A Role For Ca^{2+} In The Thermal And Urea Denaturation Of Haemocyanin From Aestivating Giant African Snails *Achatina achatina*

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Abstract

Giant African snails (*Achatina achatina)* become dormant (aestivate) under harsh environmental conditions like dry seasons. During this period the animal accumulates urea and is faced with thermal death. The stability towards thermal and urea denaturation of haemocyanin from aestivating and nonaestivating *A. achatina* has been investigated using UV spectroscopy.

Thermodynamic parameters calculated show a complex process with two sets of activation energy (Ea); 4.64kcal/mol, 14.39kcal/mol for nonaestivating and 5.77kcal/mol, 17.66kcal/mol for aestivating snails. With aestivating snails having a higher melting temperature 70°C, compared to 60°C for nonaestivating snails. The results also indicate that calcium concentration is increased during aestivation and this increase is responsible for the high conformational stability (ΔG_O^{H2O}) of haemocyanin from aestivating snails that is 1.8kcal/mol for nonaestivating and 1.5kcal/mol for aestivating snails in the absence of Ca^{2+} as against 1.9kcal/mol and 2.0kcal/mol for nonaestivating and aestivating snails respectively in the presence of 15mM Ca^{2+}. Thus Ca^{2+} plays an important role in the stability of haemocyanin during aestivation.

Keywords: Snails, *Achatina achatina*, haemocyanin, aestivation, thermal and urea, denaturation, thermostability and conformational stability.

Introduction

There are few environments in which organisms are not subject to some kind of environmental stress (lack of food, oxygen, temperature extremes, dehydration, high salt concentration etc). When an organism is placed in a situation of environmental stress, it must adapt, avoid, become dormant or dies. The giant African snail, *A. achatina* is capable of prolonged aestivation. Aestivation occurs in this snail and other terrestrial pulmonates, as a means of surviving adverse climatic conditions which occur in the dry season of tropical areas (Precht *et al.*, 1973; Hodasi 1979; Umezuruike, *et al.*, 1983). Numerous behavioural, physiological and biochemical adaptations support prolonged aestivation in snails (Guppy, *et al.*, 1994; Brooks, *et al.*, 1997). The most crucial adaptations are those that aid water retention within the body or increase dehydration tolerance and metabolic rate depression.

Haemocyanins (Hcs) are extracellular copper proteins that serve to transport oxygen in *A. achatina* and in other species of molluscs and arthropods (Ellerton, *et al.*, 1993; Van Holde, *et al.*, 1982, 1995). Although molluscan and arthropodan Hcs appear to have evolved from a molecule similar to the enzymes tyrosinase and phenoloxidase (Schneider, *et al.*, 1984; Decker, *et al.*, 2000; Van Holde *et al.*, 2001), they have diverged sufficiently such that the proteins are structurally similar only at their oxygen binding sites (Ling, *et al.*, 1994). The molecular architecture and arrangements of subunits in the native Hc from the two phyla show great differences and have been described in several reviews (Markl, 1996; Van Holde, *et al.*, 1982; 1992, 1995).

Molluscan Hc exist in several structurally distinct subclasses. In most

cases, molluscan Hc exist in the haemolymph as very large aggregates assembled as 10-mers or 20-mers of polypeptide chains (Herskovits, *et al.*, 1991; Van Holde *et al.*, 1982, 1995). Hc from gastropods are enormous and occur as complex structures called multidecamers (3.5-9 x 10^6 Da). In electron micrographs, these gastropodan Hc appear as cylinders of 300Å diameter and varying lengths. These cylinders have a three-tiered wall and 5- or 10- fold symmetry of the collar in the central cavity (Stoeva, *et al.*, 1997). Monomer units of decamers or multidecamers with molecular weights of 350-440kDa contain seven or eight domains depending on the species (Burggren, *et al.*, 1991; Decker *et al.*, 2000; Van Holde *et al.*, 2001). Each domain represents a functional unit of about 50kDa molecular weight and is equivalent to an active site (oxygen binding site).

