Conformational Dynamics at the Active Site of α-Chymotrypsin and Enzymatic Activity

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The role of dynamical flexibility at the active site of a proteolytic enzyme α-chymotrypsin (CHT) has been correlated with its catalytic activity. The temperature-dependent efficiency of catalysis reveals a bell-shaped feature with a peak at 37 °C, the typical body temperature of homeothermal animals. The overall structural integrity of the enzyme in our experimental temperature range has been confirmed from dynamic light scattering (DLS) and circular dichroism (CD) studies. We have followed the dynamical evolution at the active site of CHT with temperature using picosecond-resolved fluorescence anisotropy of anthraniloyl probe (covalently attached to the serine-195 residue) and a substrate mimic (inhibitor) proflavin. The conformational dynamics at the active site is found to have a distinct connection with the enzyme functionality. The conformational flexibility of the enzyme is also evidenced from the compressibility studies on the enzyme. The site selective fluorescence detected circular dichroism (FDCD) studies reveal that the conformational flexibility of the enzyme has an effect on the structural perturbation at the active site. We have also proposed the possible implications of the dynamics in the associated energetics.

Introduction

It is now well established that the conformational flexibility of biomolecules is essential for many biological events. This has triggered research on biomolecular dynamics. A survey of the existing literature reveals that the studies on biomolecular dynamics mainly rest on the pillars of molecular simulations[1,2] (theoretical) and NMR[3] (experimental) measurements along with a few studies on neutron scattering.[4] Theoretical studies suggest that low frequency (1−200 cm^{-1}) motions in biomolecules have biological significance, whereas NMR techniques bring out the importance of slow collective motions of biomolecules for their activity. However, these two techniques probe two different time zones of macromolecular dynamics; simulation studies mainly concentrate on subpicosecond/picosecond scale molecular motions, whereas NMR techniques explore macromolecular motions in the millisecond/microsecond regime. As a result, the intermediate nanosecond dynamics in biomolecules is less explored and understood. Fluorescence spectroscopic techniques can prove useful to understand biomolecular dynamics over a broad time scale. The fluorescence spectroscopic studies[5−7] on DNA dynamics by Murphy et. al provide elegant examples.

The role of protein conformational dynamics in enzyme catalysis is a relatively new field of research. In a pioneering work, Kern et al.[8] have proposed that the intrinsic protein dynamics is associated with its catalytic efficiency. In the study, they have used NMR relaxation dispersion measurements to compare the motions of prolyl cis−trans isomerase cyclophilin-A in the native state with those during turnover. The results show that the dynamics of the protein that are crucial for the catalytic activity are also present in the native protein and are hence the intrinsic dynamics of the protein. In another study,[9] Loria et. al have shown that the conformational mobility of a histidine residue, distant from the active site of the enzyme α-lytic protease, is essential in coordinating the motions involved in the rate-limiting enzymatic step. The role of collective motions in enzyme activity is proposed in the study by Karplus et al.[2] This has been followed by a recent theoretical study[10] on the effect of protein conformational dynamics on the enzyme-catalyzed reactions, where two-dimensional reaction free energy surfaces of the catalytic reaction have been constructed, using the protein conformational coordinate as an axis. In the present work, we attempt to use fluorescence spectroscopic studies to explore the internal dynamics at the active site of a proteolytic enzyme, α-chymotrypsin.

α-Chymotrypsin (CHT) is a proteolytic enzyme associated with the hydrolysis of peptide bonds in the mammalian digestive system. The structure of CHT,[11,12] the specificity of substrates,[13] the mechanism of hydrolysis, and the energetics[14,15] are well-documented in the literature. The studies on the enzymatic activity of CHT identify a catalytic triad comprising the residues histidine-57, serine-195, and aspartate-102 present in the active site of the enzyme. However, the role of the dynamics of specific protein

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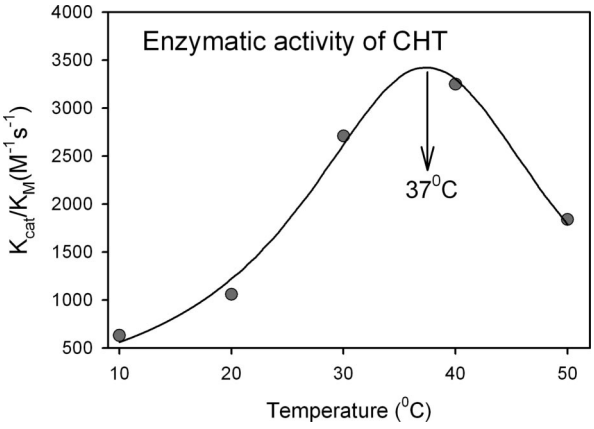

Figure 1. The variation of the catalytic efficiency of CHT with temperature. The solid line is a Gaussian fit.

