VOLUME CHANGE ASSOCIATED WITH THE LOW- TO PHYSIOLOGICAL-TEMPERATURE CONFORMATIONAL CHANGE IN THERMOMYCES LANUGINOSUS XYLANASE

A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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COLLEGE OF ARTS AND SCIENCE

BY

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To the Dean of the Graduate School:

I am submitting herewith a thesis written by Heather Hollowell entitled "Volume Change Associated with the Low-to Physiological-Temperature Conformational Change in *Thermomyces Lanuginosus* Xylanase." I have examined this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Masters of Science with a major in Chemistry.

Mark Britt

Mark Britt, Ph.D., Major Professor

We have read this thesis and recommend its acceptance:

Mark Britt

Department Chair

Accepted:

Jennifer Martin

Dean of the Graduate School

DEDICATION

To my father,

may you have finally found peace.

ACKNOWLEDGMENTS

I would first like to thank my advisor, Dr. Mark Britt, for his guidance through my research. I am thankful for your patience and positive words through this process. Your eagerness and enjoyment for teaching chemistry is truly inspiring. Thank you for teaching me how to be a better chemist and I would like to believe a better person. Thank you for believing in me and encouraging me along the way. You have been beyond a doubt one of the most influential people in my life and academic career. I am blessed to have worked for you. One could not ask for a better mentor and advisor.

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Finally, I would like to thank my friends and family. To my friends, thank you for always pushing me and believing in me when I didn't believe in myself. My appreciation is beyond words for your encouragement and support. To my mom, you have been the constant voice in my head pushing me to be a better person and for that I am thankful beyond words. You taught me that I can do anything through hard work and perseverance. Thank you for always believing in me.

ABSTRACT

HEATHER HOLLOWELL

VOLUME CHANGE ASSOCIATED WITH THE LOW- TO PHYSIOLOGICAL-TEMPERATURE CONFORMATIONAL CHANGE IN THERMOMYCES LANUGINOSUS XYLANASE

MAY 2010

Thermomyces lanuginosus is a thermophilic fungus with an optimal thriving temperature of 55°C. We have detected a conformational change in *Thermomyces lanuginosus* xylanase at 24°C and at pH = 7.0 and 1.0 atm pressure. This conformational change is revealed by an abrupt change in heat capacity as detected by slow-scan-rate differential scanning calorimetry and occurs at a temperature between that where crystals are formed for x-ray structure determination (4°C) and the physiological temperature (55°C). A volume change $\Delta V \approx -190$ L/mol was calculated associated with the conversion from the low-temperature conformation to the higher-temperature conformation. The volume of the enzyme crystal structure, presumed to be the physiological structure, is 26 L/mole[14]. We hypothesize this large, negative volume change indicates the low temperature form is either a molten globule or a random coil.

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CHAPTER I

INTRODUCTION

Enzymes are protein polymers of amino acid residues that give the enzyme a unique structure and specific activity. They are mostly globular and folded into a functional three - dimensional structure. The structure of an enzyme is important to the function it will have in certain environments. X-ray crystallography is used to determine the three - dimensional structure of an enzyme and it is generally assumed that the x-ray enzyme structure is the same as the physiological structure, even though the x-ray structure analysis is usually performed at temperatures well below the enzymes' optimal thriving temperatures, typically at 4°C.

The thermal unfolding of enzymes and the molten globule structures associated with a few of these unfolding events are well reported. For example, bovine carbonic anhydrase is known to unfold through a molten globule state and this unfolding is a four-state event. The native, folded structure unfolds to a molten globule then to another intermediate and then to its final unfolded state[1]. Molten globule structures are loosely packed secondary structures that are different from both the native and denatured structures. There have also been extensive studies on the thermodynamics of unfolding. The signs and magnitudes of the thermodynamic quantities can reveal details of the conformational change[2].

Conformational changes are induced by changes in temperature, pH, and ion concentration. Nondenaturational conformational changes that occur during the unfolding process are generally unknown, but in our laboratory substantial evidence of major conformational changes occurring between the temperature where crystals are formed for x-ray structure determination and the physiological temperature of the enzyme have been reported. For example, a study of the enzyme bovine carbonic anhydrase has demonstrated a conformational change that occurs at 30°C, which is between the temperature of 4°C where the crystals for x-ray structure determination were formed and this organism's optimal thriving temperature of 38.3°C[3-4]. The large conformational change occurring between these two temperatures highly suggest that the x-ray crystal structure and the physiological structures should not be regarded as the same.

In our laboratory several other enzymes have been studied to further verify that the x-ray crystal structure and the physiological structure cannot be the same. The conformational changes occurring between the x-ray crystal structure temperature and the physiological temperature of enzymes such as bovine adenosine deaminase, hen egg white lysozyme, papaya papain, and baker's yeast phosphoglycerate kinase have been previously analyzed as shown in Table 1[5-12]. Of the five enzymes our laboratory has investigated we find the temperature of the nondenaturational conformational change is 11 ± 8°C below the organismal optimal thriving temperature.

The question was then posed if a nondenaturational conformational change could be detected in a thermophilic enzyme and if the thermophilic enzyme would exhibit

analogous properties to the mesophilic enzymes previously studied in our laboratory. Therefore, this study will investigate the pressure-dependence of the temperature of the low-temperature, nondenaturational conformational change of a thermophilic enzyme.

Thermomyces lanuginosus is a thermophilic fungus with an optimal thriving temperature of 55°C. The x-ray structure determination was at 4°C[13-14]. *Thermomyces lanuginosus* xylanase has a molecular weight of about 25 kDa and a molar volume at 4°C[14] of 26 L/mole. This enzyme was chosen for study because of its low cost. Xylanases are often used in the feed, food, pulp, and paper industries. It is most commonly used in chlorine-free bleaching paper mills and is ideal because of its stability and tolerance to extreme temperatures and extreme pH values[14].

According to Table 1, the temperatures of the nondenaturational conformational changes found for the mesophilic enzymes previously studied occur approximately on average 11°C below that of the physiological temperature. Therefore, the hypothesis was that *Thermomyces lanuginosus* xylanase would exhibit a nondenaturational conformational change, T_{ndcc}, around 44°C and like the mesophilic enzymes the conformational change would be significant.

