FURTHER CHEMICAL STUDIES ON THE TROPOMYOSINS OF LAMELLIBRANCH MUSCLE WITH SPECIAL REFERENCE TO *PECTEN MAXIMUS*

KENNETH BAILEY AND J. C. RÜEGG
Department of Biochemistry, University of Cambridge (Great Britain)
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SUMMARY

Two types of tropomyosin have been isolated in homogeneous, crystalline form from the adductor muscle of *Pecten maximus*. The globulin type (TM_A identical with paramyosin) has been prepared from the smooth portion, and the water-soluble variety (TM_B) from both tonic and phasic parts. Solubility and electrophoretic characteristics of the two forms are presented. The variations found in the amino acid composition of these and other molluscan TM's (both lamellibranch and cephalopod) are no greater than might be expected from species variations, but the A type as a class differs from the B in the smaller net anionic charge at physiological pH and in the replacement of some lysine groups by arginine. These two factors probably explain the different solubilities of TM_A and TM_B. The probable role of TM_A in contributing to the tonicity of smooth adductor muscles is discussed in terms of the modification of its properties in presence of ATP.

INTRODUCTION

For some years we have made a detailed chemical and physicochemical study of a globulin form of tropomyosin (TM) especially abundant in the smooth (tonic) portions of lamellibranch adductor muscle[1–5]. This form of TM was presumed by various lines of evidence to be identical with paramyosin[1,3,4], a component of certain isolated fibrils which take up electron stain in a characteristic geometrical pattern[6]. This identity was completely established when isolated crystals of TM were shown to stain in a similar way[7].

In addition to the globulin form of TM, we observed in the adductor of *Pecten* and also in the arm muscle of *Octopus* a much more soluble variety[3], crystallising in plates or sometimes in needles, and quite independently. KOMINZ, SAAD AND LAKI[8,9] also reported two types distinguished by their salting-out range in ammonium sulphate. That precipitating below 40 % saturation was called TM_A and that above, TM_B, corresponding to the globulin and water soluble types respectively. Analytical data on these various TM preparations from annelids, cephalopods, gastropods and lamellibranchs[3,8,9] indicate (a) that invertebrate TM's generally contain more arginine and less lysine, more aspartic and less glutamic than vertebrate TM; (b) that the net charge at physiological pH, whether investigated by direct electrophoresis or by

analysis, is greater for the B form than the A; (c) the lys/arg ratio for the B type is higher than that for A, whilst the glu/asp ratio is lower. The diminished solubility of TM_A can partly be understood in terms of (b) and (c)[10]. The small net negative charge will not so greatly oppose aggregation, and, for reasons which are not fully understood, proteins with a high arginine content are generally globulins; moreover all proteins, including rabbit TM[11,12], subjected to the guanidination of the lysine ε-amino group, diminish in solubility.

In the present paper we report further analyses which support the above conclusions. The new data relate particularly to the TM's of *Pecten maximus*, but *Pinna* and *Octopus* protein are also included since the previous analyses[3] have been computed in a slightly different manner. Methods for the rigorous purification of *Pecten* TM_B are given, together with electrophoretic data which illustrate the charge differences between TM_A and TM_B (see also KOMINZ, SAAD AND LAKI[8]). Physical data on TM_B of *Pecten* will be reported separately.

MATERIALS AND METHODS

Protein preparations

Tropomyosin A: The methods for *Pinna* and *Octopus* protein have already been described[3]; that for *Pecten* (from the tonic adductor) follows closely the "ethanol" method as described for *Pinna*[1,3].

Tropomyosin B from smooth adductor: The preparation of homogenous material is quite difficult and the several steps are set out below; they combine isoelectric crystallisation, ethanol precipitation and salting-out procedures.

1. 0.5–1 kg of smooth adductors are rinsed with water and coarsely minced. The debris is washed with 3 volumes of water, squeezed out in muslin and dried in ethanol and ether. It is then stirred with 20 volumes *M* KCl, the pH adjusted to 7 with $NaHCO_3$ and allowed to extract overnight at 4°.

2. The fibre is centrifuged away and the extract dialysed against several changes of 0.2 *M* KCl, when TM_A crystallises in needles and may be purified by recrystallisation if needed.