Table 1. Kinetics and Energetics of CHT Catalyzed Hydrolysis of AMC

temp (°C)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	ΔG^a (kJ/mol)	ΔH (kJ/mol)	ΔS (kJ/mol K)
10	0.66	1.05	631	−5.54	54.68	0.21
20	4.13	3.91	1050	−6.94	45.18	0.18
30	12.8	4.73	2720	−9.46	35.36	0.15
40	21.7	6.79	3250	−10.17	25.21	0.11
50	8.05	4.37	1840			

[a] $\Delta G = -RT \ln[(k_{cat}/K_M)(h/KT)]$.

residues to the catalytic activity of CHT has not been previously explored. In this study we have covalently labeled serine-195 with an anthraniloyl probe and measured the picosecond-resolved anisotropy to explore the dynamics of the serine residue in the active site of CHT and correlate the observed dynamics with the temperature-dependent catalytic efficiency of the enzyme. The conformational flexibility at higher temperatures is also evidenced from temperature-dependent compressibility studies on the enzyme. The impressions of such dynamics on the active site conformation of the CHT have been explored through fluorescence-detected circular dichroism (FDCD) studies. The overall structural integrity of the protein in the temperature range studied has been adjudged from circular dichroism (CD) and dynamic light scattering studies. We have also proposed the possible implications of the dynamics to the associated energetics.

Materials and Methods

Phosphate buffer, bovine pancreatic α-chymotrypsin (CHT), Ala-Ala-Phe-7-amido-4-methylcoumarin (AMC), *p*-nitrophenyl anthranilate (NPA), and proflavin (PF) are from Sigma. All the solutions are prepared in 100 mM phosphate buffer (pH 7.0) using water from a Millipore system. To prepare anthraniloyl CHT (ANT-CHT), we have followed the method described in the literature.[16] A stock solution of NPA in acetonitrile is added in small aliquots to CHT solution at 5 °C with continuous stirring, until the concentration of the probe in solution exceeds that of the protein by an order of magnitude. The reaction is allowed to complete by overnight stirring of the NPA−CHT solution at 5 °C. The resultant solution is filtered and dialyzed extensively to remove the free probe in solution. The PF−CHT solutions are prepared by adding a requisite amount of probe to the protein solution and stirring for 1 h. For the enzymatic activity studies at different temperatures, the concentration of CHT is <1 μM, and the concentration of substrate AMC is varied in the range 50−400 μM. The details of the measurement of enzymatic

