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Stability of Segments of Rabbit α-Tropomyosin

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Abstract

Rabbit α-tropomyosin was cleaved into two pieces at the cysteine residue of each chain. The products were separated by chromatography and characterized by amino acid analysis, molecular weight determination in benign and denaturing solvents, optical rotation and circular dichroism. When the cleavage reaction was carried out under mild conditions which preserve the two-chain structure there was considerable loss of α-helix in each segment.

Thermal stability studies, monitored by optical rotation and circular dichroism, showed that the transition temperature of the *N*-terminal fragment at pH 7·6 was approximately 17°C higher than that of the *C*-terminal fragment. In acid solutions there is little difference in the thermal stability of the two segments. The least stable part of the molecule is concluded to be between residues 133 and 205 and this includes the troponin-binding site. The relative stabilities found for segments of rabbit α-tropomyosin differ from recent published conclusions and this may be a result of the different methods used to study the loss of the α-helical conformation.

The two tropomyosin fragments, unlike the parent tropomyosin, do not inhibit actomyosin adenosinetriphosphatase when mixed with troponin. The fragments did not show any of the aggregation properties of tropomyosin and did not combine with actin. The *N*-terminal fragment did not complex with troponin but there was some evidence for an interaction between the *C*-terminal fragment and troponin.

Introduction

The unfolding of tropomyosin by heat or guanidine hydrochloride has recently been explained by the existence of domains which differ sufficiently in stability to give separate stages in the denaturation profiles (Woods 1976). This model does not distinguish between two possibilities. These are the existence of either a small number of separate domains of the molecule which unfold sequentially and involve long sequences of α-helix or a large number of short lengths of helix which unfold all along the molecule. One approach to this problem is to cleave the molecule into fragments either by enzymes or by specific chemical means and examine the stability of the separated fragments. This has been applied successfully to the helical proteins paramyosin (Cowgill 1972; Halsey and Harrington 1973) and the myosin rod and its smaller segments, light meromyosin and subfragment II (Burke *et al.* 1973).

Eckard and Cowgill (1976) have examined fragments produced by the tryptic digestion of rabbit tropomyosin and found that the *C*-terminal portion of the molecule was the most stable part. This is contrary to predictions on molecular stability deduced from the α-helix-favouring potential of the amino acids (Woods 1976) and also contrary to some other experimental evidence (Parry 1975). This paper reports the separation and characterization of fragments of α-tropomyosin prepared by

cleavage at its single cysteine residue by the method of Jacobson *et al.* (1973). Rabbit skeletal tropomyosin consists of species with two types of polypeptide chains, α and β, in the ratio of about 3·5:1 (Cummins and Perry 1973). The main difference between them is that the β-chain contains two cysteine residues and the α-chain only one. Tropomyosin containing only α-chains, prepared by chromatography on hydroxylapatite (Eisenberg and Kielley 1974), was therefore used in this study and is referred to as α-tropomyosin.

Materials and Methods

Isolation of Proteins

The hind leg and back muscles from rabbits were used for the preparation of ethanol–ether dried muscle powders (Bailey 1948). The dried powder was extracted overnight at 4°C with 1 M KCl, 25 mM tris–HCl, 10 mM EDTA, 0·1 mM dithiothreitol, pH 8 (1 litre per 500 g of original muscle). After filtration through cheese cloth the residue was further extracted with half the volume of 1 M KCl for 2 h. The combined extracts were precipitated at pH 4·6 by the addition of 1 M HCl, the precipitate dissolved in 0·1 M KCl and the pH brought to 8 with tris. The fraction precipitating between 40 and 60% saturation with $(NH_4)_2SO_4$ was collected, dissolved in water and dialysed successively against several changes of water, 1 M KCl and finally 1 M KCl, 5 mM potassium phosphates at pH 7. α-Tropomyosin was separated by chromatography on an hydroxylapatite (Bio Gel HTP, Bio-Rad Laboratories) column (3·8 by 20 cm) equilibrated with 1 M KCl, 5 mM potassium phosphates at pH 7. 500–700 mg of protein were applied to the column which was eluted with a linear gradient to 0·2 M potassium phosphates in 1 M KCl at pH 7. The conditions were similar to those described by Eisenberg and Kielley (1974) except that dithiothreitol was not included in the buffer. Chromatography was carried out at room temperature in the presence of 0·02% sodium azide. Fractions which gave a single band on sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis were pooled, and the protein recovered by precipitation with HCl at pH 4·6 and rechromatographed on hydroxylapatite on a smaller column (2·5 by 38 cm).

Troponin was isolated from the pH 4·6 supernatant from the tropomyosin preparation. After adjustment to pH 7 the fraction which precipitated between 40 and 70% $(NH_4)_2SO_4$ saturation was collected. The acid precipitation and $(NH_4)_2SO_4$ fractionation were repeated, and this time the cut from 40 to 60% saturation was kept. In order to remove any proteolytic degradation products the troponin was further purified by chromatography on DEAE cellulose by the procedure of Van Eerd and Kawasaki (1973). The product contained only the three subunits of troponin when examined by SDS–polyacrylamide gel electrophoresis.

Actin free from tropomyosin was prepared from an acetone powder of rabbit muscle by the method of Spudich and Watt (1971). Myosin was prepared from the hind leg and back muscles by extraction at 2°C with Guba–Straub solution. After two precipitations by dilution to 0·05 ionic strength the myosin was dissolved in 0·5 M KCl, adjusted to pH 6·9 with $NaHCO_3$ and fractionated with $(NH_4)_2SO_4$, keeping the fraction between 40 and 50% saturation. Actomyosin was obtained by mixing myosin and actin in a 4:1 weight ratio. It was precipitated by dilution with 9 volumes of 2 mM tris–HCl, pH 7·6, and the precipitate was washed twice with the same solution and finally dissolved in 0·5 M KCl.

