulis D 2008 *J Biochem Bioph Meth* 70 1043-1047
8. Gorania M, Seker H and Haris P I 2010 *Conf Proc IEEE Eng Med Biol Soc* 10 820-3
9. Karantzeni I, Ruiz C, Liu C C and Licata V J 2003 *Biochem J* 374 785–92
10. Davoodi J, Wakarchuk W W, Surewicz W K and Carey P R 1998 *Prorein Sci* 7 1538-1544
11. Lepock J R, Ritchie K P, Kolios MC, Rodahl A M and Heinz K A 1992 *J Biochem* 31 12706-712
12. Zhadan G G and Shnyrov V L 1994 *Biochem J* 299 731-3
13. Galisteo ML and Sanchez-Ruiz J M 1993 *Eur Biophys J* 22 25-30
14. Freire E, van Osdol W W, Mayorga O L and Sanchez-Ruiz J M 1990 *Annu Rev Biophys* 19 159-188
15. Gahsteo M L, Mateo P L and Sanchez-Ruiz J M 1991 *Biochem* 30 2061-66
16. Ruiz-Sanz J, Ruiz-Cabello J, Mateo P L and Cortijo M 1992 *Eur Biophys J* 21 71-76
17. Sanchez-Ruiz J M 1992 *Biophys J* 61 921-35
18. Sanchez-Ruiz J M, Lopez-Lacomba J L, Cortijo M and Mateo P L 1998 *Biochem* 27 1648-52
19. Sanchez-Ruiz J M and Mateo P L 1987 *Biol Rev* 11 15-45
20. Ana I, Azuaga F S, Esteve P and Pedro L M 1996 *Biochem* 35 16328-16335
21. Gudiksen K L, Gitlin I, Moustakas D T and Whiteside G M 2006 *Biophys* 91 298– 310
22. Krishnamurthy V M, Bohall B R, Semetey V and Whitesides G M 2006 *J Am Chem Soc* 128 5802–12
23. Carlsson U and Jonsson B H 2000 *The carbonic anhydrases: new horizons*, Chegwidden W R, Carter N D, Edwards Y H, Birkhauser B (ed.), pp. 241 –259.
24. Christianson D W and Fierke C A 1996 *Chem Res* 29 331–39
25. Karplus M and McCammon J A 2002 *J Struct Biol* 9 646 – 652
26. http://www.gromacs.org/Documentation/Manual.
27. Ajloo D, Taghizadeh E, Saboury A A, Bazyari E and Mahnam K 2008 *J Biol Macromol* 43 151–158
28. Ajloo D, Hajipour S, Saboury A A and Zakavi S 2011 *Bull. Korean Chem. Soc* 32 3411-3420
29. Dasmeh P, Searles D J, Ajloo D, Evans D J and Williams S R 2009 *J Chem Phys* 131 214503-214507
30. Ghadamgahi M and Ajloo D 2011 *J Porphyr Phthalocya* 15 240-256
31. Matulis D, Kranz J K, Salemme F R and Todd M J 2005 *Biochem* 44 5258-66
32. Brandts J F and Lin L N 1990 *Biochem* 29 6927-40
33. Karantzeni I, Ruiz C, Chin-Chi L and LiCata V 2003 *J. Biochem* 34 785-792.
34. Vogl T, Jatzke C, Hinz H J, Benz J and Huber R 1997 *Biochem* 36 1657–1668