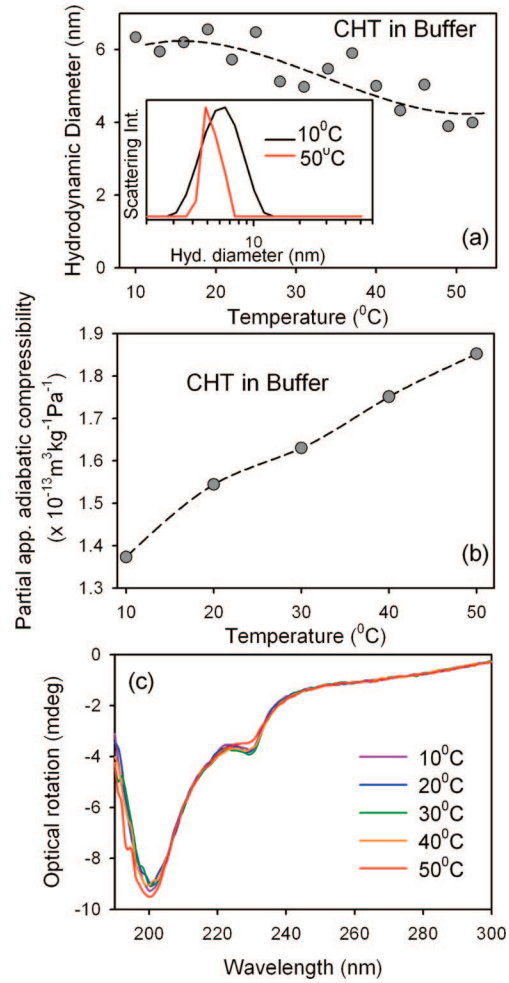

Figure 2. (a) Hydrodynamic diameter and (b) partial apparent adiabatic compressibility of CHT at different temperatures. The broken lines are a guide for the eye. The DLS profiles of CHT at two different temperatures (inset of part a). (c) The circular dichroism (CD) spectra of CHT at different temperatures.

activity and calculation of rate constants are reported elsewhere.[17] The circular dichroism (CD) studies are done with 2 μM CHT solution using a quartz cuvette having a path length of 1 mm. For fluorescence-detected CD (FDCD) studies, the probe protein ratio is maintained in such a way that ensures complete complexation of the probe with the protein and also gives sufficient fluorescence intensity. The path length for the cell used for FDCD studies is 1 cm.

Temperature-dependent steady-state absorption and emission are measured with a Shimadzu UV-2450 spectrophotometer and a Jobin Yvon Fluoromax-3 fluorimeter, respectively, with a temperature controller attachment from Julabo (model F32). The CD and the FDCD studies are done in a JASCO 815 spectrometer with an attachment for the temperature-dependent measurements (Peltier). The deconvolution of the CD signals at different temperature into relevant secondary structures has been done using the CDNN software provided by the vendor. Fluorescence transients are measured and have been fitted by using a commercially available spectrophotometer (LifeSpec-ps) from Edinburgh Instruments [excitation wavelength is 375 nm for anthranilate and 409 nm for PF, and 60 ps is the instrument response function (IRF)] with an attachment for temperature-dependent studies. The observed fluorescence transients are fitted by using a nonlinear least-squares fitting procedure to the function $[X(t) = \int_0^t E(t')\, R(t-t')\, dt']$ comprising convolution of the IRF $[E(t)]$ with a sum of exponentials $[R(t) = A + \sum_{i=1}^{N} B_i e^{-t/\tau_i}]$ with pre-exponential factors (B_i), characteristic lifetimes (τ_i), and a

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Table 2. Temperature Dependent Structure of CHT and Anthraniloyl CHT

	hydrodynamic diameter (nm)		partial app compressibility,	secondary structure					
				CHT			ANT-CHT		
temp (°C)	CHT	ANT-CHT	ϕ_k(M^3 kg^{-1} Pa^{-1} × 10^{-13})	% α-helix	% β-sheet	% random	% α-helix	% β-sheet	% random
10	6.34	5.98	1.37	10	32	33	11	33	28
20	6.55	6.01	1.54	10	32	33	11	32	28
30	4.97	5.02	1.63	10	32	33	11	32	28
40	5.00	5.20	1.75	10	32	33	11	32	28
50	3.89	4.02	1.85	8	33	33	10	33	29

background (A). Relative concentration in a multiexponential decay is finally expressed as $c_n = B_n/\sum_{i=1}^{N} B_i \times 100$. The quality of the curve fitting is evaluated by reduced χ^2 and residual data.

Dynamic light scattering (DLS) measurements are done with a Nano S Malvern instrument employing a 4 mW He−Ne laser (λ_{ex} = 632 nm) and equipped with a thermostated sample chamber. The volume and compressibility of water associated with the protein have been estimated using the density and sound velocity values measured by a density meter (DSA5000 from Anton Parr) with an accuracy of 5×10^{-6} g cm^{-3} and 0.5 ms^{-1} in density and sound velocity measurements, respectively. The details of the DLS measurements and calculation of the partial apparent adiabatic compressibility are illustrated elsewhere.[18] For anisotropy [$r(t)$] measurements, emission polarization is adjusted to be parallel or perpendicular to that of the excitation, and anisotropy is defined as $r(t) = [I_{para} - GI_{perp}]/[I_{para} + 2GI_{perp}]$. G, the grating factor, is determined following a longtime tail matching technique.[19] The time-resolved anisotropy of a fluorophore/probe reveals the physical motion of the probe in a microenvironment. We fit the anisotropy decay by using a multiexponential decay model, where the time constants reflect the rotational correlation time of the probe in the microenvironment.