For Hc to be biologically active, it must be folded into a unique three-dimensional structure. This unique structure is distorted by a number of osmolytes and extremes of temperature. Aestivating snails accumulate large amounts of urea due to excessive protein catabolism and cessations in urination (Horne, 1971; Hodasi, 1979; Van Holde, *et al.*, 1992). Aestivating snails are also exposed to high environmental temperatures. These factors could affect the structural and biological properties of Hc by facilitating its unfolding to the denatured state.

The stability of molluscan and arthropodan Hc towards pH changes, thermal and chemical denaturants have been investigated extensively using differential scanning calorimetry (DSC) and spectroscopic (UV, CD, fluorescence, light scattering spectroscopy) techniques (Herskovits, 1988; Sterner, *et al.*, 1995; Dolashka, *et al.*, 1996; Hubler, *et al.*, 1998; Favilla, *et al.*, 2000A, 2000B). From these studies it has been noticed that the oligomerization of Hc, which is mediated by Ca^{2+}, is responsible for the high stability of Hc towards denaturants. This is because Ca^{2+} considerably increases Hc's melting temperature and free energy of stabilization in water. Also demonstrated by the above studies is the fact that the subunits in the oligomers of Hc are held together by hydrophilic and polar interactions.

At present, little information is available about denaturation parameters and adaptations of Hc from aestivating giant African snail, *A. achatina* towards thermal and urea denaturation. The thermal and urea denaturation of Hcs from *Rapana thomasiana* (Dolashka, *et al.*, 1996), *Eurypelma californicum* (Hubler, *et al.*, 1998) and *Carcinus aestuarii* (Favilla, *et al.*, 2000a and 2000b) have been studied. It was considered very necessary in this study to compare the denaturation parameters and stability of Hc from aestivating snails towards thermal and urea denaturation. The stability of Hc in the presence of some haemolymph osmolytes (Ca^{2+}) was also investigated in the hope that these osmolytes may help to stabilize Hc of this snail to the physiological stress to which it is subjected.

Materials And Methods

Materials: Tris [tris (hydroxymethyl) amino methane] and urea were purchased from BDH chemicals Ltd Poole, England. All other chemicals and reagents used were of analytical grade.

Snails (A. achatina) were bought from the local market and kept inside a cage covered with wire mesh at 25-27^{0}C. The snails were fed for a week with fresh leaves of *Telferia occidentalis* after which one group of fifty snails continued to receive adequate supply of fresh leaves and water, and another group of fifty snails were starved and forced to aestivate by being transferred to a dry cage without food and water. These snails were allowed to aestivate for four months.

Isolation and purification of *A. achatina* haemocyanin: The snails (aestivating and nonaestivating) were washed, de-shelled, the pervisceral haemoceal, which is posterior to the buccal mass, is punctured and haemolymph collected. To facilitate collection, the snails were placed on small funnels containing Whatman filter papers. The crude haemolymph was

centrifuged for 30 minutes in a cooling box (4^0C) at a speed of 5000 g. After centrifugation, the Hc was concentrated twice by ammonium sulphate precipitation (30% and 55% respectively) followed by excessive dialysis in 0.05M Tris-HCl buffer pH 7.5 as described by Lamy *et al* (1977). The concentrated Hc served as the stock Hc solution. Protein concentration of the stock was determined by a modified Lowry method described by Schacterle and Pallack (1972) and the stock frozen for further use.

Spectroscopic measurement of the thermal denaturation of haemocyanin: Thermal denaturation of Hc (from aestivating and nonaestivating snails) was monitored by changes in absorbance at 340nm with a Unicam SP 500, series 2 spectrophotometer at temperatures between 40-70^{O}C, in a 1ml cuvette with a path length of 1cm.

The effect of temperature on Hc within the range of 40-70^{O}C was determined at pH 7.5 in 0.05M Tris-HCl buffer. Into 9ml of the buffer allowed to equilibrate at the required temperature for 10 minutes, 1ml of stock Hc solution was then introduced (making a protein concentration of 0.3mg/ml). At suitable intervals, 1ml of the sample was withdrawn and its absorbance at 340nm recorded.