3. The supernatant of stage 2 is adjusted to pH 5.9 with acetate buffer pH 5.4 and the precipitate discarded. The pH is now lowered to 5 with dilute acetic and the precipitate spun down. It is redissolved in 0.1 *M* KCl containing a little $NaHCO_3$ to raise the pH to 7.5 and any undissolved protein is separated and discarded.

4. To the rather turbid solution at 0°, ice-cold ethanol is added to 20 % and the precipitate discarded. Further addition to 40 % causes precipitation of a gel which is spun down and redissolved in 0.1 *M* KCl.

5. Ammonium sulphate (AS) is added at pH 7 to 30 % saturation and the precipitate spun off. To the clear supernatant more AS is added slowly to 35–40 % saturation when the TM crystallises in needles.

6. These are dissolved by dilution and dialysed against 40 % saturated AS (neutralised) when crystals appear once more. They are redissolved in water, dialysed against several changes of AS (32 g/l containing 0.02 acetate pH 5.7), and when finally equilibrated the pH is lowered to 5.3 to start crystallisation. The yield is about 1.2 % of the wet wt.

Tropomyosin B from striated adductor

Here, the minced, dried fibre obtained at stage 1 is extracted with 20 volumes *M* KCl overnight, the residue separated, and AS added to a final saturation of 30 %. The precipitate of TM_A is discarded and the supernatant is brought to 60 % saturation. The precipitate is filtered off on paper, dissolved in water, dialysed against 0.2 *M* KCl and treated as in stages 3–5. The crystals obtained by salting out are redissolved in water, and recrystallisation is effected by dialysing against AS solution (16 g/l, pH 5.4). The yield is about 0.5 % of the wet wt.

Amino acid analysis

The proteins were dialysed against water, precipitated isoelectrically, washed with water and dried in ethanol and ether. They were hydrolysed in boiling 5.8 *N* HCl for 24 h, and, after evaporating to dryness, were fractionated on Amberlite CG 120 according to the improved column procedure of MOORE, SPACKMANN AND STEIN[13,14]. The first column of 150 cm separates all amino acids other than arg, his and lys, and the second of 15 cm separates the latter and ammonia. The yields of ser and thr were corrected for destructive losses. None of the proteins contains tryptophan (tested by the method of SPIES AND CHAMBERS[15]) or proline, and determinations of total P indicated absence of nucleic acid.

In our hands, the ammonia derived from the column, even after correction for that derived from breakdown of ser and thr, is usually greater than the true amide N liberated by the method of BAILEY[16]. For this reason direct assay of amide N has been carried out on all samples.

RESULTS

General

The crystalline forms of *Pecten* tropomyosin are shown in Fig. 1. The needles of the A type (insets 1 and 3) are quite similar to those obtained by us from oyster and *Pinna*; the B types may appear as plates (inset 4) or as needles (inset 2). These latter are of some interest since TM has never before been crystallised by a salting-out procedure.

TABLE I

ELECTROPHORETIC MOBILITIES OF PECTEN TM_A AND TM_B

Conditions	*TM_A (tonic)*	*TM_B (striated)*	*TM_B (smooth)*
I = 0.5, pH 6.8 (0.4 *M* KCl + phosphate)	2.45	4.8	5.0
I = 0.15, pH 8.0 (0.14 *M* KCl + glycylglycine)	2.75	—	7.5
I = 0.15, pH 8.0 (0.09 *M* KCl + 0.005 *M* ATP + glycylglycine)	3.3	—	7.9
I = 0.3, pH 7.0 (0.25 *M* KCl + histidine)	2.6	—	—
I = 0.3, pH 7.0 (0.2 *M* KCl + 0.005 *M* ATP + histidine)	3.1	—	—

* Ascending boundary, 10^{-5} cm^2 /V-sec.