To investigate the possibility of a significant, nondenaturational conformational change, first the temperature of the nondenaturational conformation change was determined using slow-scan-rate differential scanning calorimetry under physiological conditions. The transition state thermodynamics were then calculated at the temperature of the transition at 1 atm pressure. Next the volume change, ΔV, associated with the low-

to physiological temperature conformational change was calculated by the study of the pressure dependence on the temperature of the nondenaturational conformational change. If there is a significant conformational change then it is likely the volume change will be significant. The last objective for this work is to calculate the activation volume, $\Delta V^{\ddagger}$, from the analysis of the pressure-dependency of the kinetics of the transition to gather support for our volume change studies.

Evidence of a conformational change occurring between these two temperatures has been detected and the conformational change interestingly occurs well below the hypothesized transition temperature for *Thermomyces lanuginosus* xylanase. The volume change, ΔV, and the activation volume, $\Delta V^{\ddagger}$, suggest a major conformational change.

Table 1

Conformational Changes, T_{ndcc}, Occurring Between X-Ray Crystal Structure Temperature, $T_{crystal}$, and Physiological Temperature, T_{phys}, for all Previously Studied Enzymes by Our Laboratory.

Enzyme	$\mathbf{T_{crystal}}$	$\mathbf{T_{ndcc}}$	$\mathbf{T_{phys}}$
Adenosine Deaminase[5,6]	4°C	30°C	38.3°C
Bovine Carbonic Anhydrase[3,4]	4°C	30°C	38.3°C
Lysozyme[7,8]	-173°C	15°C	41.4°C
Papain[9,10]	4°C	15°C	24°C
Phosphoglycerate Kinase[11,12]	4°C	24°C	27.5°C

CHAPTER II

MATERIALS AND METHODS

Thermomyces lanuginosus xylanase (product number X2753) and the sodium phosphate buffer components were obtained from Sigma Aldrich. All measurements were performed in 150mM phosphate buffer and at pH=7.0. Solutions with concentrations greater than 2.0 mg/mL of a known volume were prepared and were allowed to dialyze against a thousand-fold excess of 150mM phosphate buffer for four hours in a 4°C cold room. While taking the solution from the dialysis kit, close attention was paid to the new volume in order to determine if there is a change in concentration during dialysis. A 1.0 mL sample of a 2.0 mg/mL solution was then prepared by dilution using the dialysate. Measurements of the temperature of the conformational change are determined by slow-scan-rate differential scanning calorimetry[5] using a Calorimetry Sciences Corporation Nano DSC III. Each 2.0 mg/mL solution was then degassed and scanned at different rates ranging between 0.02°C/min – 0.05°C/min from 15°C - 60°C. The instrument allows for application of pressures up to 5.0 atmospheres. Therefore, conformational changes were investigated at applied pressures of 1.0, 2.0, 3.0, 4.0, and 5.0 atmospheres. All runs were then baseline corrected by running dialysate versus dialysate at the same scan rate, pressure, and range of temperature as the individual

samples. Conformational change was revealed by an abrupt change in heat capacity and was analyzed using the calorimetry software.

Microcal Origin Software (version 7) was used to create all plots used in this work. The apparent transition temperatures were plotted against scan rate and were linearly extrapolated to zero scan rate to obtain the equilibrium temperature of the nondenaturational conformational change, T_{ndcc}, at each applied pressure. The curve fitting for each plot was performed in Origin.

First order rate constansts (k_1) were calculated from $k_1 = r_{scan}/\Delta T$ at T_t where ΔT is the temperature interval of the transition centered about the transition temperature, T_t[5]. The transition temperature, T_t, was determined by fitting the pre-transition (y_{pre}), transition (y_t), and post transition (y_{post}) of a baseline corrected scane to a linear line and solving for the *x* value that satisfies $y_{post} - y_t = y_t - y_{pre}$ as shown in Figure 1. The transition state thermodynamics are calculated at 24.0°C, the temperature of the nondenaturational conformational change at 1 atm pressure.

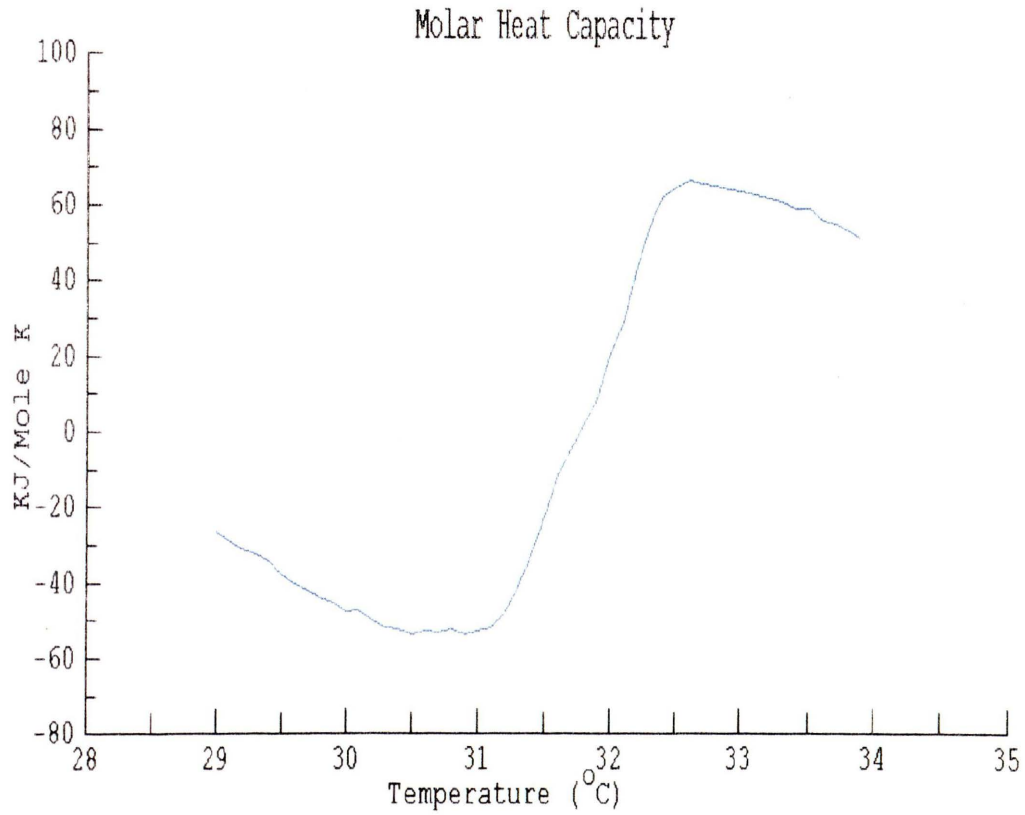

Figure 1: Data of 0.03°C/min scan at 1 atm, revealing the abrupt change in heat capacity. For this scan rate and pressure, the transition temperature is 31.7°C.