Spectroscopic measurement of urea denaturation of haemocyanin: Studies on the urea denaturation of Hc (aestivating and nonaestivating) was monitored at 340nm with a Unicam SP 500, series 2 spectrophotometer at 25^{0}C in a 3ml cuvette with a path length of 1cm.

10M urea was freshly prepared in a 0.05M Tris-HCl buffer, pH 7.5.The 10M urea was pipette into nine different test tubes such that the concentration of urea in the tubes ranges from 0.5-8M. The nine tubes were prepared in three sets. Into each set of tubes were added 5, 10 and 15mM Ca^{2+} respectively. Ca^{2+} was added as $CaCl_2$. Into each set of tubes, equal volumes (0.5ml) of stock Hc solution were added and the final volume made up to 5ml. This gave a final protein concentration of 0.3mg/ml in each tube. When Hc was added, the absorbance of each tube was taken immediately and after 24 hrs of incubation at room temperature.

For the control experiment, another set of nine tubes were prepared as described above with the absence of Ca^{2+}.

Calculation of the conformational stability of haemocyanin: The equilibrium for the urea denaturation of Hc was satisfactorily described with a two-state model (Tanford, 1970; Pace, 1986). From this model, the fractional denatured population (F_D) in each condition was determined by

$$F_D = \frac{A - A_N}{A_D - A_N} \quad \ldots\ldots (1)$$

Where; A is the observed value of the absorbance at 340nm at different concentrations of urea. A_N and A_D are the absorbance of the native and the denatured states respectively. A_N and A_D in the transition region were determined by a usual linear extrapolation method (Pace, 1986).

The free energy of denaturation of Hc in the presence of urea, $\Delta G_{(d)}$ was estimated from the equation

$$\Delta G_{(d)} = -RT\ell nk_{(d)} \quad \ldots\ldots (2)$$

where; R is the universal gas constant (1.987 cal $mol^{-1}k^{-1}$) and $k_{(d)}$ is the equilibrium constant for the transition of Hc from the native to the denatured state.

$$K_{(d)} = \frac{A - A_N}{A_D - A} \quad \ldots\ldots (3)$$

The conformational stability of Hc (ΔG_O^{H2O}) in the absence of the denaturant was determined from a plot of $\Delta G_{(d)}$ versus urea concentrations.

Calculation of activation parameters: Half lives for the thermal denaturation of Hc at various temperatures were extrapolated from the percentage stability curves. This was based on the fact that the time for the stability of the protein to be reduced by 50% is equivalent to the half lives of the protein at a given temperature. This half lives ($t_{1/2}$) were used to calculate the first order denaturation rate constant (k) at various temperatures for the equation.

$$k = \ell n2/t_{1/2} \quad \ldots\ldots (4)$$

Activation energies (Ea) were calculated from the Arrhenius equation.

$k = e^{-Ea/RT}$ ..... (5)

by plotting lnk against 1/T. T, k and R represent the absolute temperature, the calculated kinetic rate constant and the universal gas constant (1.987 Cal $mol^{-1}k^{-1}$) respectively.

Other activation parameters, entropies of activation ($\Delta S^{\neq}$), enthalpies of activation ($\Delta H^{\neq}$) and Gibbs free energies of activation ($\Delta G^{\neq}$) were calculated from the equations.

$A = \frac{K_B T e^{\Delta S^{\neq}/R}}{h}$ ..... (6)

$\Delta H^{\neq} = Ea - RT$ ..... (7)

$\Delta G^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq}$ ..... (8)

respectively where K_B is the Boltzman constant ($3.298 \times 10^{-24} Cal^{-1}K^{-1}$) and h is the Plancks constant (1.584×10^{-34} cal.s).

Results

Effect of urea: The transition of Hc from non aestivating and aestivating snails from the native to the denatyred state induced by urea is similar to that of other globular proteins in that it follows a simple two state transition model (Tanford, 1970; Pace *et al.*, 1990). Upon exposure of Hc to urea concerntration of 3M gradual decreases were observed in their absorbance, Fig.1, thus the rate of denaturation was slower at lower concentration of urea but increased sharply at higher concentrations (6M, 7M, and 8M).