Cleavage at Cysteine Residue and Separation of Fragments

The method of Jacobson *et al.* (1973) was employed. The protein (5 mg/ml) at pH 8 in 0·2 M tris–HCl was treated with 1 mM dithiothreitol at room temperature for 30 min. A fivefold excess of 2-nitro-5-thiocyanobenzoic acid (Degani and Patchornik 1971) over total thiol was added, the pH adjusted to 8 and the mixture allowed to stand for 30 min. The mixture was then acidified with glacial acetic acid to pH 4 and dialysed against several successive changes of 30% (v/v) acetic acid. The protein was freeze dried, dissolved in 0·1 M tris–HCl, pH 9, and left at room temperature for 7–8 h. The reaction mixture was then dialysed against water and the appropriate buffer prior to the separation of the fragments. In initial experiments both the conversion of the cysteine to *S*-cyanocysteine and the cleavage reaction were carried in the presence of 4 M guanidine hydrochloride. Chao and Holtzer (1975) have shown that denaturation at the sulphydryl sites is complete at 1·5 M guanidine hydrochloride and at this concentration the two-chain structure of tropomyosin is still intact (Pont

and Woods 1971). Therefore, in order to preserve the two-chain structure as far as possible, the reactions were carried in guanidine hydrochloride concentrations between 1·5 and 2·0 M. However, the yields of split products were lower (*c.* 40%) compared to *c.* 80% when 4 M guanidine hydrochloride was incorporated in the reaction mixture. In some experiments (indicated in the results) the modification was carried out sequentially using 5,5′-dithiobis(2-nitrobenzoic acid) and then KCN (Jacobson *et al.* 1973).

The split products were separated by chromatography on hydroxylapatite as for the preparation of α-tropomyosin. The protein was recovered from the pooled fractions by precipitation with 7% (v/v) trichloroacetic acid since precipitation with HCl at pH 4·6 was incomplete. Some fractionations were also carried out on a DEAE cellulose column (2 by 20 cm) equilibrated with 7 M urea–50 mM tris–HCl, pH 8, and elution was with a linear gradient to 0·2 M NaCl in the buffer.

Physical Methods

Sedimentation experiments were carried out with a Beckman Model E ultracentrifuge equipped with both Rayleigh interference and Schlieren optics. Molecular weights were measured by the meniscus depletion method (Yphantis 1964) employing column heights of 3 mm. The point-average molecular weights down the column were calculated by the Roark and Yphantis (1969) computer program. A value of 0·739 ml/g was used for the partial specific volume, $\bar{v}$, of the fragments in salt solutions and 0·728 ml/g in 6 M guanidine hydrochloride. These values are for tropomyosin measured by Kay (1960) on dialysed solutions in benign and denaturing solvents respectively. The theoretical values of $\bar{v}$ for the fragments calculated from the amino acid compositions are almost identical to those for tropomyosin. Molecular weights were measured in 30 mM tris–HCl, pH 7·6, at several ionic strengths by the addition of NaCl and in 6 M guanidine hydrochloride.

Optical rotatory dispersion (ORD) measurements were made with a Perkin Elmer spectropolarimeter Model 141 at wavelengths of 365, 405, 436, 546 and 578 nm. Values of b_0 from the Moffit equation were determined by a least-squares linear fit to the data (Moffitt 1956). The mean residue weights were calculated from the amino acid compositions and a λ_0 value of 212 nm was used. The temperature transitions were carried out as described in a previous paper (Woods 1976). Measurements of ORD at lower wavelengths and of circular dichroism (CD) were made with a Jasco instrument ORD/UV5 with a modified CD attachment and in some later experiments the temperature transitions were followed by the ellipticity at 222 nm, $[\theta]_{222}$, or the mean residue rotation at the Cotton trough at 232 nm (m'_{232}).

Ultraviolet absorption measurements were made with a Beckman DB spectrophotometer. Protein concentrations were measured refractometrically using a value of 0·188 ml/g for the specific refractive increment for tropomyosin (Kay 1960) and its fragments.

Electrophoresis in the presence of SDS was carried out in 10% polyacrylamide gels by the technique of Weber and Osborn (1969). Electrophoresis was also carried out in the tris–glycine discontinuous buffer system (in the absence of SDS) of Davis (1964) in 7·5% polyacrylamide gels but without a stacking gel.

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 modified Beckman Spinco 120 C amino acid analyser. Lanthionine was estimated according to the procedure of Inglis and Nicholls (1968).

Synthetic Actomyosin Adenosinetriphosphatase

The Mg^{2+}-adenosinetriphosphatase activity of synthetic actomyosin in the presence of mixtures of troponin and tropomyosin or its derived segments was measured under the conditions used by Greaser and Gergely (1971). Experiments were carried out with the addition of either Ca^{2+} or EGTA and the assay conditions are given in Table 3.

Actin Binding

The ability of tropomyosin and the fragments to bind to F-actin was tested by the cosedimentation method of Hitchcock *et al.* (1973). The samples were mixed with F-actin (1 mg/ml) in 0·05 M NaCl, 0·01 M imidazole, 2 mM $MgCl_2$ at a molar ratio of tropomyosin or fragments:actin of 1:5 and centrifuged at 150 000 *g* for 3 h. The pellets were rinsed in buffer, redispersed in the same buffer and sedimented again. The pellets were then examined by SDS–polyacrylamide gel electrophoresis.

