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The Conformational Stabilities of Tropomyosins

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Abstract

The stability to denaturation by heat and guanidine hydrochloride of seven vertebrate (including skeletal, cardiac and smooth muscle) tropomyosins and three invertebrate tropomyosins was examined. The transition profiles were discontinuous and in many cases distinct plateaux were observed which indicated the presence of unique partially unfolded states at intermediate temperatures and guanidine hydrochloride concentrations.

The denaturation by guanidine hydrochloride could be described in the majority of cases by a model in which the native state unfolds to a partially unfolded stable intermediate which then unfolds to the completely denatured state. On this basis it was possible to estimate the free energies of unfolding in water. It was shown that part of the α-helical structure of tropomyosin is only marginally stable and the free energy of unfolding in water of this segment is less than values found for globular proteins, whereas another segment (or segments) has a stability comparable to that found for globular proteins. The stepwise unfolding may be explained in terms of the coiled-coil interactions in tropomyosin.

Differences in stability were found between tropomyosins from different muscles of the same species as well as between species, no two tropomyosins giving the same denaturation profiles. The invertebrate tropomyosins showed a wider range of stabilities, that from scallop striated muscle being far more easily denatured than all the others. No correlation was found between the stability of tropomyosin and the type of regulatory system of the muscle. A comparison of the results from vertebrate and invertebrate species suggests that there has been no selection for proteins of higher or lower stability during the evolutionary time scale.

Introduction

The denaturation of fibrous proteins by heat or chemical reagents frequently takes place in separate stages (Noelken and Holtzer 1964; Riddiford 1966; Harrap 1969; Woods 1969*a*; Woods and Pont 1971) and such transitions are more difficult to analyse than those which occur in a single stage. Some of the single-step transitions in globular proteins (Tanford 1968; Pace 1975) have all the characteristics of a two-state process. Even where the denaturation has been shown to deviate substantially from a two-state mechanism, analysis in terms of a two-state model has led to useful conclusions about stability. On the other hand a two-state model was shown to be inadequate to describe the single-step denaturation of rabbit tropomyosin by guanidine hydrochloride (Pont and Woods 1971). This was supported by molecular weight measurements which indicated that the dissociation into subunits did not parallel the unfolding as determined by optical rotation. Spin-label (Chao and Holtzer 1975) and tyrosine fluorescence studies (Cowgill 1968; Satoh and Mihashi 1972; Mihashi 1972) on rabbit tropomyosin provide further evidence that the

denaturation is not of the two-state sort and suggest the presence of α-helical segments of varying stability.

The extent to which the results for the denaturation of rabbit tropomyosin apply to other tropomyosins has not been investigated previously, although some preliminary studies on crayfish tropomyosin (Woods 1968) and abalone tropomyosin (Woods and Pont 1971) showed that they differed in stability from rabbit tropomyosin. In the work reported in the present paper the stabilities of vertebrate tropomyosins from different muscle types from the same animal and from different species are examined with respect to their denaturation by heat and by guanidine hydrochloride. Many of the denaturation curves show a distinct plateau region. Therefore they have been divided into two separate steps and a two-state model applied to each stage. Some results are also reported on tropomyosins from molluscan muscles where the regulation of contraction is different from that in vertebrate muscle (Kendrick-Jones *et al.* 1970; Lehman *et al.* 1973; Lehman and Szent-Györgyi 1975).

Materials and Methods

Preparation of Proteins

The vertebrate tropomyosins were isolated from ethanol–ether dried muscle powders by the method of Bailey (1948). The isoelectric precipitation (pH 4·6 in the presence of 1 M KCl) and ammonium sulphate fractionation between the limits 48–60% saturation were carried out three times. The starting materials were the hind leg and back muscles from rabbits, the back muscle from beef, dog and beef hearts, chicken leg, chicken breast and chicken gizzard.

The invertebrate species used were: oyster, *Crassostrea commercialis*; abalone, *Notohaliotis ruber*; scallop, *Pecten alba*. The white and yellow parts of the oyster adductor muscle, the striated part of the scallop adductor muscle and the foot muscle of the abalone were used. The proteins were prepared by the method of Bailey and Ruegg (1960) as described in a previous publication (Woods and Pont 1971).

All the tropomyosins were examined by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (Weber and Osborn 1969) and the results are shown in Fig. 1. Rabbit tropomyosin was separated into two fractions by chromatography on hydroxylapatite (Bio Gel HTP, Bio-Rad Laboratories) as described by Eisenberg and Kielley (1974). One fraction gave a single band of α-chains on SDS–polyacrylamide gel electrophoresis, and the other comprising both α- and β-chains (Cummins and Perry 1973) gave a double band. Attempts to fractionate beef skeletal tropomyosin and chicken gizzard tropomyosin on hydroxylapatite were not successful which suggests that these tropomyosins may consist of a single species with non-identical subunits. No attempt was made to fractionate the chicken leg tropomyosin.