Results and Discussion

The enzyme CHT catalyzes the hydrolysis of peptide bonds in mammalian digestive systems. In the present study, we have studied the temperature-dependent hydrolysis of the substrate peptide Ala-Ala-Phe-7-amido-4-methylcoumarin (AMC) by CHT. Figure 1 shows the k_{cat}/K_M value for the hydrolysis of AMC. k_{cat}/K_M is a measure of the catalytic efficiency of an enzyme. It is evident from figure 1 that the catalytic efficiency of the enzyme shows a maximum at 37 °C, coinciding with the normal body temperature of homeothermal animals. The rise and subsequent fall of the activity of CHT associated with the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester is reported.[20] In the study, the catalytic activity of the enzyme exponentially falls off after 40 °C and is thought to be associated with the thermal denaturation of the protein. The catalytic parameters associated with the hydrolysis of AMC by CHT are tabulated in Table 1. It is seen that both the k_{cat} and K_M values associated with the enzyme catalysis increase up to 40 °C and then fall. In enzyme catalysis, k_{cat} gives an idea of the turnover rate and K_M gives an indication of the formation of the enzyme−substrate complex. The inverse of the K_M is associated with the possibility of dissociation of the enzyme−substrate complex. The fall in the turnover rate and the increase in association possibility of the enzyme−substrate complex at 50 °C suggest that, at the higher temperatures, even when enzyme−substrate complex is formed favorably, product formation is hindered. The result might indicate conformational perturbation of the enzyme−substrate complex at higher temperatures.

Since proteins change their structure with temperature, the possible association of structural perturbations to the catalytic activity cannot be ruled out. In addition, proteolytic enzymes undergo autocatalysis in solution,[21] the rate of which increases at higher temperatures. The fall in catalytic activity above 37 °C can therefore be due to the decrease in the effective enzyme concentration as a result of autocatalysis. In order to explore the globular tertiary structures of the protein at different temperatures, and also the possibility of autocatalysis, we use the dynamic light scattering (DLS) technique. DLS gives the hydrodynamic diameter of suspended particles in solution. The DLS studies are carried out with different initial concentrations of the protein ranging from 1 to 200 μM. The DLS experiments (Figure 2a) reveal the hydrodynamic diameter of CHT to be ∼6 nm, which agrees well with the dimensions of the hydrated protein.[22] The DLS measurements done with a high concentration of protein (>150 μM) show a prominent peak at ∼1 nm above 50 °C. The size of 1 nm agrees well with the dimensions of an autocatalytic fragment of the CHT.[21] However, such fragments do not appear for lower concentration (<50 μM) of the protein (the concentrations that are used for the measurement of enzymatic activity), at any temperature. This indicates that the fall in enzymatic activity of the protein at high temperatures is neither due to the loss in effective enzyme concentration due to autocatalysis nor due to massive unfolding of the enzyme (characteristic of thermal denaturation).

Figure 2a and Table 1 show the hydrodynamic diameter of CHT ([CHT] = 2 μM) at different temperatures. It becomes evident from the inset of Figure 2a that the average hydrodynamic diameter of the protein decreases from ∼6 nm at 10 °C to ∼5 nm at 50 °C. The decrease in hydrodynamic diameter in proteins at the high temperature can be associated with the thinning of the hydration shell, corresponding to the loss of hydration waters at higher temperatures.[18] The change in the number of hydration waters surrounding a protein is also reflected in the change in the partial apparent adiabatic compressibility (ϕ_k) of the protein solution at different temperatures. Figure 2b shows the ϕ_k at different temperatures. An increase in ϕ_k values at higher temperatures suggests progressive loss of the hydration waters with increasing temperature,[18] resulting in the thinning of the hydration layer and concomitant decrease of the hydrodynamic diameter. However, the absence of scattering peaks at higher hydrodynamic diameters (inset of Figure 2b, representative of the denatured protein[22]) stands against a massive uncoiling of the protein at higher temperature. The DLS results thus confirm that there is no substantial change in the tertiary structure of the CHT that might be associated with the catalytic activity. In order to explore the secondary structure of CHT at different temperatures, the circular dichroism (CD) of the protein is monitored. The CD signal gives an idea about the overall secondary structure