Results

Isolation of N-*terminal Fragment by Chromatography in 7* M *Urea*

The material for this experiment was prepared by modification of α-tropomyosin by the two-step procedure with 5,5′-dithiobis(2-nitrobenzoic acid) and KCN. The reaction and cleavage were carried in the presence of 4 M guanidine hydrochloride. Under these conditions the protein is dissociated into its constituent polypeptide chains. After cleavage the protein was dialysed against 7 M urea–50 mM tris–HCl,

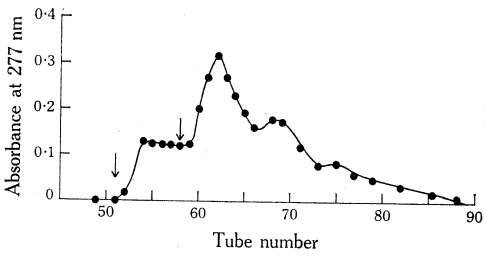

Fig. 1. Chromatography of tropomyosin cleavage products on DEAE cellulose in 7 M urea, 50 mM tris–HCl, pH 8. A linear gradient of NaCl in the starting buffer from 0 to 0·2 M was applied. Column size 2 by 20 cm, fraction size *c.* 10 ml. Tubes 50–58 comprised pure *N*-terminal fragment and were pooled (see text).

pH 8, and chromatographed on DEAE cellulose equilibrated with the same buffer (Fig. 1). The material which was eluted off the column first gave a single band on SDS–polyacrylamide gel electrophoresis, corresponding to a polypeptide chain of molecular weight about 20 000. All the other tubes contained both the cleaved

Table 1. Amino acid composition of tropomyosin segments

Amino acid	*N*-Terminal fragment Residues per 189 residues			*C*-Terminal fragment Residues per 94 residues	
	Found[A]	Found[B]	Expected[C]	Found[B]	Expected[C]
Lys	25·7	26·8	26	13·1	13
His	1·0	1·1	1	1·0	1
Arg	12·0	11·4	12	2·2	2
Asp	18·9	19·5	19	10·2	10
Thr	3·1	3·7	3	4·8	5
Ser	7·4	7·8	8	6·7	7
Glu	49·2	47·0	48	21·8	22
Gly	3·1	2·9	3	0·2	0
Ala	26·9	26·2	27	9·1	9
Val	5·5	6·2	6	3·1	3
Met	4·6	4·8	5	1·0	1
Ile	7·5	7·4	8	3·9	4
Leu	21·1	21·7	21	11·9	12
Tyr	2·1	2·3	2	4·0	4
Phe	Trace	0·3	0	1·0	1

[A] Prepared by chromatography on DEAE cellulose in 7 M urea.
[B] Prepared by chromatography on hydroxylapatite in 1 M KCl.
[C] Values expected from the sequence (Stone *et al.* 1975).

products and tropomyosin. Subsequent rechromatography with a shallower salt gradient did not produce further fractionation. The protein from tubes 50–58 was recovered for characterization. Its molecular weight determined by sedimentation equilibrium was 44 000 in 1 M NaCl at neutral pH. Some heterogeneity was evident;

the range of molecular weight values in a meniscus depletion experiment varied from 39 000 to 48 000. In 8 M urea the molecular weight was 20 000. The value of b_0 from a Moffitt plot was −418 deg cm^2/dmol corresponding to about 65–70% α-helix. The amino acid analysis of the fragment is given in Table 1. The almost complete absence of phenylalanine, and the tyrosine, histidine and glycine contents identify this fragment as arising from the *N*-terminal end of the molecule when compared with the expected values from the sequence (Stone *et al.* 1975). The molecular weight results are in agreement with this. Although chromatography in 7 M urea gave a pure *N*-terminal fragment it was considered preferable to isolate the fragments under non-denaturing conditions, therefore chromatography on hydroxylapatite was preferred.

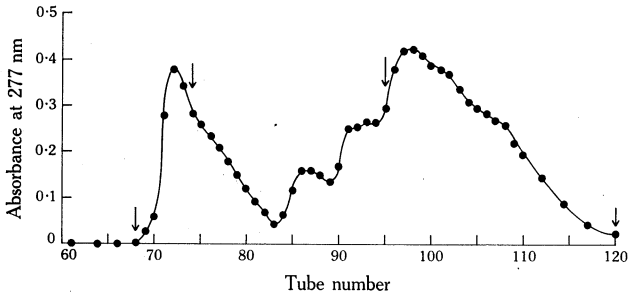

Fig. 2. Chromatography of tropomyosin cleavage products on hydroxylapatite in 1 M KCl–5 mM potassium phosphates, pH 7. A linear gradient to 0·2 M potassium phosphates in 1 M KCl was applied. Column size 2·5 by 36 cm, fraction size *c.* 7·5 ml. Gels of selected tubes are shown in Fig. 3. Arrows indicate the pools made for recovery of fractions referred to in text.