Optical Rotation

Optical rotatory dispersion measurements were made with a Perkin Elmer spectropolarimeter Model 141, at wavelengths of 365, 405, 436, 546 and 578 nm. Values of a_0 and b_0 from the Moffitt equation were determined by a least-squares linear fit to the data (Urnes and Doty 1961). The dispersion of the refractive index for the buffer salts as a function of temperature was assumed to be the same as that for water which was taken from International Critical Tables (1930). The refractive indices of guanidine hydrochloride were taken from Krivacic and Urry (1971). Small time effects were present in both the guanidine hydrochloride and heat denaturation studies (Woods 1969*a*; Pont and Woods 1971). The guanidine hydrochloride solutions of the protein were prepared and allowed to stand for 3 h before measurements were made. The times chosen to make measurements were such that any residual time effects were small enough to be neglected. For the thermal denaturation studies the temperature was raised in increments of 5°C and readings were made after 15 min; for smaller increments in temperature the time was correspondingly reduced. Changes in concentration due to thermal expansion were neglected. The buffer was 0·2 M NaCl, 10 mM tris–HCl, pH 7·6, with either 50 mM β-mercaptoethanol or 1 mM dithiothreitol except where otherwise indicated.

Amino Acid Analysis

Samples were hydrolysed *in vacuo* for 24 and 72 h at 108°C with 6 M HCl containing 2 mM phenol. Hydrolysates were lyophilized and the amino acids estimated with a Beckman Spinco 120C amino acid analyser. Cysteine was estimated according to the procedure of Inglis and Liu (1970). The amide nitrogen was determined by the method of Inglis *et al.* (1974) and tryptophan by the method of Liu and Chang (1971).

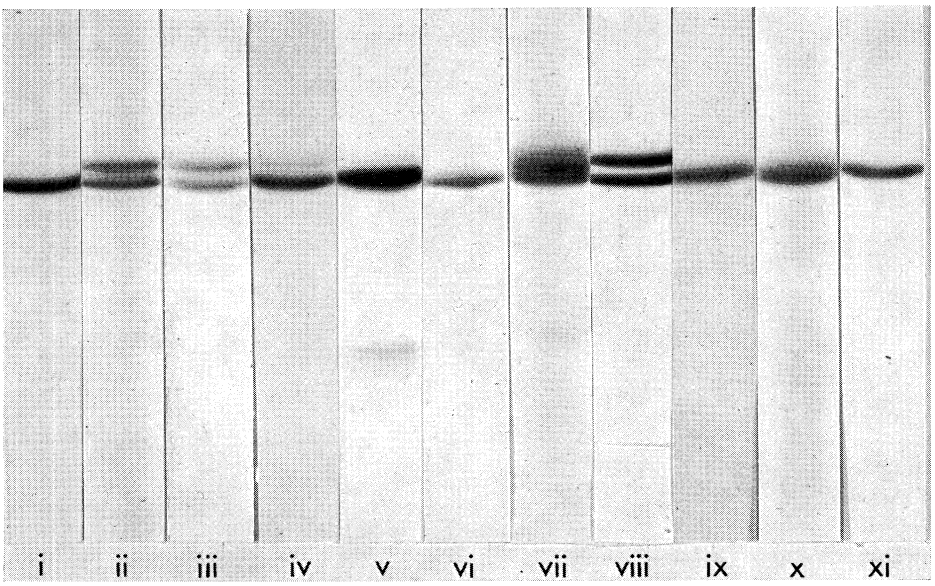
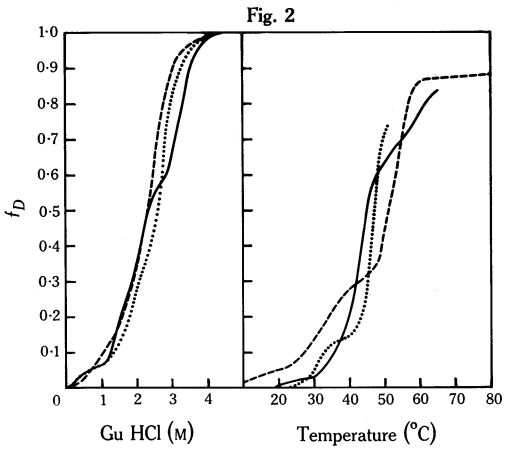


Fig. 1. Gel electrophoresis patterns of tropomyosins. Electrophoresis was carried out in 10% polyacrylamide gels in 0·05 M sodium phosphate buffer at pH 7 containing 0·1% SDS according to the method of Weber and Osborn (1969). (i) and (ii) Rabbit skeletal fractions from hydroxylapatite; (iii) beef skeletal; (iv) beef cardiac; (v) dog cardiac; (vi) chicken breast; (vii) chicken leg; (viii) chicken gizzard; (ix) abalone; (x) oyster; and (xi) scallop tropomyosin.