CHAPTER III

RESULTS AND DISCUSSION

The pressure dependence of the temperature of the conformational change of *Thermomyces lanuginosus* xylanase was investigated at applied pressures of 1.0, 2.0, 3.0, 4.0, and 5.0 atmospheres. The scans are reversible if the solution used for the each scan is recovered from the calorimeter cell and stored at 4°C for 72 hours and the scan is repeated. An almost identical trace is obtained. Waiting for 48 hours does not give a reproducible result. An example of a raw, non-baseline corrected scan produced by the calorimeter is shown in Figure 2. After a baseline is obtained at the same pressure and scan rate, the scan is then baseline corrected and the apparent transition temperature for each individual scan is obtained. Figure 3 shows the increase in apparent transition temperature with the increase of scan rate by representative normalized slow-scan-rate DSC scans for the low-to physiological conformational change at 1.0 atm pressure. Then at each pressure the apparent transition temperature, T_t, obtained from slow-scan-rate differential scanning calorimetry was plotted against the scan rate of the instrument and extrapolated to zero scan rate to obtain the equilibrium temperature of the conformational change, T_{cc}, at the respective pressure (Figure 4). The transition temperatures, T_t, for all scan rates and pressures used in obtaining the temperature of the conformational changes are shown in Table 2. The equilibrium temperatures for the conformational changes at

each pressure are 24.0°C for 1.0 atm, 14.8°C for 2.0 atm, 12.8°C for 3.0 atm, 16.6°C for 4.0 atm, and 24.0°C for 5.0 atm. There is an obvious trend with transition temperature, T_t, and the transition temperature interval, ΔT, and the increase in scan rate as shown in Table 3.

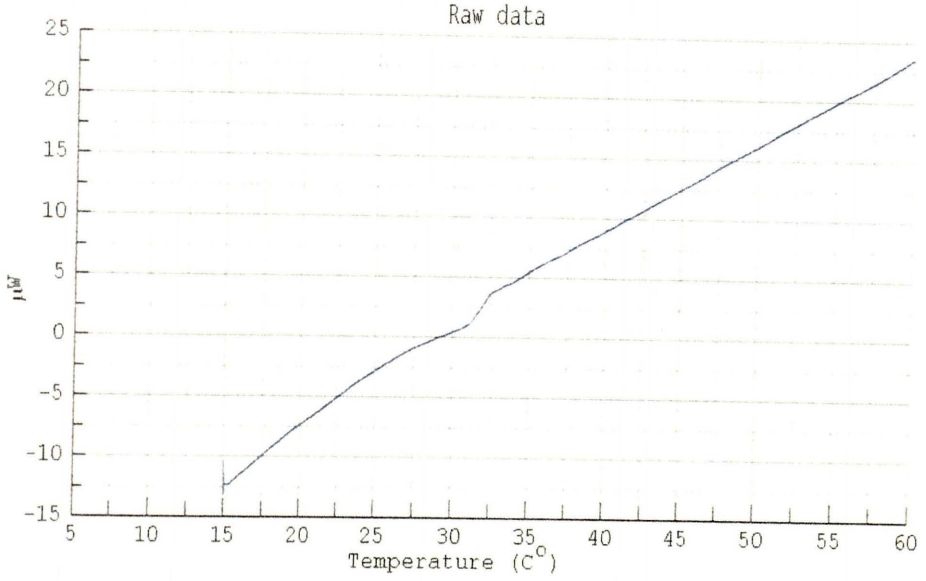

Figure 2: Representative raw DSC scan at 0.03°C/min and 1 atm. Scan is not baseline corrected.

Table 2

Transition Temperatures (°C) as a Function of Scan Rate Used to Find Temperatures of Conformational Changes at Each Pressure.

	Scan Rate (°C/min)						
Pressure(atm)	**0.02**	**0.025**	**0.03**	**0.035**	**0.04**	**0.045**	**0.05**
1.0		29.55	31.73		33.17	34.75	
2.0		26.64		34.59	36.70		39.85
3.0		26.64		32.56	34.76	37.94	
4.0	30.36	31.45	35.72	39.64			
5.0		34.24	36.15	39.32		42.40	