Separation of Fragments by Chromatography on Hydroxylapatite

In these and subsequent experiments the tropomyosin was modified and cleaved in the presence of 1·8 M guanidine hydrochloride. The results of chromatography on hydroxylapatite in 1 M KCl are shown in Fig. 2. SDS–polyacrylamide gels of tubes from the column are shown in Fig. 3. Tubes 68–74 were pooled and concentrated to give the pure fastest moving band. Tubes 96–120, which corresponded to unchanged tropomyosin, were also pooled and the protein recovered. The b_0 value of this fraction

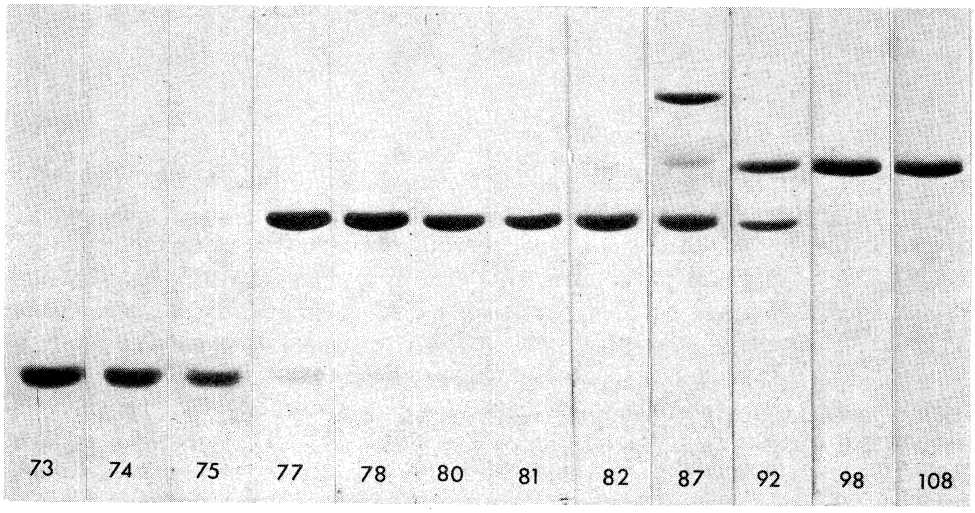

Fig. 3. SDS–polyacrylamide gels of tubes from hydroxylapatite chromatography shown in Fig. 2. Tube numbers are shown at the base of each tube.

was -640 deg cm^2/dmol, which is identical to that of the original tropomyosin. The middle fraction (tubes 75–95) was rechromatographed and those tubes which showed mainly the band of molecular weight 20 000 on SDS–polyacrylamide gel electrophoresis were pooled and rechromatographed. In addition the fractions which contained the band moving slower than tropomyosin (corresponding to tube 87 in Fig. 3) were recovered. The origin of the higher-molecular-weight material is not known. The conditions of cleavage are favourable for the β-elimination of thiocyanate which yields the dehydroalanyl residue which can then react with any free SH groups to form a lanthionine residue, thus crosslinking the two chains. However, analysis for lanthionine by the procedure of Inglis and Nicholls (1968) at high column loadings failed to detect the presence of this amino acid.

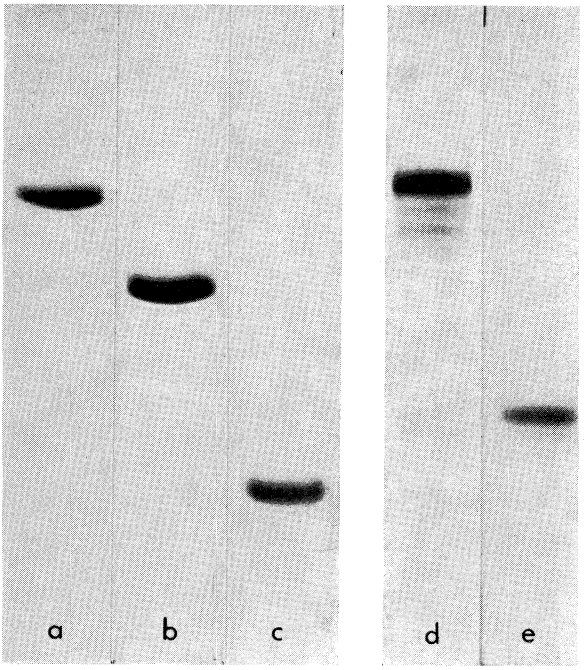

Fig. 4. Gel electrophoresis of purified fractions. (*a*)–(*c*) SDS–polyacrylamide gels, (*d*) and (*e*) tris–glycine polyacrylamide gels. (*a*) α-Tropomyosin, (*b*) and (*d*) *N*-terminal fragment, (*c*) and (*e*) *C*-terminal fragment.

Characterization of the Fragments

Gel electrophoresis of the two purified fragments is shown in Fig. 4. On SDS–polyacrylamide gels the purified fragments move with molecular weights corresponding to about 20 000 and 10 000. On high gel loadings the larger fragment shows a faint band moving just ahead of the main band and also some contamination with the smaller fragment. The small fragment shows no contamination with the larger piece. In the tris–glycine electrophoresis system both of the fragments show some heterogeneity which probably arises from charge differences. This is not evident in the SDS–polyacrylamide gels where separation is on the basis of size. The heterogeneity may be due to heterogeneity of the original protein, as Cummins and Perry (1973) have shown that on isoelectric focussing α-tropomyosin consists of two components. It is also possible that some amide groups could be lost during the cleavage (pH 9 for 8 h) leading to charge heterogeneity.

The origin of these fragments is confirmed by the amino acid analysis given in Table 1 which also shows the values expected from the sequence (Stone *et al.* 1975) for fragments arising by cleavage at the cysteine residue. The expected amino acid analyses have been calculated for 189 residues and 94 residues respectively. Although there are 95 residues in the *C*-terminal fragment the product of the cleavage reaction at its *N*-terminal end (2-imido-thiazolidine-4-carboxylic acid) does not give a colour with the ninhydrin reagent. If the modification and cleavage are specific and the subsequent separation procedures are ideal then there should be no glycine in the *C*-terminal fragment and no phenylalanine in the larger *N*-terminal piece. The *C*-terminal fragment does contain a trace of glycine which suggests possible contamination with the *N*-terminal part even though gel electrophoresis at high loadings did not reveal any larger fragment. The *N*-terminal fragment shows some phenylalanine and this confirms the gel electrophoresis which shows that this fragment is not completely free of the smaller one. However, the contents of arginine, glycine, phenylalanine, methionine and tyrosine of the two fragments are conclusive evidence of their origin. The absorbance at 277 nm of a 10 mg/ml solution of the *C*-terminal fragment was 5·6, and of the *N*-terminal fragment 2·0. The ratio of these two values is slightly less than expected from the tyrosine contents.