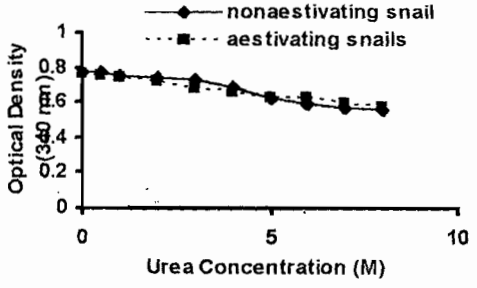

Fig. 1: Urea denaturation curve of haemocyanin from snails in the absence of calcium ions.

The Gibbs free energy of denaturation ($\Delta G_{(d)}$) from both Hc varied linearly with urea concentrations and lines of best fits were used to calculate the conformational stability (ΔG_O^{H2O}). From Fig 1, Hc from aestivating snails is very sensitive to urea denaturation as supported by small conformational stability

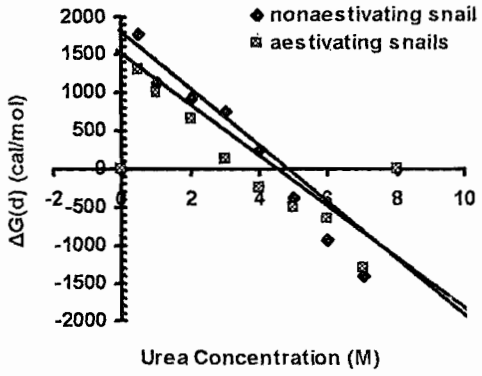

Fig 2: ΔG(d) vs [urea] at 340nm of haemocyanin from snails in the absence of calcium ions

of Hc from aestivating snails (1.5kcal/mol as against 1,8 kcal/mol for non aestivating snails.(Fig 2).

Ca^{2+} is an allosteric ligand of Hc and is required for the structural stability and biological functions of Hc (10). It was thus thought that the increase in Ca^{2+} in the haemolymph of snails during aestivation is expected to play an important role in increasing the conformational stability of Hc towards denaturants like urea. The conformational stability of Hc was thus determined in the presence of 5mM, 10mM, and 15mM Ca^{2+} and represented on table 1. From this table, reasonable changes were noticed in the stability of Hc when there were exposed to various concentrations of Ca^{2+}. For non aestivating snails, minimal increases and decreases were notice when their Hc was exposed to various concentration of Ca^{2+}. For aestivating snails, increases were noticed on the stability of their Hc when exposed to Ca^{2+}. A marked increase was noticed with Ca^{2+} concentrations of 15mM which is close to the concentration obtained when the snails aestivated for four months.

Table 1: Summary of parameters calculated for the urea denaturation kinetics of Hc from snails

Experimental Conditions	Nonaestivating Snails			Aestivating Snails		
	$C_{1/2}$ (M)	m (kcal/molM^{-1})	ΔG^0 (kcal/mol)	$C_{1/2}$ (M)	m (kcal/molM^{-1})	ΔG^0 (kcal/mol)
Control	4.40	4.50	1.80	3.30	3.50	1.50
5mM Ca^{2+}	3.20	3.80	1.28	4.00	3.40	1.41
10mM Ca^{2+}	2.70	5.10	1.98	1.90	3.90	1.38
15mM Ca^{2+}	2.48	3.50	1.91	4.50	4.80	2.00