Results

The results are presented in Figs 2–6 and are given as the fraction of protein denatured, f_D, as a function of temperature and guanidine hydrochloride concentration. The fraction unfolded, f_D, is given by $(b_0 - b_0^{\text{helix}})/(b_0^{\text{coil}} - b_0^{\text{helix}})$, where b_0^{helix} and b_0^{coil} are the characteristic values for the native protein (high ionic strength and low temperature) and the completely denatured form (6 M guanidine hydrochloride) respectively. No dependence of b_0 on temperature in the post- or pretransition regions was observed. Guanidine hydrochloride denaturation always led to complete unfolding of the polypeptide chain with values of b_0 between 0 and -30, whereas thermal denaturation in all cases gave a product that retained from 10 to 15% of the helical conformation, even in the case of the much less stable scallop tropomyosin. If mercaptoethanol or dithiothreitol was omitted the transitions were broader and occurred at slightly higher guanidine hydrochloride concentrations and temperatures as found previously for rabbit tropomyosin (Woods 1969*a*; Satoh and Mihashi 1972). In some temperature studies difficulties arose due to turbidity near 50°C when a reducing agent was present. This was found occasionally with rabbit tropomyosin and always with chicken gizzard tropomyosin and so for the latter protein no thiol

was added for the temperature transition studies. The preparations of chicken leg and chicken breast tropomyosins always became turbid at about 50°C irrespective of the presence of a thiol and so complete temperature transition profiles could not be obtained.



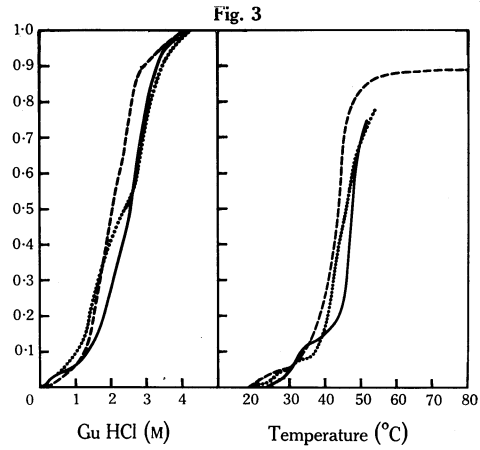
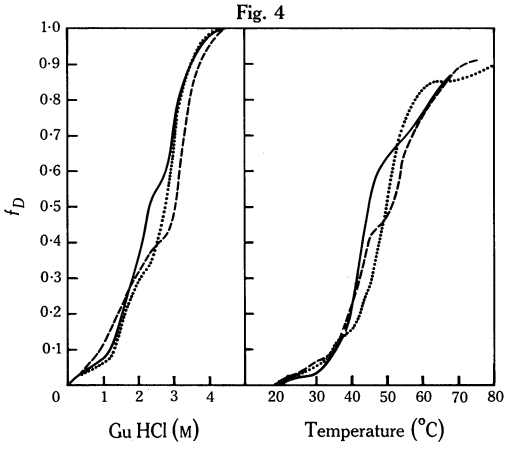


Fig. 2. Fraction of tropomyosin denatured calculated from b_0 values as a function of guanidine hydrochloride (GuHCl) concentration and temperature for beef skeletal (——), rabbit skeletal (– – –) and chicken breast (· · ·) tropomyosins.

Fig. 3. Transition curves for chicken breast (——), chicken gizzard (– – –) and chicken leg (· · ·) tropomyosins.



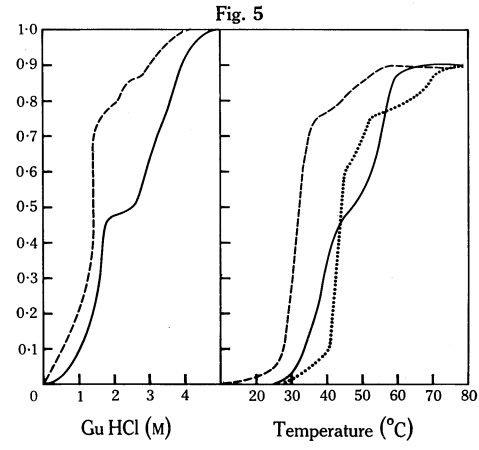


Fig. 4. Transition curves for beef skeletal (——), beef cardiac (– – –) and dog cardiac (· · ·) tropomyosins.

Fig. 5. Transition curves for abalone (——), scallop (– – –) and oyster (· · ·) tropomyosins.