The molecular weight of the *N*-terminal fragment determined by sedimentation equilibrium in 1 M NaCl was 44 500 (cf. value expected from sequence is 43 460) with little evidence of heterogeneity. In 6 M guanidine hydrochloride the molecular weight showed a slight decrease down the column and the value extrapolated to the meniscus was 19 500. This is somewhat less than the expected value (21 730) but can be explained by the presence of 15–20% of the *C*-terminal fragment, a result which is consistent with the amino acid analysis and gel electrophoresis patterns. The *C*-terminal fragment gave a value of 23 900 for the molecular weight in 1 M NaCl (cf. expected value of 22 120) with no indication of heterogeneity. In 6 M guanidine hydrochloride the molecular weight was 11 300 (cf. expected value of 11 060) with also no evidence of heterogeneity.

Below ionic strength 0·6, tropomyosin polymerizes by an end-to-end association (Tsao *et al.* 1951). The molecular weights of the fragments were the same at ionic strength 0·06 as found in 1 M NaCl, indicating that the polymerizing properties of the parent tropomyosin were lost by cleavage at the cysteine residue. The fragments were not precipitated by magnesium chloride under conditions where tropomyosin forms ordered aggregates (Caspar *et al.* 1969), and the 5–7% increase in molecular weight was not considered to be significant. Examination of the solutions with an electron microscope after the addition of magnesium chloride did not show any evidence of tactoids.

Optical Activity and Temperature Transitions of the Fragments

The results of the optical rotatory dispersion and circular dichroism measurements are summarized in Table 2 which gives the percentage α-helix for tropomyosin and the two derived fragments at 5 and 20°C. Since α-tropomyosin has been shown to be more than 90% helical at 25°C (Wu and Yang 1976) the values of b_0, m'_{232} and $[\theta]_{222}$ at 5°C were taken to represent 100% helix. The values of these parameters for α-tropomyosin in 6 M guanidine hydrochloride were taken to be representative of the unordered form and, in order to calculate the percentage of α-helix, it was assumed

that only the α-helix and unordered forms were present. All the parameters indicate that the helical contents of α-tropomyosin and the two derived segments are higher at 5 than at 20°C. This difference is reproducible on repeated measurement and is outside the experimental error of the optical measurements.

Table 2. α-Helical content of tropomyosin fragments

	Temperature (°C)	Percentage α-helix b_0	m'_{232}	$[\theta]_{222}$
α-Tropomyosin	5	100	100	100
	20	94	93	95
N-terminal fragment	5	74	68	69
	20	69	60	63
C-terminal fragment	5	88	81	81
	20	82	74	72

The thermal denaturation curves for the two fragments are shown in Fig. 5, the transition being followed by the changes in $[\theta]_{222}$ in one experiment and in the b_0 value in another. Both methods show that the *N*-terminal fragment is of higher stability than the *C*-terminal fragment, the temperatures at the mid-points of the transitions being approximately 48 and 31°C respectively. The circular dichroism

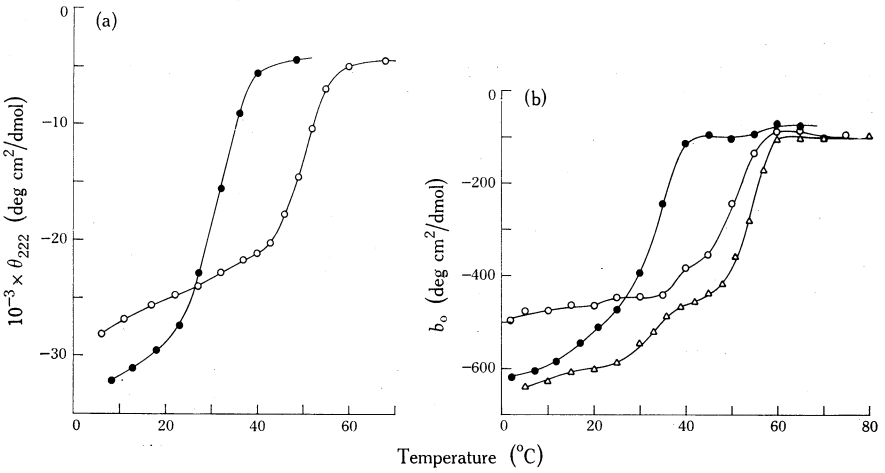

Fig. 5. Thermal denaturation of tropomyosin and the two fragments at pH 7·6 in 0·2 M NaCl, 10 mM tris–HCl, pH 7·6. (*a*) $[\theta]_{222}$. (*b*) b_0. ● *C*-Terminal fragment. ○ *N*-Terminal fragment. △ α-Tropomyosin.

results indicate a small loss of α-helix after heating and cooling to 5°C. Fig. 5(*b*) also shows the curve for α-tropomyosin where it is seen that the discontinuity in the transition profile correlates with the transition profiles of the two segments. Fig. 6 shows the temperature transition curves for the fragments and tropomyosin in 0·02 M HCl, the transition being followed by the change in mean residue rotation at 232 nm.

Biological Activity and Interaction of the Fragments with Actin and Troponin

Table 3 shows the results of the Ca^{2+} sensitivity experiments where it is seen that troponin in combination with α-tropomyosin inhibits the adenosinetriphosphatase activity in the absence of Ca^{2+}. When α-tropomyosin is replaced by the *N*-terminal fragment no inhibition occurs. When the *C*-terminal fragment replaces α-tropomyosin there is a small inhibition which is of doubtful significance.