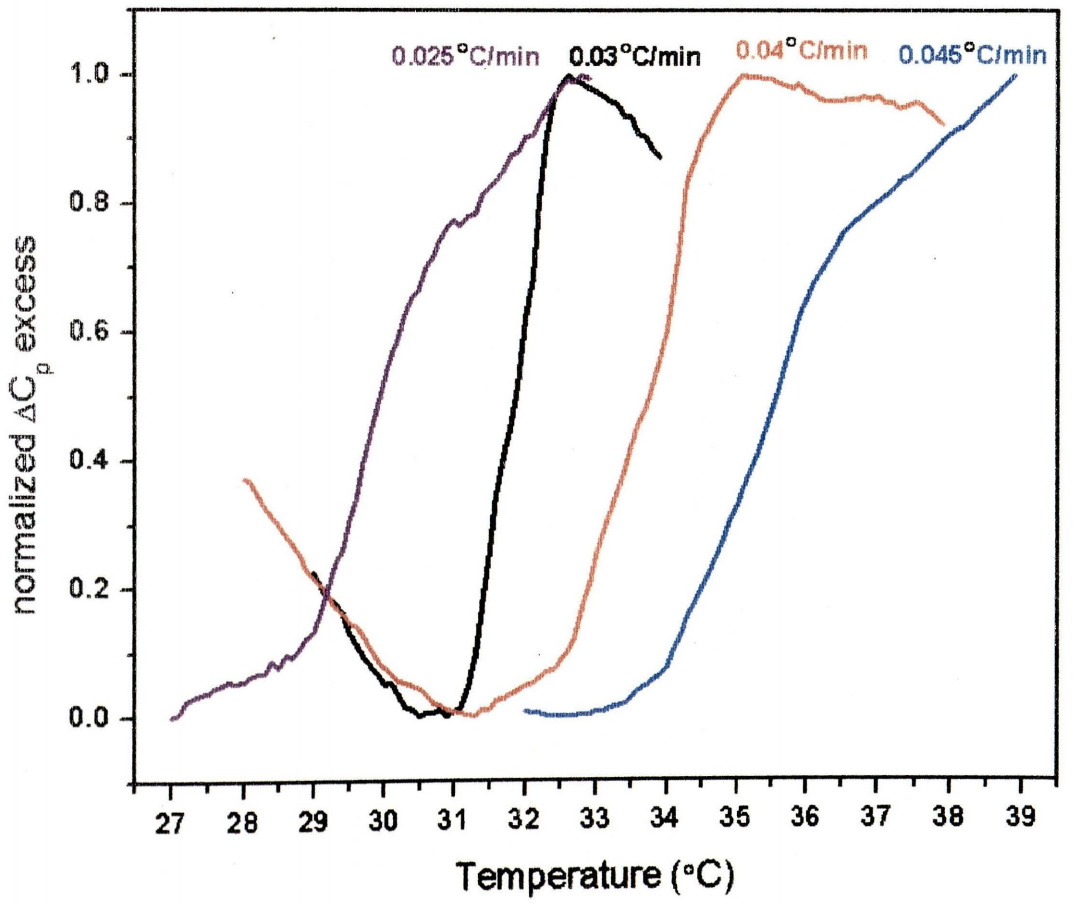

Figure 3: Representative normalized slow-scan-rate DSC scans for the low to physiological conformational change as shown by an abrupt change in ΔC_p. P = 1.0 atm.

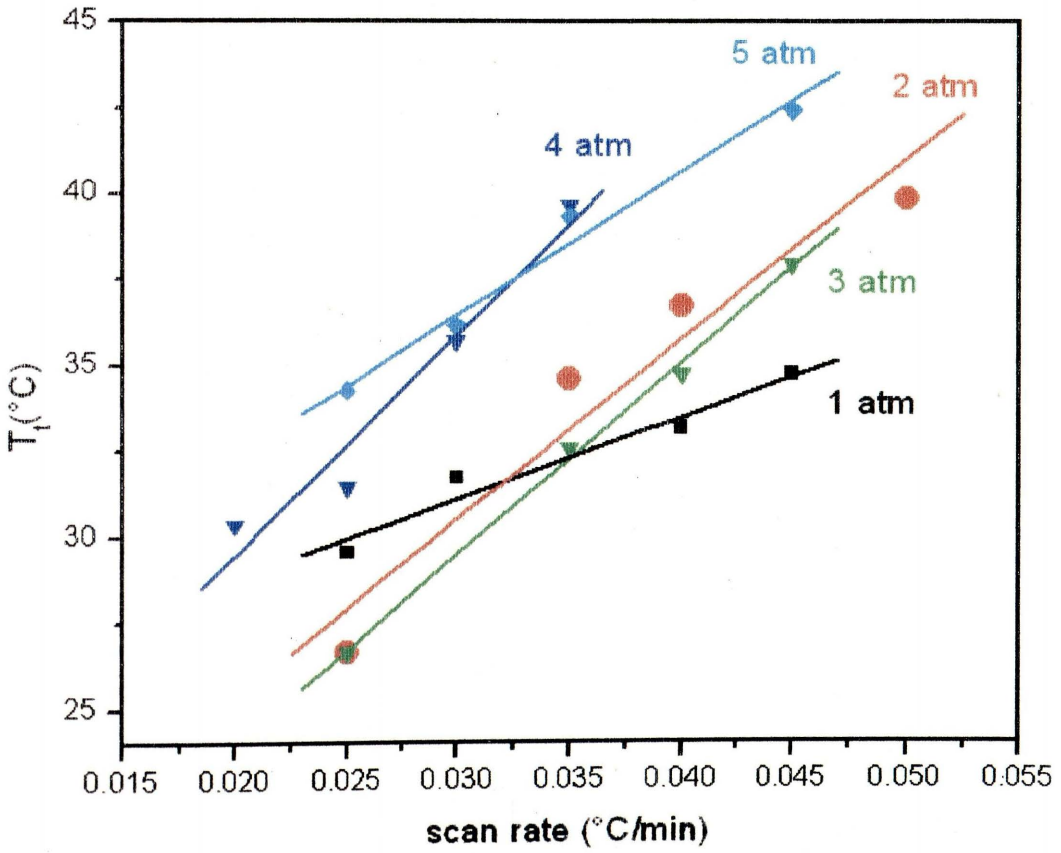

Figure 4: Linear extrapolations to zero scan rate of the apparent transition temperatures give the temperature of the conformational change at each pressure. These temperatures are used to construct the partial phase diagram. T_t for 1.0 atm = 24.0°C (R^2 = 0.978), 2.0 atm = 14.8°C (R^2 = 0.969), 3.0 atm = 12.8°C (R^2 = 0.999), 4.0 atm = 16.6°C (R^2 = 0.975), and 5.0 atm = 24.0°C (R^2 = 0.989).

An Arrhenius plot was constructed for the purpose of calculating the transition state thermodynamics (Figure 5). With the assumption that $\Delta C_p \neq 0$ but is constant, the data was modeled with a second order polynomial. Using $\ln(k) = -2.293 \times 10^8 (1/T)^2 + 1.504 \times 10^6(1/T) - 2473$, a value of 6.15×10^{-5} s^{-1} was calculated for the first order rate constant at 24.0°C. The activation free energy is calculated at 24.0°C from $\Delta G^{\ddagger} = -RT [\ln(kh/(k_B T)]$ where k is the rate constant, h is Planck's constant, and k_B is Boltzmann's constant. $\Delta G^{\ddagger} = 97$ kJ/mole. The activation enthalpy was calculated from $\Delta H^{\ddagger} = E_a - RT$ where E_a is the activation energy calculated from the slope or derivative of the quadratic fit to the Arrhenius plot at 1/(297 K). The activation enthalpy, $\Delta H^{\ddagger}$, calculated at 24.0°C is equal to 330 kJ/mole. The activation entropy, $\Delta S^{\ddagger}$, is calculated from $(\Delta H^{\ddagger} - \Delta G^{\ddagger})/T$ at 24.0°C and is equal to 0.79 kJ/mol · K. Heat capacity, $\Delta C_p^{\ddagger}$, is calculated from $(\Delta(\Delta H^{\ddagger}))/T$ and is equal to -42 kJ/mole · K.