In this and previous work with rabbit tropomyosin (Woods 1969*a*; Woods and Pont 1971) some variability in the original b_0 values and in the early stages of the denaturation profiles have been noted for different preparations. This variability is reduced, but not completely eliminated, if the results are plotted as the fraction

unfolded rather than b_0 values or percentage α-helix. There was no evidence that the conclusions drawn from the results were affected by preparation variability.

The transition curves in guanidine hydrochloride were always reversible and the results obtained by dilution from higher concentrations always fell on the same curve, within experimental error, as those obtained by the addition of guanidine hydrochloride to aqueous tropomyosin solutions. On the other hand as reported previously for rabbit tropomyosin (Woods 1969*a*), reversibility became progressively less on heating above 50°C. Scallop tropomyosin which unfolded at a much lower temperature than all the others could be 85% unfolded reversibly by heating to 45°C. When this tropomyosin was subjected to several heating–cooling cycles between 5°C and 45°C the reverse transitions were identical, within experimental error, but reversibility was progressively lost if the tropomyosin was heated to high temperatures.

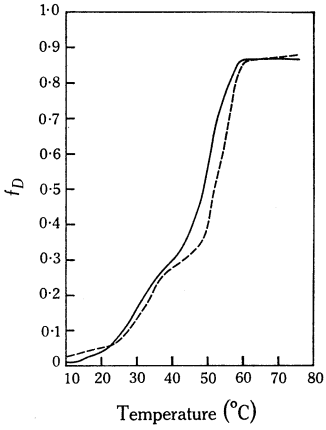


Fig. 6. Temperature transitions for double-banded tropomyosin (——) and single-banded tropomyosin (– – –) from rabbit skeletal muscle.

A feature of many of the profiles presented in Figs 2–6 is that the transitions are discontinuous. Distinct plateaux are observed in many cases, indicating the presence of unique partially unfolded states at intermediate temperatures and guanidine hydrochloride concentrations. Thus all the tropomyosins behave like rabbit tropomyosin in that they do not unfold as a single cooperative unit. Generally, where the profile shows a distinct plateau, this occurs at about the same degree of unfolding for both guanidine hydrochloride and thermal denaturation. It is not appropriate to describe the whole process by the concentration of denaturant ($C_{\frac{1}{2}}$) or by the temperature ($T_{\frac{1}{2}}$) at which the molecule is half unfolded. The free energy of unfolding in the absence of denaturant, $\Delta G_D(H_2O)$ (Tanford 1968, 1970) has proved a useful measure of the stability of globular proteins and Pace (1975) has summarized the results obtained to date. Since no corresponding data have been obtained for fibrous proteins because of the multistage nature of the unfolding, an attempt has been made to treat the unfolding of tropomyosin as the sum of two independent transitions.

The model adopted to describe the results is $N \rightleftharpoons X \rightleftharpoons D$ where N is the native state, X is a partly unfolded stable intermediate and D the denatured state which is the random coil for guanidine hydrochloride denaturation, and an incompletely denatured state (ID) for thermal denaturation. Each of the transitions is analysed in terms of a two-state mechanism characterized by apparent equilibrium constants K_{NX} and K_{XD}. The intermediate X was taken to be the point at which a distinct plateau occurred in the denaturation profile. Where no plateau occurred, for

example gizzard tropomyosin and the thermal denaturation of the two cardiac tropomyosins, the transitions were treated as a single step. The profile of rabbit tropomyosin denatured with guanidine hydrochloride did not show such a well-defined plateau but the transition was divided into two steps on the basis that subunits could not be detected in solution until the guanidine hydrochloride concentration reached about 2 M (Pont and Woods 1971).