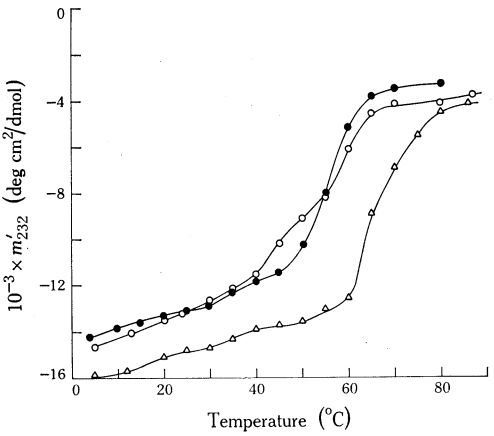

Fig. 6. Thermal denaturation of tropomyosin and its fragments in 0·02 M HCl followed by mean residue rotation at 232 nm.
● *C*-Terminal fragment.
○ *N*-Terminal fragment.
△ α-Tropomyosin.

Table 3. Relative adenosinetriphosphatase [ATPase] activities of skeletal actomyosin plus troponin with tropomyosin or its cleavage products

Assay conditions: 25 mM tris–HCl, pH 7·5; 25 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 0·01 mM $CaCl_2$ or 1 mM EGTA in a total volume of 2 ml, incubation time 15 min at 25°C. Actomyosin concentration was 0·3 mg/ml, 0·16 mg/ml troponin and 0·16 mg/ml tropomyosin or its fragments

Additions to actomyosin	Relative ATPase activity (EGTA/Ca^{2+})
None	0·95
Troponin	0·97
Troponin, α-tropomyosin	0·34
Troponin, *N*-terminal fragment	1·06
Troponin, *C*-terminal fragment	0·85

The results of the actin pelleting experiments (Fig. 7) indicate that neither of the fragments complex with actin whereas α-tropomyosin is found in combination with actin in the pellet. The interactions of tropomyosin and the fragments with troponin were investigated by polyacrylamide gel electrophoresis. These experiments were inconclusive because troponin gave several bands, one of which moved at about the same position as the *N*-terminal fragment; α-tropomyosin, which associates at the low ionic strength of the gel buffers, also showed a wide smear. It appeared, however, that the movement of the fragments was unaffected by the presence of troponin. Sedimentation velocity experiments on mixtures were therefore carried out and these

are shown in Fig. 8. The bimodal boundary (Fig. 8*a*) observed for troponin is similar to patterns reported by others (Wakabayashi and Ebashi 1968; Lovell and Winzor 1977), and arises as a result of troponin association. Lovell and Winzor (1977) have shown that troponin undergoes concentration-dependent dissociation into its constituent subunits as well as self-association. The extent of association was found to be dependent on ionic strength, pH and temperature. These dissociation–association reactions complicate the interpretation of interactions with tropomyosin and account for the failure of the gel electrophoresis experiments to give an unequivocal answer.

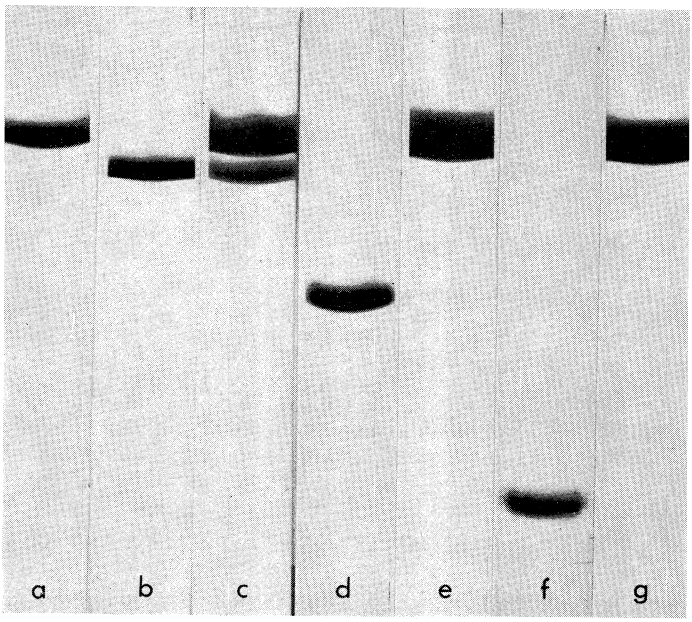

Fig. 7. Sedimentation study of binding of α-tropomyosin and its cleavage products to actin (see Methods). SDS–acrylamide gels of the pellets and controls. (*a*) F-Actin. (*b*) α-Tropomyosin. (*c*) F-Actin + α-tropomyosin pellet. (*d*) *N*-Terminal fragment. (*e*) F-Actin + *N*-terminal fragment pellet. (*f*) *C*-Terminal fragment. (*g*) F-Actin + *C*-terminal fragment pellet.

When troponin was mixed with α-tropomyosin an obvious increase in viscosity occurred, followed by formation of a loose gel after standing. A new hypersharp peak appeared in the ultracentrifuge pattern (Fig. 8*b*) in addition to faster moving material. There was a very marked decrease in the material moving with the sedimentation coefficient of tropomyosin. On the other hand, when the fragments were mixed with troponin no obvious viscosity increase occurred and peaks corresponding to them could be readily identified in the mixture (Figs 8*c* and 8*d*). Area measurements showed an increase in area of the *N*-terminal fragment peak compared with the control. This is to be expected because of the Johnston–Ogston effect (Johnston and Ogston 1946). The area under the *C*-terminal fragment peak was less than the control and this indicates the possibility of some interaction between this fragment and troponin.