The enthalpy change ΔH at 1 atm was calculated from the integration of the area enclosed by the baseline and abrupt change in heat capacity for each individual scan (Figure 6). For these calculations the raw, non-baseline corrected scans were used. The scans produced from the calorimeter gave a plot of microwatt, μW, versus time, in seconds. A Joule is defined as the work required to produce one watt of power for one continuous second. Therefore, enthalpy could be calculated from the raw scans using this conversion and the formula weight of the enzyme, which is 25,000 g/mole. Once the enthalpies for each scan, ΔH_{app}, were calculate they were plotted against scan rate and then extrapolated to zero scan rate to find the overall enthalpy change ΔH at 1 atm.

As shown in Figure 7, the enthalpy change is 630 kJ/mole. The regression of how ΔH_{app} changes with scan rate is shown in Figure 8. These values are scan rate dependent as is typical for this type of measurement.

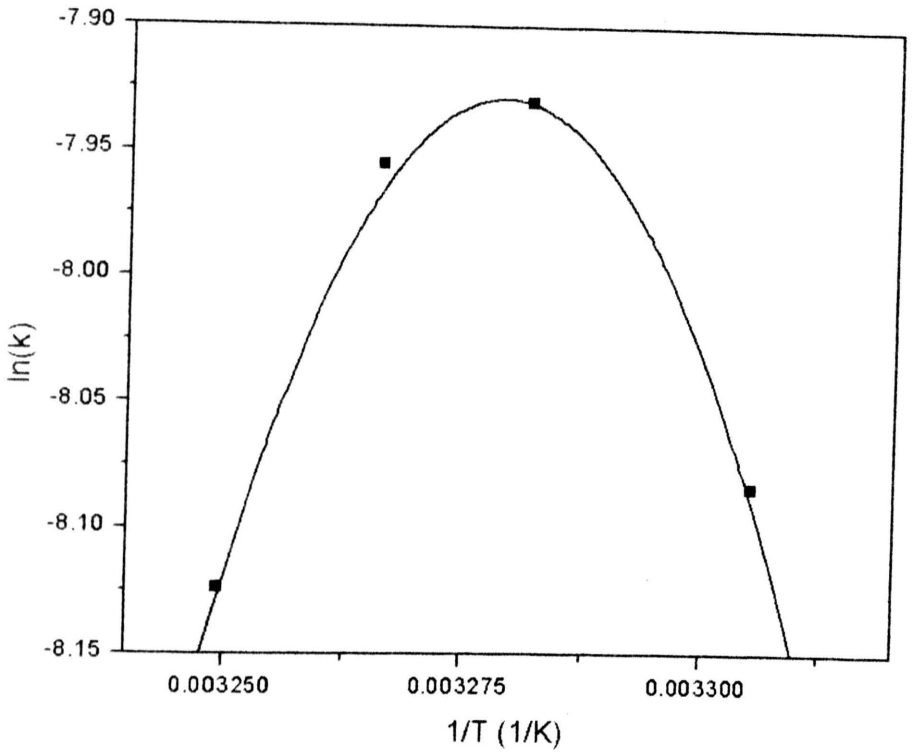

Figure 5: Arrehenius plot of the rate constants from Table 3 at 1 atm. Data trend is modeled to $\ln(k) = -2.293 \times 10^8 (1/T)^2 + 1.504 \times 10^6 (1/T) - 2473$. $R^2 = 0.998$.

Table 3

DSC Scan Characteristics of Runs Shown in Figure 1.

r_{scan} (°C/min)	T_t	ΔT	k (s^{-1})
0.025	29.55	1.35	3.086×10^{-4}
0.03	31.73	1.39	3.597×10^{-4}
0.04	33.17	1.90	3.509×10^{-4}
0.045	34.75	2.53	2.964×10^{-4}