The steepness of the transitions with respect to guanidine hydrochloride concentration, C, was investigated by assuming a function of the form, K = constant C^n (Tanford 1968) where K is the appropriate equilibrium constant and n is the slope of the logarithmic plot near the midpoint of the transition. Generally these logarithmic plots were non-linear for the first transition (N to X) and linear for the second transition (X to D). Values of n for the N to X transition were in the range 3–6 and for the X to D transition they varied between 9 and 15. These values indicate a lower cooperativity for the N to X transition than for the X to D transition. If one wishes to determine $\Delta G_D(H_2O)$ the foregoing procedure is inappropriate and Tanford (1970) has pointed out that $\log K$ may be expected to be closer to a linear function of C than of $\log C$. The main advantage of plotting $\log K$ against C is that such a plot can be extrapolated to $C = 0$ to yield values of the equilibrium constant, and hence $\Delta G_{app}(H_2O)$, in the absence of denaturant. The terms ΔG_{app} and K_{app} are used here to indicate that the mechanism of denaturation is uncertain. Pace (1975) recommends that ΔG_{app} be plotted against C and the data fitted to the equation $\Delta G_{app} = \Delta G_{app}(H_2O) + mC$, where m describes the dependence on guanidine hydrochloride concentration. The values of $C_{\frac{1}{2}}$ (the midpoints), $\Delta G_{app}(H_2O)$, and m are given in Table 1 for the two separate postulated steps in the denaturation profiles. A representative plot of $\log K_{app}$ as a function of guanidine hydrochloride concentration is given in Fig. 7 for the two transitions of rabbit tropomyosin. The plots of $\log K_{app}$ (or ΔG_{app}) *v.* C were linear provided $0{\cdot}1 < K_{app} < 10$ and there was no difficulty in extrapolating to zero concentration of denaturant. The one exception was the transition of scallop tropomyosin between 0 and 1·9 M guanidine hydrochloride concentration. The plot of $\log K_{app}$ *v.* C was non-linear and in this case led to some uncertainty in the value obtained for $\Delta G_{app}(H_2O)$. Only one step in this transition was analysed since after the first stage scallop tropomyosin appears to unfold further in a series of small steps.

The thermal transition profiles were also treated by considering the unfolding to take place in two distinct stages, $N \rightleftharpoons X \rightleftharpoons ID$, where X is again a partly unfolded stable intermediate which is not necessarily identical with the intermediate postulated for guanidine hydrochloride denaturation, and ID is the incompletely denatured state. Equilibrium constants for each point of the thermal transitions were calculated from the value of the fraction unfolded and the values of $\Delta G_{app}(H_2O)$ (Table 1) were obtained by extrapolation to 20°C of plots of ΔG_{app} *v.* temperature (T). Van't Hoff plots of $\log K_{app}$ *v.* $1/T$ were also plotted to obtain more information on the nature of the transition. In three instances—the chicken gizzard tropomyosin transition which was treated as a single step, and the main transitions of oyster and scallop tropomyosins—there was evidence of a maximum in the apparent transition enthalpies when plotted against temperature, indicating that these transitions do not follow two-state behaviour (Lumry *et al.* 1966). The thermal denaturation shows greater deviation from a two-state mechanism than denaturation by guanidine hydrochloride

and does not lead to complete unfolding of the polypeptide chains, a feature which is also common to the denaturation of globular proteins (Pace 1975). Less reliance can therefore be placed on derived thermodynamic quantities.

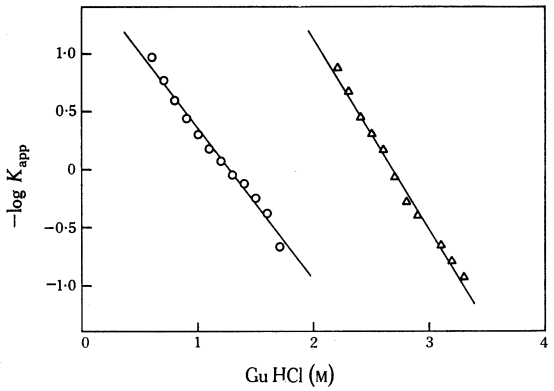


Fig. 7. The negative logarithm of K_{app} as a function of guanidine hydrochloride concentration for rabbit tropomyosin analysed as two transitions from 0 to 1·8 M GuHCl (○) and from 1·8 to 4·5 M GuHCl (△).

Table 1. Parameters defining the gunidine hydrochloride and temperature denaturation of tropomyosins

Tropomyosin	Stage[A]	GuHCl denaturation			Temp. denaturation	
		$C_{\frac{1}{2}}$ (M)	$\Delta G_{app}(H_2O)$[B] (kJ/mol)	m (kJ mol^{-1} M^{-1})	$T_{\frac{1}{2}}$ (°C)	$\Delta G_{app}(H_2O)$[B] (kJ/mol)
Rabbit skeletal	(1)	1·26	8·8	7·1	30·8	6·3
	(2)	2·70	25·9	9·5	54·1	36·8
Beef skeletal	(1)	1·66	11·3	6·1	41·7	11·3
	(2)	3·33	36·4	10·9	59·0	30·9
Beef cardiac	(1)	1·36	8·8	6·5	39·9	6·7
	(2)	3·41	39·7	12·1	56·5	20·1
Dog cardiac	(1)	1·60	11·3	7·0	48·3[C]	10·0
	(2)	3·00	29·3	10·0		
Chicken breast	(1)	1·80	11·7	5·3		
	(2)	2·83	33·0	8·1		
Chicken leg	(1)	1·50	11·3	7·6		
	(2)	3·08	34·3	9·7		
Chicken gizzard[C]		2·04	14·6	7·0	42·8	8·8
Abalone	(1)	1·43	10·5	7·4	37·6	15·0
	(2)	3·40	20·9	7·0	54·8	47·7
Scallop	(1)[D]	1·16	7·9[E]	5·8[E]	30·8	7·5
Oyster	(1)[D]				41·9	16·8

[A] Numbers (1) and (2) refer to the separable stages in the transition.
[B] $\Delta G_{app}(H_2O)$ was measured at 20°C.
[C] There were no obvious separable stages in the profiles and the transition was treated as a single step.
[D] Only the first stage in the transition was analysed and this comprised 75% of the total change.
[E] Plot of log K *v.* C was non-linear leading to uncertainty in $\Delta G_{app}(H_2O)$ and m.