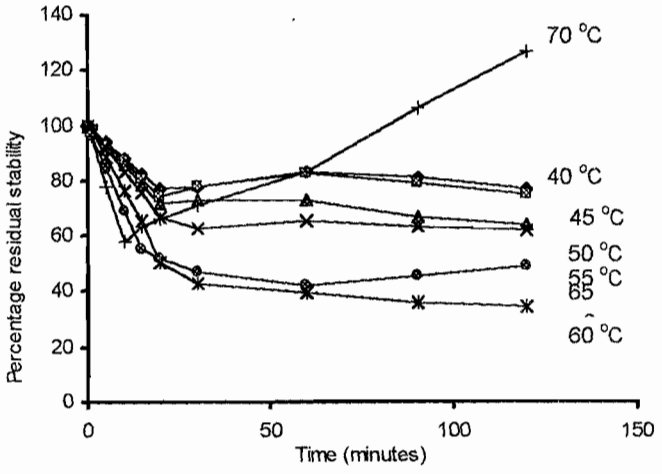

Fig. 3a: The effect of temperature on the percentage residual stability of haemocyanin from nonaestivating snails at 340 nm, pH 7.5, Protein concentration 0.3 mg/ml

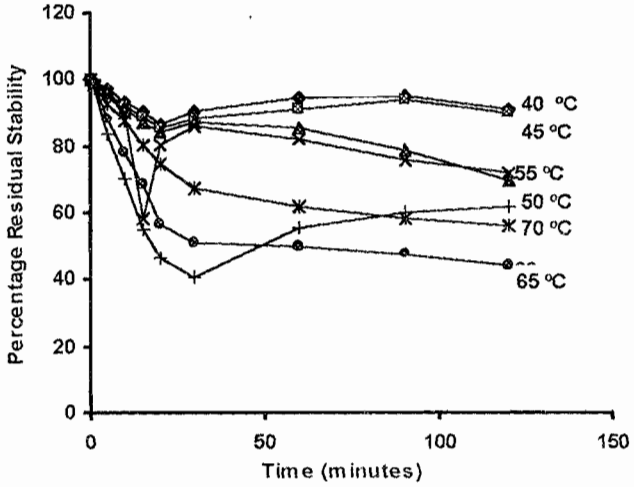

Fig. 3b: The effect of temperature on the percentage residual stability of haemocyanin from aestivating snails at 340 nm, pH 7.5 and protein concentration 0.3 mg/ml

Thermostability of haemocyanin: Fig 3a and 3b shows the temperature dependence of percentage stability of Hc as a function of time for Hc from non aestivating and aestivating snails respectively. From these curves the following observations were made:

The thermal denaturation of Hc from non aestivating and aestivating snails obey a simple first order denaturation process at 60-70 °C for aestivating and 55 - 70 °C for non aestivating snails.

Unfolding kinetics from 40-50°C for nonaestivating and 40-55°C for aestivating snails are biphasic with a first order denaturation process obeyed only in the first 20 min of incubation. This indicates that there were no intermediate in the denaturation pathway for the first 20 min of incubation after which intermediate were introduced giving rise to the biphasic nature of the curves at lower temperatures.

The melting temperature (T_m) of Hc from both groups of snails was determined by making a plot of percentage stability at 30 min of incubation against their respective temperatures (Fig 4). From these plots it was observed that Hc from non-aestivating snails showed a drop in stability at 50°C with complete denaturation at 60°C (melting temperature), while Hc from aestivating snails showed a drop in stability at 55°C with a melting temperature at 70°C.

Activation parameters: The half-lives for the thermal denaturation of Hc from non-aestivating and aestivating snails at various temperatures were determined by extrapolating the percentage stabilities before 20 min of incubation to a 50 % stability point. These half-lives were used to calculate the first order denaturation

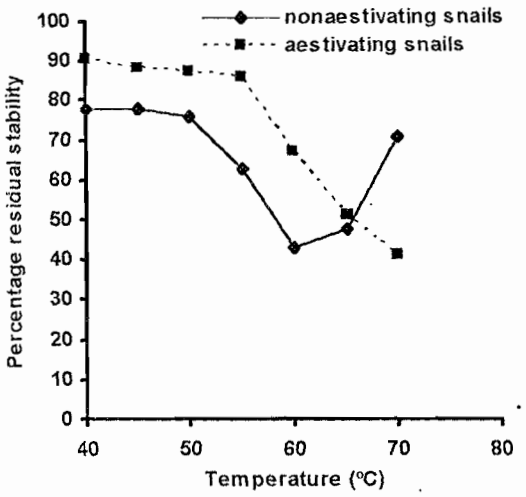

Fig. 4: The effect of temperature on the percentage residual stability of haemocyanin from snails after 3 minutes of incubation