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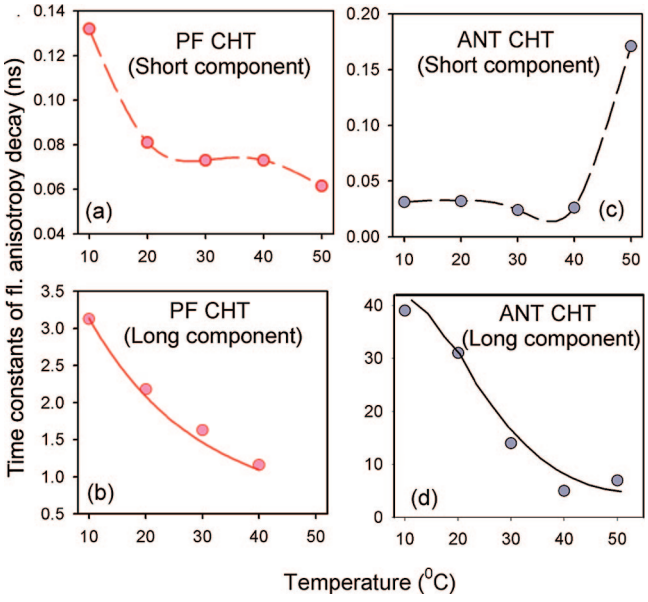

Figure 3. The rotational correlation times of fluorescence anisotropy of PF in CHT (a and b) and anthraniloyl-CHT (ANT−CHT) (c and d). The solid lines are the fit of the Stokes−Einstein−Debye equation; the broken lines are a guide for the eye.

Table 3. Fluorescence Anisotropies of CHT Bound Probes at Different Temperatures

	rotational correlation time (ns)				
	anthraniloyl group			PF	
temp (°C)	τ_1	τ_2	τ_3	τ_1	τ_2
10	39	0.034	12	3.1	0.13
20	31	0.032	9	2.2	0.08
30	17	0.024		1.6	0.07
40	5	0.028		1.1	0.07
50	7	0.171			0.06

of the protein. The deconvolution of the CD signal at 20 °C reveals 10% helix, 32% β sheet, and 33% random coils present in the protein (Table 2), which is consistent with earlier reports.[23] Figure 2c shows the optical rotation of the protein at different temperatures. The essential similarity (Table 2) of the CD spectrum of the protein at different temperatures rules out the possibility of the contributions of major structural perturbations to its catalytic activity.

It is to be noted that a hydration layer, comprised of rigidly bound water molecules, surrounding the protein restricts its conformational flexibility.[24] The increase in the ϕ_k values at high temperatures (Figure 2b and Table 2) suggests loss of rigidly bound water molecules associated with the hydration layer. The loss of the rigidly bound hydration shell at higher temperatures is, therefore, associated with the increased conformational flexibility of the overall protein. The conformational flexibility of the protein is likely to reflect itself in the dynamics of protein residues and substrates attached to the protein.[25] Since serine-195, histidine-57, and aspartate-102 are associated with the catalytic activity of the enzyme, it seems logical that the motions of these residues are involved in the enzymatic activity. It is to be noted that NMR of isotopically labeled proteins to give information about the local dynamics is often not feasible due to the molecular size of the enzymes. The dynamics of covalently labeled fluorophores, estimated from fluorescence techniques, provides perhaps the best alternative for the study of local dynamics in proteins. Here we have monitored the dynamics of the fluorescent anthraniloyl group covalently attached to the serine-195 residue to study the dynamics of the serine residue using the fluorescence anisotropy technique. The dynamics of the substrate is mimicked by proflavin in the active site of the protein. Figure 3a,b reflects the change in the fast and slow components of the rotational relaxation of PF at the protein active site. The slow component in the rotational dynamics of PF represents the microviscosity limited rotation of the probe in the protein cavity. This restricted rotation of PF has been used to calculate the microviscosity of the protein cavity according to the Stokes−Einstein−Debye equation.[26] The microviscosity at the active site of the protein is thus estimated to be 5 cP at 10 °C. The corresponding viscosity of buffer at the same temperature is 1.3 cP. At higher temperatures, the microviscosity decreases, manifesting in the faster rotation of PF. At 50 °C, the microviscosity of the active site becomes buffer-like and PF tumbles off in a single rotational mode. It is to be noted that PF is not detached from the enzyme at this temperature. This is evidenced from the fluorescence lifetime of the probe in the protein at 50 °C (∼4 ns), which is much slower compared to that of the probe in buffer (<60 ps). The shorter (130−30 ps) (Table 3) time component in the decay of fluorescence anisotropy of PF reflects the subslip rotational motion of the probe,[26,27] which decreases monotonically with temperature.