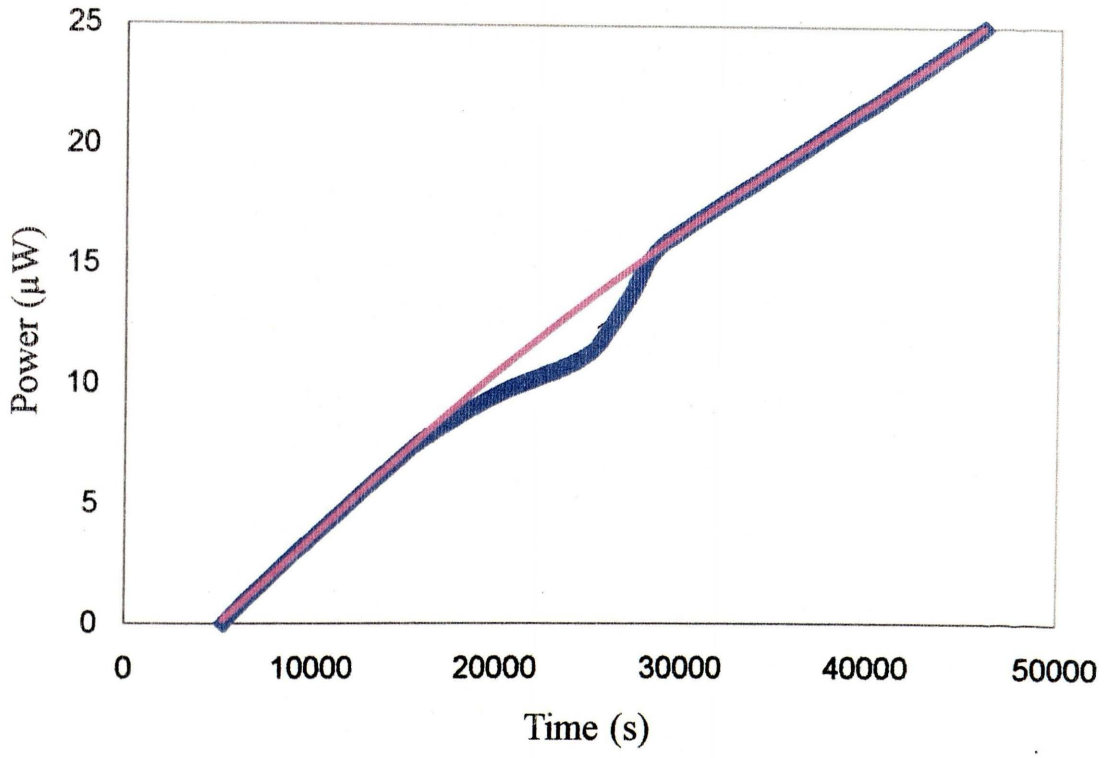

Figure 6: A representative plot of power versus time to obtain the apparent ΔH, calculated from the integration of the area enclosed by the baseline and abrupt change in heat capacity. Plot is of 0.045°C/min scan at 1 atm. ΔH_{app} = -619 kJ/mole.

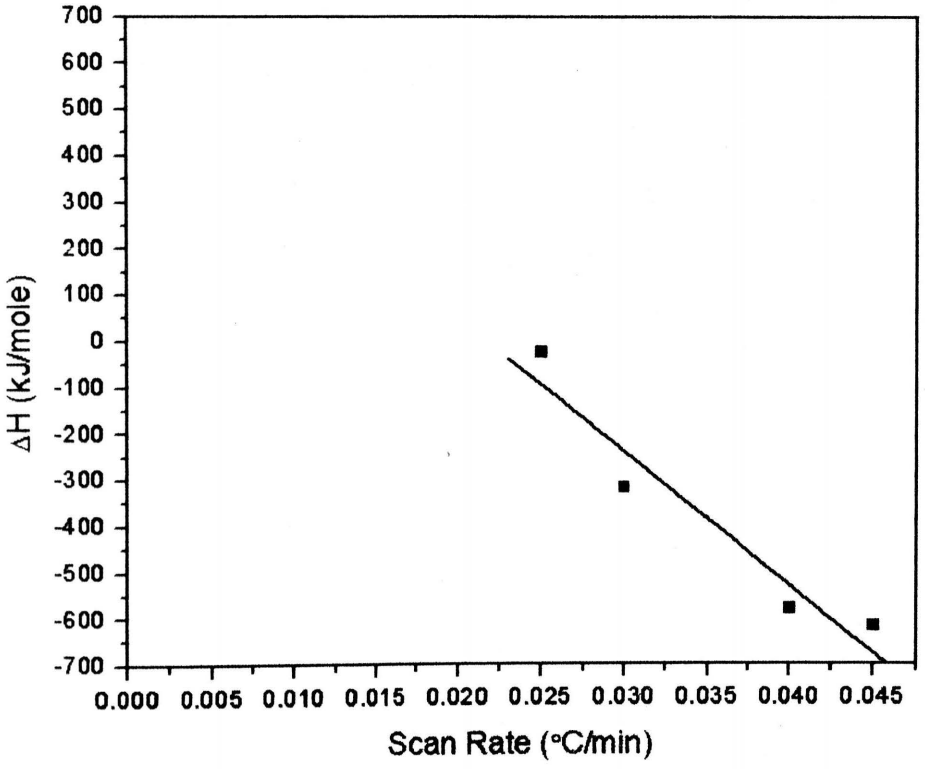

Figure 7: Apparent ΔH versus scan rate. Linear extrapolated to zero scan rate to give the equilibrium ΔH for the low to physiological conformational change. $\Delta H_{L \rightarrow P} = 630 \pm 210$ kJ/mol. $R^2 = -0.962$.

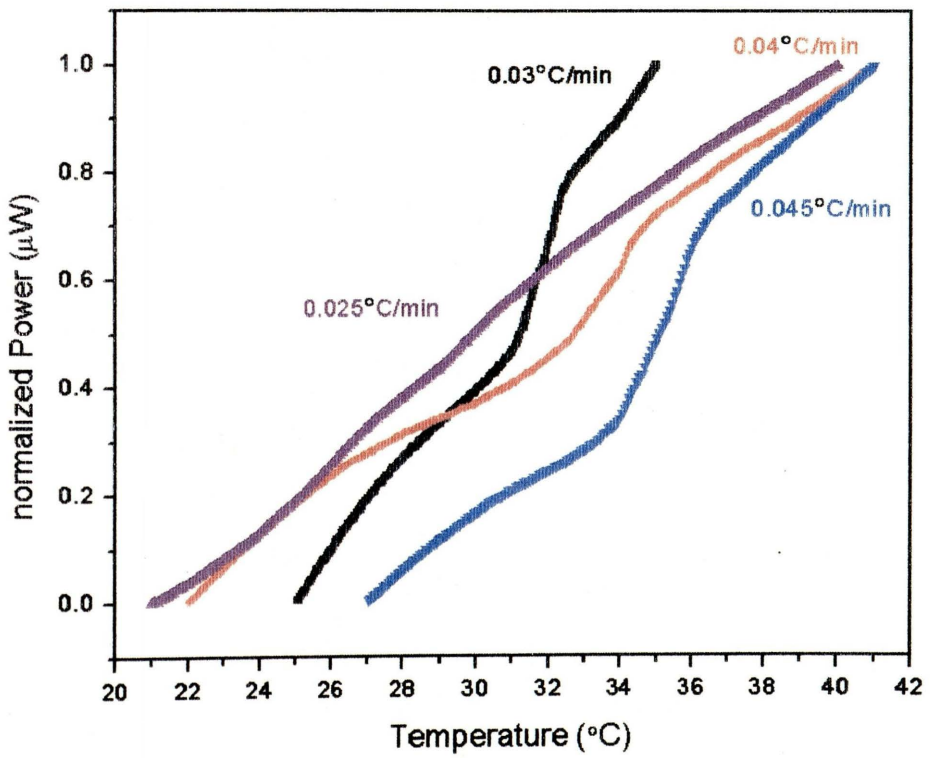

Figure 8: Regression of apparent enthalpy change ΔH_{app} versus temperature to show ΔH dependency on scan rate. P = 1.0 atm.