Discussion

Cleavage of α-tropomyosin at its single cysteine residue and separation by hydroxylapatite chromatography gave the two fragments expected from the sequence. The molecular weight studies suggest that the two-chain structure has been maintained during the cleavage and separation. The two polypeptide chains of tropomyosin have been shown to be in the register (Johnson and Smillie 1975; Lehrer 1975; Stewart

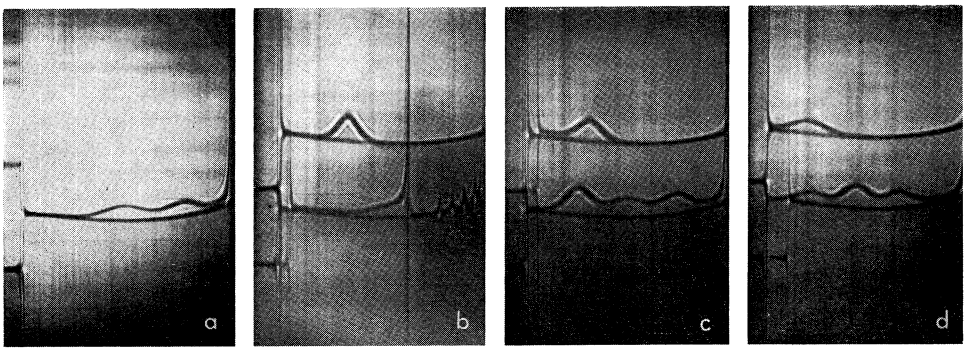

Fig. 8. Sedimentation velocity patterns of troponin and mixtures of troponin with tropomyosin and its *N*-terminal and *C*-terminal fragments. Upper patterns show controls of tropomyosin and the fragments. Buffer was 0·4 M NaCl–50 mM tris–HCl, pH 7·6; speed 60 000 rev/min; photos were taken 115 min after reaching speed; the concentration of troponin in each case was 7·8 mg/ml. (*a*) Troponin. (*b*) Troponin + tropomyosin (2·3 mg/ml). (*c*) Troponin + *N*-terminal fragment (2·1 mg/ml). (*d*) Troponin + *C*-terminal fragment (1·8 mg/ml).

1975*a*), and since the conditions of modification and cleavage were chosen to maintain the two-chain structure then the fragments should also have this symmetrical arrangement. The amino acid analyses and gel electrophoresis indicate that the larger *N*-terminal fragment is contaminated to the extent of about 18–20% with the smaller one and the *C*-terminal fragment may also be not quite pure. The thermal unfolding results at pH 7·6 indicate quite clearly that the *N*-terminal end of the molecule is more stable than the *C*-terminal part. Since the isolated *N*-terminal fragment is only 70% α-helical (taking the values for 5°C given in Table 2), the maximum number of residues involved in the transition is about 132 per chain. There is some unfolding between 5 and 35°C (Fig. 5) but the greater part of this probably arises from the presence of some of the *C*-terminal segment. Since α-tropomyosin is almost 100% α-helical it seems reasonable to assume that the more stable section is towards the *N*-terminus, away from the cleavage point. The less stable *C*-terminal fragment is about 83% helical at 5°C and the unfolding transition therefore involves about 78 residues per chain. It is interesting to note from the transition curves (Fig. 5) that only 5% or less of the *C*-terminal fragment unfolds above 40°C, indicating the absence of the *N*-terminal part and therefore the finding of glycine in this fragment (Table 1) may be adventitious.

The cleavage reaction results in loss of some α-helical structure. Optical rotatory dispersion measurements show that the conversion of the cysteine to *S*-cyanocysteine does not appreciably alter the helix content of α-tropomyosin. On subsequent cleavage ORD measurements indicate a loss of about 20% of α-helix in the mixture of fragments and unchanged tropomyosin. A small loss of α-helix may be expected,

involving possibly 5 or 6 residues due to end effects, but about 50–60 residues per chain in the *N*-terminal part and about 15–20 residues per chain in the *C*-terminal part had lost their α-helical conformation. Eckard and Cowgill (1976) found a similar loss of α-helical structure in tropomyosin fragments prepared by tryptic digestion. However, other helical muscle proteins can be split by enzymes or chemical means without loss of structure. The rod fragments of myosin (myosin rod, light meromyosin and subfragment II) all appear to be 100% α-helical (Burke *et al.* 1973). Paramyosin, on the other hand, can be split into two fragments — the larger one is of molecular weight 140 000 and is completely α-helical, and one of molecular weight 60 000 has lost 15% of its helical conformation (Cowgill 1972, 1975*a*; Halsey and Harrington 1973). For the latter paramyosin fragment this means that about 40 residues per chain are not in the α-helical conformation. If the α-helix is only marginally stable then breakage at a particular point may cause disruption of a considerable length of the coiled-coil. On this basis the section of tropomyosin from about residues 133 to 205 would appear to be less stable than the *N*-terminal segment from 1 to 132 and the *C*-terminal part from 206 to 284.