The time constants associated with the decay of fluorescence anisotropy of anthraniloyl group are tabulated in Table 3. The anthraniloyl group is covalently attached to the serine-195 residue and hence follows its motion. The slowest component at all temperatures (Table 3) reflects the global tumbling motion of the protein, which varies with the viscosity of the solvent buffer according to the Stokes−Einstein−Debye equation, as indicated by the solid black line in Figure 3d. This long component is therefore not associated with enzyme activity. The fastest component of ∼30 ps (Table 3) is similar in time scale with the normal modes of protein residues.[1] Recently, the role of picosecond dynamics in the activity of a heme protein has been brought forward.[28] Normal mode analysis and molecular dynamics simulations of protein residues[1] have reported the existence of such low-frequency normal modes with similar time constants in a chymotrypsin-like serine protease. This mode remains constant in the temperature range 10−40 °C. However, at 50 °C, this motion of the serine residue becomes slower (Table 3), indicating lower frequency of vibrations. The loss in catalytic activity at this temperature, coinciding with the slowing down of this component, suggests that the fast dynamics of the serine residue is associated with enzymatic activity.

In addition to this global motion of the protein and the normal mode dynamics, an additional motion having a time constant of 12 ns (Table 3) is associated with the protein residue at lower temperature. The existence of this nanosecond component in the decay of rotational anisotropy of antraniloyl CHT has already been reported.[29] The motion becomes faster (9 ns) (Table 3) at high temperatures and vanishes at 30 °C, where the protein activity is near maximum. This nanosecond component in the protein

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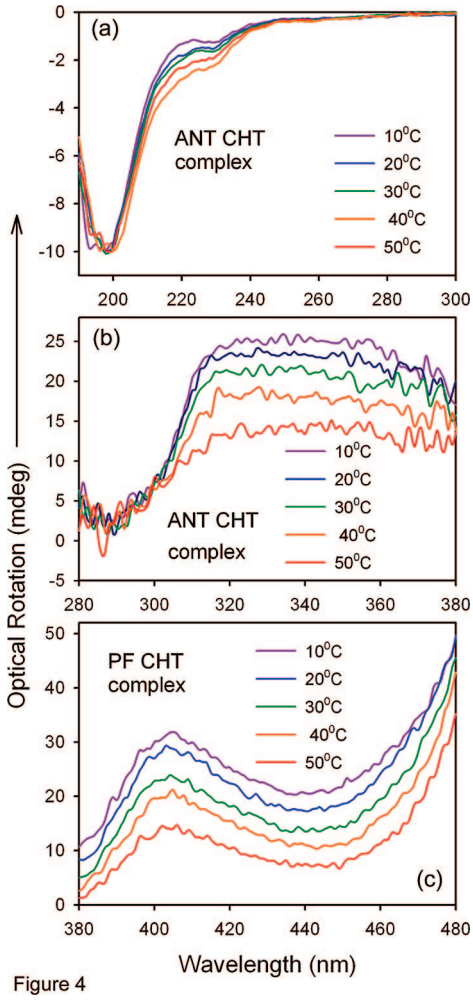

Figure 4. The CD (a) spectrum of ANT−CHT complex. The FDCD spectra of CHT using (b) ANT−CHT and (c) PF as fluorescent probes.

dynamics represents an extremely low frequency mode of the protein, which perhaps lies outside the purlieu of simulation and NMR studies. It is now well-known that the enzymatic activity is associated with the achievement of a critical active site conformation, which is effected through the conformational dynamics of the protein residues. It is also known that the active site conformation conducive to catalysis is preachieved by proteins at higher temperature. The nanosecond dynamics of the serine residue, therefore, fits as a part of the critical conformational dynamics that effects the change of the conformation of the active site residues to the one most suited for the catalytic efficiency. The absence of this component at higher temperatures suggests that the active site conformation, suitable for catalysis, is preachieved by the protein at that temperature.