A partial phase diagram of the low-to physiological-temperature conformational change was then constructed from the transition temperatures obtained from each pressure to calculate the change in volume (Figure 9). The partial phase diagram of *Thermomyces lanuginosus* xylanase resembles the typical low temperature portion protein phase diagram[15]. The change in volume, ΔV, was calculated at 24.0°C using $\partial P/\partial T=\Delta H/T\Delta V$. This graph according to mathematical rule is not a function because it produces two *x* values for a *y* value. Therefore, data points at 1.0 and 2.0 atm were used to estimate the slope of the line between these two points. With this approximation, $\Delta V \approx -190\pm 60$ L/mole. The molar volume, 26 L/mole, of the low-temperature conformer is calculated from knowledge of the empirical formula and the volumes of the atoms as calculated from the van der Waals radii. This value is in very good agreement with simple geometric volume calculations from molecular dimensions of the crystal structures[14]. Since the estimated volume change equals -190 L/mole the hypothesis is that the low temperature conformer is a molten globule. The low-temperature form could possibly be a random coil but usually have large molar volumes. The volume of the molten globule or random coil structure is approximately eight times that of the more compact conformer corresponding to a doubling of the radius from the physiological conformer.

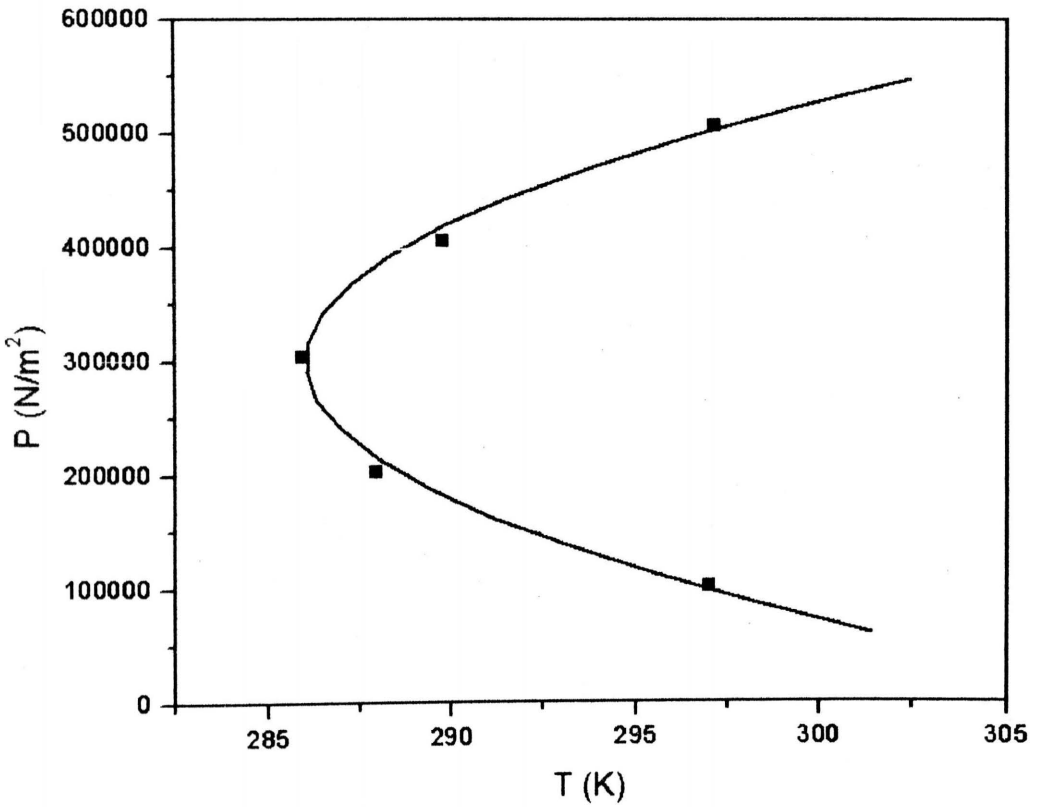

Figure 9: Partial phase diagram of the low- to physiological-temperature conformational change. Calculation of ΔV at 24.0°C from $dP/dT = \Delta H/(T\Delta V)$. $\Delta V \approx -190 \pm 60$ L/mole. $Y = 310.28 - 1.61709 \times 10^{-4} X + 2.69442 \times 10^{-10} X^2$. $R^2 = 0.988$.

The activation volume $\Delta V^{\ddagger}$ was then calculated from $\Delta V^{\ddagger} = -RT(\partial(\ln(k))/\partial P)_T$. The first order rate constants, k, as a function of pressure are obtained from Figure 10. Once the values of ln(k) were found at 24.0°C, they were plotted against pressure, in N/m^2. The activation volume, $\Delta V^{\ddagger}$, was calculated to be -65 L/mole (Figure 11). The value for the activation volume supports the value of the calculated volume change.

The usual interpretation of transition state analysis from an unfolded to a folded state is that $\Delta C_p^{\ddagger}$ and $\Delta S^{\ddagger}$, if subject to hydrophobic effects, have negative and positive values, respectively, and $\Delta H^{\ddagger}$ has a positive value[2]. According to calculations for the transition state for *Thermomyces lanuginosus* xylanase the values for $\Delta C_p^{\ddagger}$ and $\Delta S^{\ddagger}$ are negative and positive values, respectively. The enthalpy of activation $\Delta H^{\ddagger}$ is also positive. Therefore, our results are consistent with a removal of nonpolar amino acid side chains from the solvent with a resulting loss of water molecules and a reduction in volume. The hypothesis is that *Thermomyces lanuginosus* xylanase is not becoming less compact from a low-temperature folded structure, but is becoming more compact from what is hypothesized to be a molten globule or random coil structure.