Discussion

The validity of thermodynamic data derived from protein denaturation studies depends on how closely the process approaches a two-state transition. The demonstration that many of the tropomyosins show definite plateau regions in the transition

profiles suggested that the helix to coil transition could be treated as two separate two-state transitions. For the skeletal and cardiac tropomyosins, that is the first six entries in Table 1, it is seen that no two tropomyosins show the same $C_{\frac{1}{2}}$, m, or $T_{\frac{1}{2}}$ values. The first transition to the postulated intermediate state, $N \rightleftharpoons X$ involves the unfolding of one-third to two-thirds of the molecule with a low cooperativity as judged by the values of m. The values of $\Delta G_{app}(H_2O)$ are all in the range 8–13 kJ/mol and these values probably represent a lower limit since any further intermediates in the pathway will cause the slope of plots such as those in Fig. 7 to be less than the slope for a true two-state transition (Tanford 1968; Pace 1975). There are several extrapolation procedures available to obtain $\Delta G_{app}(H_2O)$ and these have been discussed by Pace (1975) who has shown that the values obtained depend on the extrapolation method employed. The linear extrapolation method used here for the guanidine hydrochloride denaturation gave values of $\Delta G_{app}(H_2O)$ which are in reasonable agreement with those derived from the thermal transitions for the stage $N \rightleftharpoons X$. Thus on the basis of these results up to 50% of the tropomyosin helix is not very stable and is only from 6 to 13 kJ/mol more stable than the unfolded state. These values are even less than those found (25–67 kJ/mol) from similar studies on globular proteins.

The physiological significance, if any, of a segment of tropomyosin of low thermal stability is not obvious. The fraction of α-helix unfolded at the body temperatures (these are given by Spector 1956) of the vertebrate species studied in this investigation varies from 0·15 for chicken breast to 0·30 for chicken gizzard tropomyosin. These denaturation studies were carried out in solution at approximately physiological conditions of pH and ionic strength. In the myofibril, tropomyosin is aggregated end-to-end in close association with actin and troponin in the thin filament, and under these conditions tropomyosin may gain additional stabilization. However, the results do suggest that in vertebrate muscles a considerable length of the tropomyosin molecule exists in the non-helical state at body temperature. The increased flexibility imparted by non-helical segments may have some relevance to its function in muscle.

For the second step in the transition, $X \rightleftharpoons D$, the values of m (Table 1) which describe the dependence on guanidine hydrochloride concentration indicate a higher co-operativity of the process. The values of $\Delta G_{app}(H_2O)$ are also higher and similar to those found for the guanidine hydrochloride denaturation of many globular proteins. For the denaturation of rabbit tropomyosin, Pont and Woods (1971) showed that subunit dissociation occurred in this region of denaturation concentration, i.e. above 1·8 M. Their evidence supported the view that the dissociation and conformation changes are linked. In Fig. 8 the data of Pont and Woods (1971) have been used to plot the weight fraction of subunits of molecular weight 34000 as a function of guanidine hydrochloride concentration and this is compared with the fraction unfolded determined from the b_0 values. The values of f_D calculated from b_0 for the change $X \rightleftharpoons D$ do not follow exactly the weight fraction of subunits produced although the agreement is much closer than when the transition was considered as a single step, i.e. $N \rightleftharpoons D$, without any intermediate stages. The broader curve for the optical rotation changes suggests that not all of the unfolding of the α-helix in this region is linked to the dissociation into subunits.