It is important at this stage to consider the structural consequences of the modification of CHT to its anthraniloyl derivative. In this regard, we have monitored the secondary and overall globular tertiary structure of anthraniloyl CHT by CD and DLS techniques, respectively. The similarity in the hydrodynamic radius of CHT and its anthraniloyl derivative (Table 2) suggests that the tertiary structure of the protein remains essentially the same on fluorescent labeling. Figure 4a shows the CD spectra of anthraniloyl CHT at different temperatures. A comparison with the temperature-dependent CD spectra and the percentage secondary structures of the native CHT (Figure 2c and Table 2) reveals that the overall secondary structure essentially remains the same; only a minor diminution of the 230 nm peak is observed, consistent with the CD spectrum of other acetylated chymotrypsin derivatives.[23,30] X-ray crystallographic studies on CHT reveal that, in the active site, there exists an ion pair between protonated isoleucine-16 and aspartate-194 (adjacent to the serine-195). The formation of this ion pair is crucial for the substrate binding and hence activity of the enzyme. In this condition, histidine-57 and serine-195 are close enough to make hydrogen bonds with each other,[31] which is essential for the catalytic activity. Structural studies have also shown that the attachment of aromatic groups to the serine residue preserves the ion pair in the active site.[11] Thus, it is expected that the attachment of antraniloyl group to serine-195 preserves the active site conformation. Although the crystal structure of antraniloyl CHT is not reported in literature, the comparison of the crystal structure of the tosyl derivative with that of the native enzyme[32] reveals no significant differences in the active site conformation of CHT upon tosylation. Since the antraniloyl group remains attached to the serine-195 residue (the serine-195 residue being directly involved in catalysis), direct measurement of the enzymatic activity of anthraniloyl CHT is not possible. However, considering the retention of the active conformation in anthraniloyl CHT,[11] it is likely to assume that the rotational dynamics of the anthraniloyl group in the active site of CHT would closely resemble the protein residue dynamics of the native enzyme.

The consequences of the conformational dynamics to the active site structure at different temperatures are monitored through FDCD studies. FDCD studies[33,34] have emerged as an efficient technique to probe the local structure of fluorescent probes in the free state and when bound to macromolecules. The technique utilizes the different absorption of left and right circularly polarized light. Here, we have measured the FDCD of anthraniloyl CHT and the well-known CHT inhibitor PF. The fluorophores methyl anthranilate (mimic of the anthraniloyl group attached to CHT) and PF in buffer do not show circular dichroism. However, when bound to the active site of the protein, they adopt a fixed geometry as predicted by the geometry of the local environment. The bound molecules therefore show circular dichroism as shown in Figure 4b,c. A chirality is, thus, induced in the protein bound fluorophores. Figure 4b,c shows the change in the optical rotation detected by the probes at different temperatures. The reduction in the optical rotation at higher temperatures clearly indicates a change in the active site conformation of the protein with increasing temperatures. The possibility that the loss in optical rotation is due to precipitation of the enzyme at high temperatures is ruled out by control absorption studies. The monotonic decreases in the FDCD signal with increasing temperatures suggest progressive decrease in the induced chirality of the bound fluorophore. This loss in induced chirality of the fluorophore with increasing temperature suggests greater conformational flexibility of the protein at higher temperatures. The increased conformational flexibility at higher temperature assists the attainment of the critical active state conformation, reflected in the increase in the catalytic activity up to 40 °C. The existence of active site conformations more suited for catalytic activity at high temperatures is reported.[35] The fall in the catalytic activity at 50 °C, where there is the least rigid binding of the substrate mimic/inhibitor due to the higher

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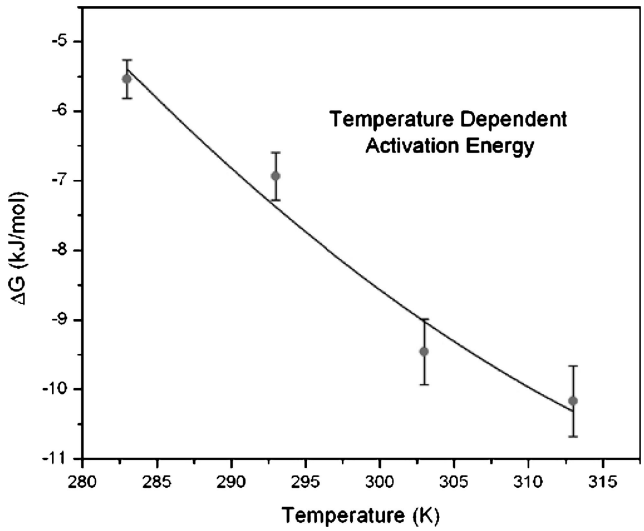