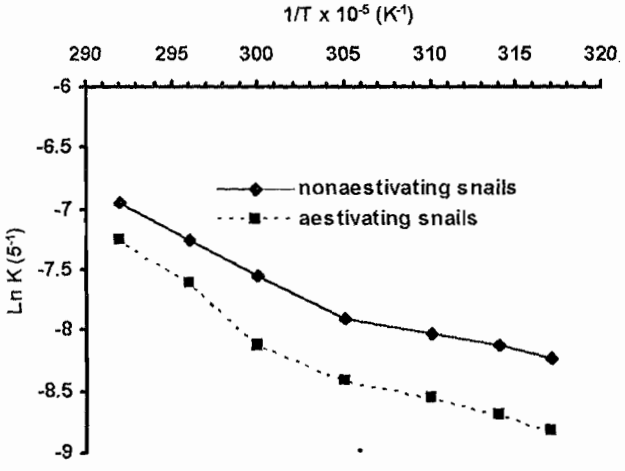

Fig. 5: Arrhenius plots for the thermal denaturation of haemocyanin from snails

rate constant (k) at various temperatures. Fig 5 shows a plot of lnk against 1/T (Arrhenius plots) for Hc from both groups of snails. These plots were clearly non linear and in both cases, the plots were drawn as two straight lines that intercept at 55 °C. The lines in Fig 5 are those of best fit for the data at low and high temperatures. Slopes of the lines in fig 5 were used to calculate the values of the activation energy (Ea) in the Arrhenius equation at both low and high temperatures (Table 2).

Discussion

The increased urea sensitivity of Hc from aestivating *A. achatina* is due to the accumulation of urea by the snails resulting from cessation of excretion during aestivation (Horne, 1971; Rees *et al.*, 1993).

The values of the conformational stability observed for Hc from both groups of snails in the absence of Ca^{2+} are typical of small globular proteins and remarkably similar to those found with proteins of similar sub unit size (Tanford, 1970). These small values are supported by the report of (Hubler *et al.*, 1998) who demonstrated that the quaternary structure of Hc from *E. californicum* is stabilised by hydrophobic and polar forces and that the urea denaturation process consist of the dissociation of the oligomeric Hc into intact sub units at lower concentrations of urea followed by denaturation of the sub units at higher urea concentration. Thus very small amounts of energy are required to break the weak intermolecular forces that keep Hc in its oligomeric form.

Another explanation for the values of the conformational stabilities is that these values are those involved in the disruption of the oxygen binding sites of Hc and not in the disruption of the secondary, tertiary or quaternary structure of the Hc. This is because the band at 340 nm for oxyHc is due to the copper (II)-peroxide complex found at the active site. A comparative denaturation study done at 280nm and 340nm revealed that denaturants (Urea and Guanidine hydrochloride) destabilize the oxygen binding sites of Hc at slightly lower concentrations than the environment of tryptophans and tyrosines (Hubler, 1998). Also compared with the dissociation of *E. californicum* Hc, the loss of oxygen and the changes in the environment of tyrosines and tryptophans occur at higher denaturant concentrations.

Variations in the concentration of Ca^{2+} demonstrate that Ca^{2+} probably plays a secondary role to increase the stability of Hc during aestivation by stabilizing the oxygen binding sites of Hc. In addition to Ca^{2+}, the high conformational stability of Hc from aestivating snails could be attributed to the co-accumulation of osmolyte(s) that counteract the effect of urea in destabilizing the structure of haemolymph proteins during aestivation (Philip, *et al.*, 1996). Such osmolytes include methylamines (trimethylamine oxide, betaine, sarcosine), polyols (inositol, mannitol, sorbitol) and certain amino acids (β-alanine, taurine).