Fig. 6 shows the thermal transition curves for single-banded and double-banded rabbit tropomyosin where it is seen that the single-banded tropomyosin has a slightly

higher thermal stability than double-banded tropomyosin although the shape of the profile is not greatly altered. The trend seems to be borne out with the beef and chicken tropomyosins, where the tropomyosins which show predominantly one band on SDS–polyacrylamide gel electrophoresis are slightly more stable than those which give a double band. The stabilities of the vertebrate tropomyosins fall in a fairly narrow range and the differences in stability, although not large, are significant and must arise from chemical differences within the molecule. Amino acid analyses (Woods 1969*b*; Bodwell *et al.* 1971; Cummins and Perry 1974) show no systematic trends between species although there are some significant differences. The tropomyosins from beef skeletal muscle and beef cardiac muscle have almost identical amino acid compositions and have identical chymotryptic peptide maps (Fine and Blitz 1975), yet both the thermal and guanidine hydrochloride denaturation curves differ. Gel electrophoresis patterns (Fig. 1) do, however, indicate differences in the subunits. Difficulties arise in comparing the stabilities of different tropomyosins because of the multistage nature of the unfolding. This is not overcome by a comparison of the $\Delta G_{app}(H_2O)$ values since the domains which give rise to separable stages in the transitions vary. Thus the domain of lower stability in rabbit tropomyosin involves about one-quarter of the residues whereas three-quarters of the residues are involved in the initial stages of the unfolding of scallop tropomyosin.

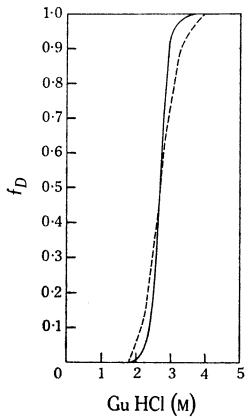


Fig. 8. Comparison of fractional change in b_0 for the second transition of rabbit tropomyosin as a function of GuHCl concentration (– – –) and weight fraction of subunits of molecular weight 34 000 (——).

The three invertebrate tropomyosins, which show only one band when examined by SDS–polyacrylamide gel electrophoresis (Fig. 1), all behaved differently. Abalone tropomyosin showed a distinct plateau in the transition at 50% loss of helix, whereas oyster and scallop were 75% unfolded before there was evidence of a plateau in the profile. Scallop tropomyosin shows a marked difference to all others studied in its ease of denaturation. The lower thermal stability may be related to the environmental temperature at which the species exists. There are several reported instances of a correlation between the stability of proteins (usually taken as the temperature at which the molecule is half unfolded) and the preferred temperature range at which species exist. These include the thermal transitions of collagen (Rigby and Robinson 1975), the denaturation of skeletal muscle myosins by heat and urea (Connell 1961), and the thermostability of fish myofibrillar ATPases (Johnston *et al.* 1973). However, more data would be required to test this hypothesis for tropomyosins.

Parry (1975) has suggested that the α-helix-favouring potential of the amino acids may be correlated with molecular stability. The only tropomyosin whose sequence is known is that of rabbit, so comparison of the helix-forming tendencies can only be made on the basis of the total amino acid composition of the proteins (Table 2), neglecting the co-operative interactions between amino acids. The helix information parameters for the amino acids are given by Robson and Pain (1971) and revised by Suzuki and Robson (E. Suzuki, personal communication). The total helix information was calculated for these three tropomyosins and is given by the sum of the values for each residue multiplied by the residue percentage. The asparagine and glutamine contents are known for rabbit tropomyosin from the sequence, and the total amide values of oyster and scallop tropomyosins were assumed to be proportionately distributed between aspartic and glutamic acids. The values found, in decinats/100 residues, were rabbit 273, oyster 239, and scallop 213. These predictions from the amino acid composition correlate with the observed stabilities to denaturation.

Table 2. Amino acid composition of some tropomyosins

Values are expressed as residues per 100 amino acid residues. n.d., Not determined

Amino acid	Rabbit[A] tropomyosin	Scallop tropomyosin	Oyster[B] tropomyosin	Helix information[C] (decinats)
Lys	13·73	10·39	10·03	+2·3
His	0·70	0	0·37	+1·2
Arg	4·93	5·59	6·51	−0·9
Cys	0·35	0	0·23	−1·3
Asp	8·45	9·86	8·23	+0·5
Asn	1·76	4·43	3·48	−5·1
Thr	2·82	7·15	4·77	−2·6
Ser	5·28	3·75	5·29	−3·9
Glu	19·72	16·57	18·58	+7·8
Gln	4·93	7·44	7·84	+1·0
Pro	0	0	0	−7·7
Gly	1·06	1·45	0·78	−8·6
Ala	12·68	11·53	11·04	+6·5
Val	3·17	4·06	3·43	+1·4
Met	2·11	1·26	1·77	+5·3
Ile	4·23	2·79	4·14	+0·6
Leu	11·62	11·09	10·77	+3·2
Tyr	2·11	1·27	1·99	−4·5
Phe	0·35	1·40	0·75	+1·6
Trp	0	0	n.d.	+1·2

[A] Taken from the sequence of the α-chain of rabbit tropomyosin (Stone *et al.* 1975).
[B] This analysis agrees with a previously published analysis for this protein (Woods and Pont 1971).
[C] Personal communication from E. Suzuki (see text). The helix information of Robson and Pain (1971) is expressed in nats.