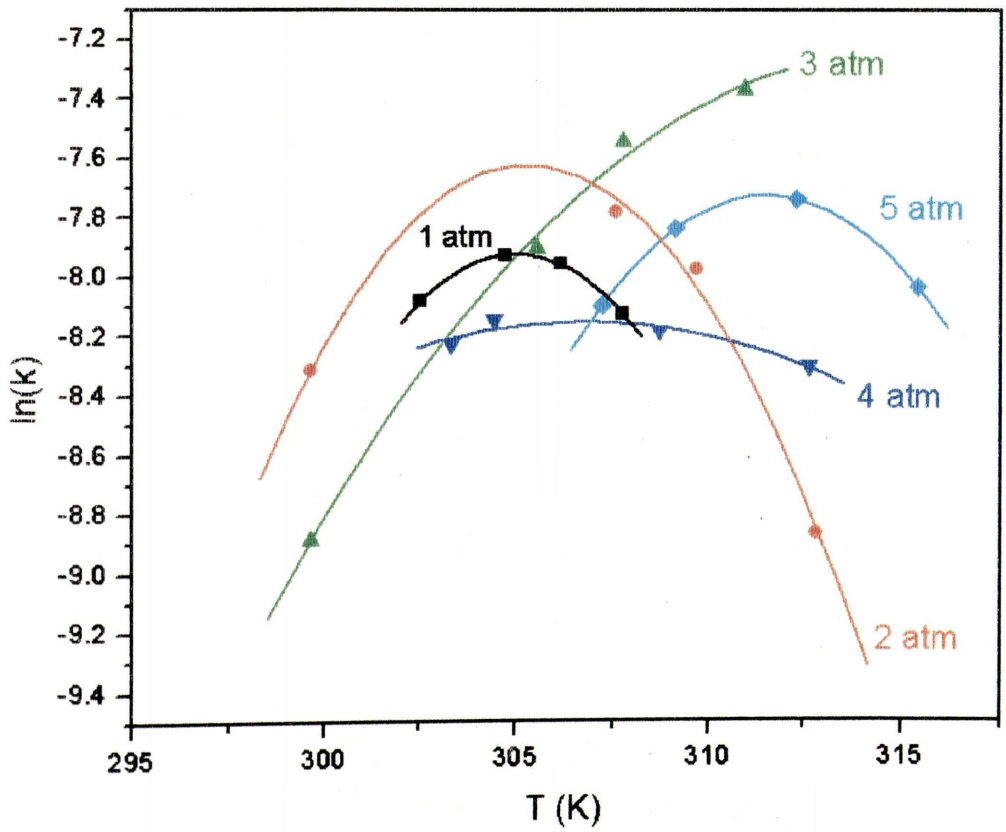

Figure 10: Plots of ln(k) vs T for the purpose of obtaining values of ln(k) at constant T for the calculation of the activation volume $\Delta V^{\ddagger}$ for the process.

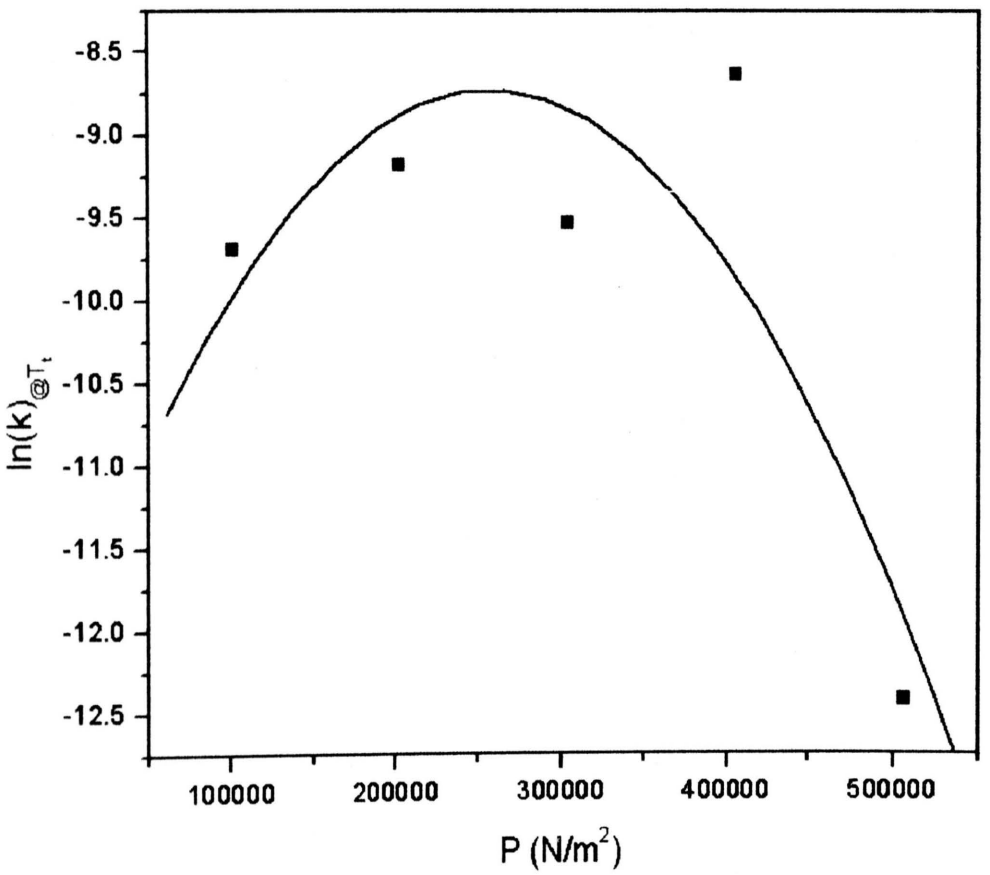

Figure 11: Activation volume calculated from $\Delta V^{\ddagger} = -RT(\partial(\ln(k))/\partial P)_T$. $\Delta V^{\ddagger} = -65 \pm 44$ L/mole. $Y = -12.10024 + 2.62664 \times 10^{-5} X - 5.11715 \times 10^{-11} X^2$. $R^2 = 0.724$.

CHAPTER IV

CONCLUSIONS

The hypothesis, namely that the temperature of the nondenaturational conformational change, T_{ndcc}, would occur at approximately 44°C, was rejected since it has been detected at 24°C. This work also set out to calculate the volume change and the activation volume change associated with the low-to physiological conformational change. The volume change, ΔV, was estimated to be -190 L/mole. This surprisingly large and negative volume suggests the low-temperature form is a molten globule or possibly a random coil.

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