Predictions on stability may be made from the α-helix-favouring potential of the amino acids (Parry 1975). The information measures for the α-helical conformation are tabulated by Robson and Suzuki (1976). By using the values given in their Table 1 for middle and terminal residues, the values obtained for the helix information, in decinats per 100 residues, are 184 decinats for residues 1–189, and 115 decinats for residues 190–284. These predictions correlate well with the higher thermal stability at pH 7·6 of the *N*-terminal fragment. The *N*-terminal portion is only 70% α-helical when isolated and we might expect its *C*-terminal part to contain less helix information. However, the helix information contained in residues 1–132 is the same as for residues 133–189 (184 decinats/100 residues). Cowgill (1975*b*) reported similar findings for the stability of segments of paramyosin isolated by proteolysis. He found that predictions on stability from the gross amino acid composition did not correlate with the observed stabilities to denaturation by heat and guanidine hydrochloride. The strength of the hydrophobic and electrostatic interactions between the two strands of the coiled-coil structure would need to be taken into account in order to predict the molecular stability (Woods 1976). The unstaggered coiled-coil structure (McLachlan and Stewart 1975) 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. The helix information parameters are derived from globular proteins and may be different for coiled-coil structures.

There are two other reports on the stability of tropomyosin segments. The cyanogen bromide fragment from the *N*-terminal region (residues 11–127) is reported to be more stable than a *C*-terminal cyanogen bromide fragment (residues 142–281) (unpublished observations of Smillie reported by Parry 1975). This is in agreement with the present work. The trypsin-resistant segments from rabbit tropomyosin have been examined by Eckard and Cowgill (1976) who showed that the *C*-terminal end was more stable than the central one-third of the molecule and that the *N*-terminal end was the least stable part of the molecule. Their results depend on the correct identification of segments isolated after tryptic digestion. There is one phenylalanine residue per chain (No. 241) in rabbit tropomyosin, so that Eckard and Cowgill's segment A should not contain this amino acid but they found 0·4 residues of

phenylalanine per 100 amino acid residues. This seems to indicate that the segments are not completely separated from each other. This could also be inferred from the tyrosine contents and their $E_{1\,cm}^{0.1\%}$ values. Eckard and Cowgill (1976) also used the change of tyrosine fluorescence to monitor the loss of helical conformation and, as emphasized by Cowgill (1972), conclusions about loss of helical conformation must be restricted to regions bearing tyrosine residues. Examination of the sequence (Stone *et al.* 1975) indicates there should be only one tyrosine residue in their segment A in a length of over 100 Å so only a small part of this sequence would be monitored by the tyrosine fluorescence method.

Eckard and Cowgill (1976) carried out their studies on the stability of their trypsin-resistant fragments at pH 2, whereas the present experiments were carried out at pH 7·6. Tropomyosin has been shown to be more stable at acid pH values (Noelken and Holtzer 1964; Lowey 1965; Woods 1969) and Fig. 5 shows that the isolated segments also have stabilities below the isoelectric point different from those observed above it. In addition the *N*-terminal segment appears to show two stages in the transition at acid pH values. The altered stability of tropomyosin at acid pH values indicates the important contribution of electrostatic interactions to helix stability. This cannot be explained solely on the basis of the net charge since in the pH range 1–3 the net positive charge for α-tropomyosin and the two segments is more than twice the net negative charge at pH 7·6. In addition stabilization of the helices by salt bridges (McLachlan and Stewart 1975) should be abolished when the carboxyl group takes on a proton. Thus we might expect a decrease in stability at low pH values similar to what is found above pH 8 when the net negative charge increases (Lowey 1965; Woods 1969).

Eckard and Cowgill (1976) concluded that the *N*-terminal third of tropomyosin was the most labile region of the molecule since this was the part that was most easily digested by trypsin. For the rod segments of myosin (Burke *et al.* 1973) and for paramyosin (Cowgill 1972, 1975*a*, 1975*b*; Halsey and Harrington 1973) the helical regions of low thermal stability correspond to the tryptic sensitive regions of the molecules. In the present work the reasonable assumption is made that the *N*-terminal part has lost the α-helical conformation from its *C*-terminal end as a result of the cleavage. The *N*-terminal region is assumed to be the heat stable part at pH 7·6 (Fig. 5). This is contrary to the conclusions from proteolysis experiments. These results indicate that conclusions on stability may be a function of the method and pH of observation. Tyrosine fluorescence, proteolysis and optical rotation appear to give a different result for tropomyosin. The conclusions from optical rotation and circular dichroism are that approximately 45% of rabbit α-tropomyosin near the *N*-terminal region is more heat stable than the 30% near the *C*-terminal end and the remaining central part of the molecule, which includes the troponin-binding site, is the least stable.

The cleavage reaction has led to a loss of the biological activity. The fragments have also lost the ability to bind to actin, in agreement with the results of Gorecka and Drabikowski (1977) for fragments prepared by tryptic digestion. The *N*-terminal fragment does not combine with troponin but there is some evidence for an interaction between the *C*-terminal fragment and troponin. Because of the association–dissociation behaviour of troponin, studies on troponin-T would be preferable. Stewart (1975*b*) has shown that the troponin-binding site of α-tropomyosin is located

near the cysteine residues (residue 190) which are 14 nm from the *C*-terminal end. Since blocking of the thiol groups has no effect on the biological activity of tropomyosin (Cummins and Perry 1973), it appears that the cysteine residues are not necessary for binding to troponin. McLachlan and Stewart (1976) have speculated that the binding site is on the *C*-terminal side of the cysteine residues and near residues 197–217. This is the region where the α-helix is probably disrupted by the cleavage reaction. The maintenance of the coiled-coil structure may be necessary for complex formation with troponin but some interactions, however, may still be preserved.

Note added in Proof

Following submission of this manuscript two papers have appeared on the fragments of α-tropomyosin produced by cleavage at the cysteine residue (Ueno and Ooi 1977; Ueno *et al.* 1977). In agreement with the present findings the Japanese workers find no troponin-binding capacity of the *N*-terminal fragment and a weak binding capacity of the *C*-terminal fragment to troponin. They also found that a mixture of the two fragments formed a complex with troponin as judged by gel electrophoresis.

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