Parry (1975), who used different helix parameters to those given in Table 2, cited experimental evidence in favour of his predictions that an *N*-terminal cyanogen bromide fragment of rabbit tropomyosin is more stable than the *C*-terminal end. Eckard and Cowgill (1976), on the other hand, found experimentally that a

C-terminal fragment prepared by tryptic digestion is more stable than the *N*-terminal end, which disagrees with the predictions made from the helix-forming tendencies of the amino acids. Whilst the helix-forming tendencies of the amino acids are undoubtedly important for the self-organization and stability of fibrous proteins, it would appear that this approach is not very satisfactory in explaining, let alone predicting, differences in stability between different proteins and between segments of the one molecule. A far better explanation seems to be in terms of the hydrophobic interactions between the two strands of the coiled-coil structure and this is now discussed.

It is often stated that the denaturation of the α-helical fibrous proteins is more like the helix–coil transition of synthetic polypeptides than the unfolding of globular proteins. Homopolypeptides do not conform to the two-state model and the unfolding is usually a progressive process of lower cooperativity compared with the denaturation of globular proteins (Brandts 1969). Tropomyosin is a rod-shaped molecule of molecular weight 68 000 and consists of two parallel α-helical polypeptide chains of about equal molecular weight (for review see Hodges *et al.* 1973). The sequence of the principal component of rabbit tropomyosin has recently been determined (Stone *et al.* 1975). Direct experimental evidence (Johnson and Smillie 1975; Lehrer 1975; Stewart 1975) as well as stereochemical arguments (McLachlan and Stewart 1975) favour a molecule in which the chains are in register. On the basis of its X-ray diffraction pattern, tropomyosin is generally regarded as a two-stranded coiled-coil structure (for review see Fraser and MacRae 1973). The coiled-coil interactions have recently been analysed by McLachlan and Stewart (1975). The sequence shows non-polar residues occurring in two series at intervals of every seven residues (Stone *et al.* 1975). The supercoiling allows close packing of the non-polar side chains of the two helices and this arrangement also allows favourable interactions between positive and negative charges between the helices. Thus the non-polar residues occur largely at the interface between the helices and the charged residues occur on the outside of the supercoil. The factors determining stability are thus seen to be really no different to those for globular proteins and we may expect similar behaviour. A few amino acid replacements could markedly alter the susceptibility of the coiled-coil structure to denaturation, and could account for the differences between the skeletal tropomyosins which are very similar in amino acid compositions and have identical chymotryptic peptide maps. McLachlan and Stewart (1975) have pointed out that the hydrophobic interactions would be stronger at some places than others and they thus account for the uneven denaturation of the tropomyosins. Part of the α-helical structure of tropomyosin is only marginally stable, the free energy of unfolding in water being less than the values usually observed for globular proteins, and there is a segment (or segments) of stability comparable to that found for globular proteins. This more stable part of the molecule appears to be concerned with the association of the two polypeptide chains to form a two-stranded α-helical molecule (Woods and Pont 1971). The existence of domains which differ sufficiently in stability to give separate stages in the denaturation profiles is not peculiar to the fibrous proteins. This has been observed in the urea denaturation of the immunoglobulin light chains (Azuma *et al.* 1972) which are considerably less stable than most globular proteins, and muscle phosphorylase *b* (Chignell *et al.* 1972). The fibrous proteins also behave similarly to globular proteins in that thermal denaturation does not bring about complete unfolding.

Although some of the tropomyosins studied here are from mixed muscle types there does not appear to be any correlation between muscle type and stability. The tropomyosins investigated were representative of red and white skeletal, cardiac and smooth muscle and include the different regulatory systems. Vertebrate skeletal and cardiac muscles are regulated by means of the tropomyosin–troponin system on the actin filament; the regulatory system of gizzard muscle is reported to be different to that of skeletal muscle (Bremel 1974; Driska and Hartshorne 1975; Ebashi 1975), and the molluscan species are regulated by small subunits of the myosin molecule (Szent-Gyorgyi *et al.* 1973). It is apparent that appreciable variation in stability is allowed without alteration in biological function. Thus scallop tropomyosin which is half unfolded at a temperature 16°C below that of rabbit tropomyosin can replace it in a synthetic rabbit regulatory system (Lehmann *et al.* 1973). The results are in fact similar to those of Puett *et al.* (1973) for myoglobins where it was shown that homologous proteins can differ appreciably in their stability and that there appeared to be no selection for proteins of higher or lower stability during the evolutionary time scale. This is not meant to imply that biological differences do not exist between tropomyosins from various sources. Studies on the effect of hybrid tropomyosin–troponin complexes in actomyosin systems *in vitro* would need to be investigated.

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