Table 2: Summary of the activation energy of Hc

Temperature Range (^{0}C)	Activation Energy (kcal/mol)	
	Nonaestivating Snails	Aestivating Snails
High (60-70)	14.39	17.66
Low (40-50)	4.64	5.77

Table 3: Summary of activation parameters calculated from the thermal denaturation kinetics of snails

Temperature (^{0}C)	Nonaestivating Snails			Aestivating Snails		
	$\Delta H^{\#}$ (kcal/mol)	$\Delta S^{\#}$ (cal/mol/K)	$\Delta G^{\#}$ (kcal/mol)	$\Delta H^{\#}$ (kcal/mol)	$\Delta S^{\#}$ (cal/mol/K)	$\Delta G^{\#}$ (kcal/mol)
50	4.00	-73.79	27.83	-	-	-
55	-	-	-	5.12	-74.65	29.60
60	13.73	-72.50	37.87	-	-	-
70	-	-	-	16.98	-73.14	42.07

It has been demonstrated that methylamines function by decreasing the solubility of protein side chain groups in contrast to urea, which increases their solubility (Yancey, *et al.,* 1979, 1980). The counteracting effect is maximal at a concentration ratio of 2:1 for urea and methylamines respectively.

The thermal denaturation of Hc from nonaestivating and aestivating snails is biphasic at lower temperatures (Table 3). This is suggestive of a change in denaturation mechanisms or pathways at these temperatures. Hence the biphasic nature of the denaturation process could be explained in two ways: Hc exists in the haemolymph of snails in two or more forms (Akintola *et al.,* 1971), these forms could have different affinities for oxygen such that the disruption of the oxygen binding sites in one form would be less stable and occurs at lower temperatures while the binding sites of the other forms are more stable and denatures only at higher temperatures. Another explanation is that since Hc exists as an oligomer, the disruption of the binding sites of the monomer units could occur at different temperatures due to cooperative binding of oxygen between the monomers.

The melting temperatures of the Hcs; 60^{0}C for nonaestivating and 70^{0}C for aestivating snails are within range with what was obtained by Dolashka *et al.,* (1996) as 65^{0}C for the native and 59^{0}C for the structural subunits of *Rapana thomasiana* Hc. The Tm of 70 ^{0}C for Hc from aestivating snails was far below the 90^{0}C obtained for Hc of *E. californicum*, a desert dwelling spider by earlier workers. (Sterner *et al.,* 1995). As observed by Sterner *et al.,* (1995) the Ca^{2+} mediated state of oligomerisation of Hc from *E. californicum* is responsible for the extreme thermal stability of its Hc. In a similar manner, the increase in haemolymph Ca^{2+} concentration during aestivation should also be responsible for higher thermal stability of Hc from aestivating *A. achatina*.

The discontinuity in Arrhenius plots (55^{0}C) for Hc from nonaestivating and aestivating snails confirms the presence of two sets of melting temperatures (fig. 4). This discontinuity also indicates the presence of two sets of activation energy: 4.64 kcal/mol, 14.39 kcal/mol for nonaestivating snails and 5.77 kcal/mol, 17.66 kcal/mol for aestivating snails. The magnitudes of these activation energies calculated are indicative of the denaturation of a microenvironment in a protein molecule and not the entire conformation of the protein. In Hc, this microenvironment are the oxygen binding sites in which the disruption of the binding sites of Hc from aestivating snails require more energy than that of Hc from nonaestivating snails. This also suggest that the copper (II)-peroxide complex is more stable in oxyHc from aestivating snails than in that from nonaestivating snails.

In conclusion, a comparative study has been done on the stability of Hc from nonaestivating and aestivating *A. achatina* in the presence of denaturants (urea and temperature). The denaturation of Hc from both groups of snails by urea is a one step transition between the native and the completely denatured states. But the thermal denaturation of these Hcs is

biphasic at certain temperatures and involves 2 sets of activation energy before complete denaturation occurs. Above all, Hc from aestivating snails is relatively more stable in the presence of denaturants than that from nonaestivating snails, with Ca^{2+} playing an important role in this stability.

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