Figure 5. The plot of ΔG against temperature. The solid line is the second-order polynomial fit (see the text).

conformational flexibility of the protein, suggests the existence of a critical conformation for catalysis. In this conformation, the normal mode dynamics of the serine residue becomes slower, as discussed earlier, altering the catalytic activity. Thus, the interplay between the two shorter dynamics associated with the motion of the anthraniloyl serine-195 residue plays a crucial role in the determination of active site conformational changes and also controls the catalytic efficiency at different temperatures.

It is interesting to observe the implication of the conformational dynamics of the protein to the energetics of catalysis. The overall free energy of activation for the enzymatic catalysis can be estimated from the catalytic efficiency (k_{cat}/K_M) according to the relation, $\Delta G = -RT \ln[(k_{cat}/K_M)(h/kT)]$.[36] The decrease in the ΔG values with an increase in temperature suggests that the enzymatic activity is thermodynamically more favorable at higher temperatures. A possible reason for the observed decrease in ΔG can be due the easier achievement of the transition state conformation through motions along the conformational coordinates at elevated temperatures, as discussed earlier. The enthalpy of the process is obtained from the nonlinear dependence of the ΔG on T (Figure 5) in terms of a second-degree polynomial equation[37]

$$\Delta G = a + bT + cT^2 \quad (1)$$

$$\Delta H = \frac{d\left(\frac{\Delta G}{T}\right)}{d\left(\frac{1}{T}\right)} = a - cT^2 \quad (2)$$

where a, b, and c are constants and T is the absolute temperature. Figure 5 shows the variation of ΔG with T. From the second-order polynomial fit, we obtain the values of a, b, and c and put them in eq 2 to calculate the values of ΔH at different temperatures (Table 1). As is observed from Table 1, there occurs a considerable decrease in the ΔH value as the temperature is raised. The increase in substrate affinity to reach the transition state is associated with a decrease in enthalpy, which might have its origin in the formation of new electrostatic and hydrogen-bonding interactions, which can act synergistically.[38] The result is consistent with the fact that the affinity of an enzyme−substrate complex to proceed from the ground-state to the transition state is largely enthalpic in origin.[39] It could be noted that when water molecules are involved as a substrate in the course of the reaction, the contribution of water to the energetics is significant.[40] In the present system also, the water present in the hydrophobic cavity of the enzyme might contribute to the observed decrease in ΔH. It is to be noted that the decrease in ΔH at different temperatures is *not* associated with a corresponding increase in ΔS, which can be expected in cases where the ΔG itself changes with temperature. Examination of Table 1 reveals that the ΔS values remain more or less constant, suggesting that the entropic contribution is less significant in the present process. The observation clearly indicates the role of conformational dynamics at high temperatures to assist the protein catalysis by lowering the energy of the transition state.

Conclusion

The dynamics of a specific protein residue in the active site of the proteolytic enzyme α-chymotrypsin (CHT) has been correlated with its temperature-dependent catalytic efficiency. The catalytic efficiency k_{cat}/K_M of the enzyme at different temperatures shows a maxima at 37 °C, coinciding with the normal body temperature of homeothermals. The overall secondary structure of the protein determined from circular dichroism and globular tertiary structure from dynamic light scattering, however, shows no significant change in the studied temperature range, ruling out the possibility of a massive structural reorientation of the overall protein being responsible for the altered catalytic activity. The temperature-dependent rotational dynamics of the protein and probe at the active site of CHT projects out the crucial dynamics of active site serine-195 residue responsible for catalysis. The minor structural perturbations at the active site due to conformational flexibility at high temperature are also evidenced from FDCD studies. The energetic parameters associated with the catalytic processes at different temperatures indicate that the conformational dynamics enhances the catalytic activity at higher temperatures by lowering the transition state energy.

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