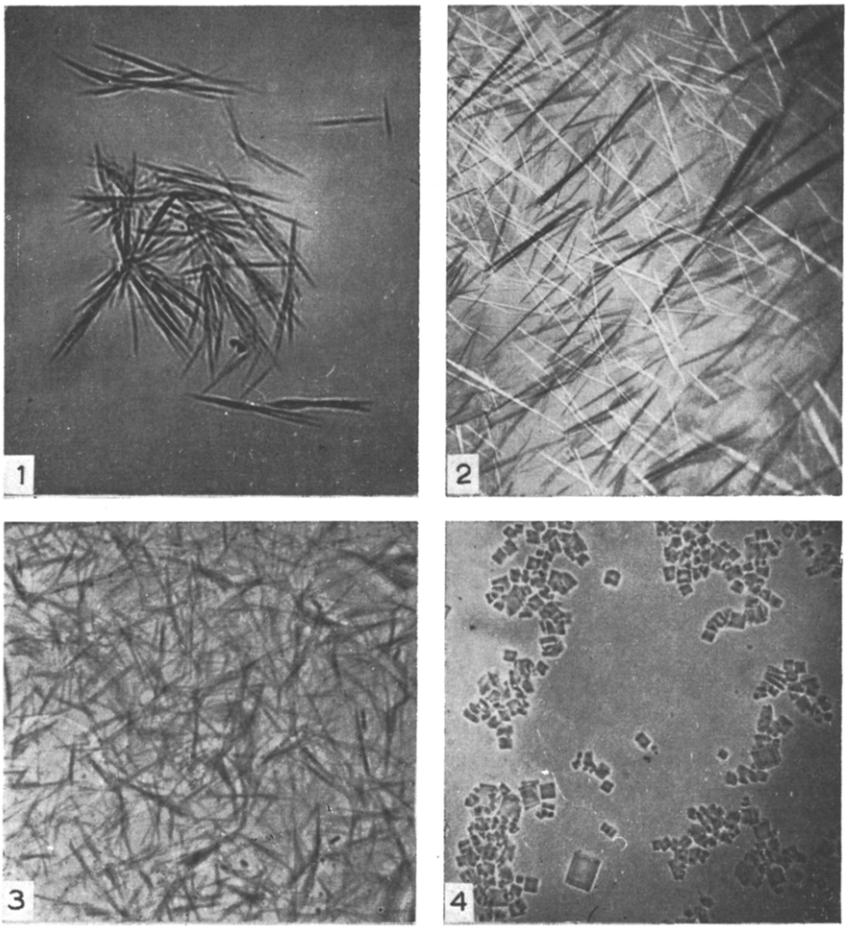

Fig. 1. Crystal forms of TM_A and TM_B from *Pecten maximus*. (1) TM_A from striated portion; (2) TM_B from smooth portion (obtained by salting-out); (3) TM_A from tonic portion; (4) TM_B from striated portion.

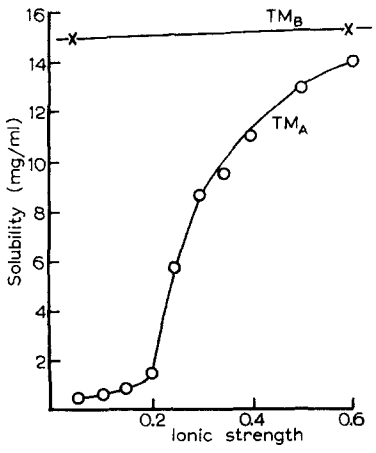

Fig. 2. Solubility of TM_A and TM_B of *Pecten maximus*. Equilibrated at 4° in 0.05 *M* TRIS, pH 7.0. Solubility determined by absorption at 278 mμ.

The electrophoretic mobilities (Perkin-Elmer apparatus, Model 38) of the various TM's are given in Table I, and agree well with values reported by KOMINZ, SAAD AND LAKI[9]. The increase of mobility in presence of ATP is commented upon later.

The marked difference in solubility of TM_A and TM_B is shown in Fig. 2. Below ionic strength 0.2, TM_A is very insoluble at pH 7, but between value of I 0.2–0.3, the salting-in curve is very steep.

Analytical

The analyses have been computed by converting the yield of α-amino N into weights of anhydro amino acid. Since the recovery of N from the columns is less than 100 % (usually about 95 %), it appears more accurate to derive the reference weight of protein, not from the N content of the sample placed on the column, but from the total weight of anhydro amino acids derived from it. The total recovery of N from the column divided by the total weight of anhydro amino acid gives the theoretical N content of the protein which may be checked against the experimental.

Table I gives the detailed analyses of TM_A from *Pecten* striated muscle and of TM_B from both striated and smooth adductors; it includes also the revised values for TM_A of whole *Pinna* adductor and of TM_A of *Octopus* arm muscle. It will be seen that the variations in the amount of any one amino acid, allowing for some divergence due to analytical error, are no more than might reasonably be expected from species variations. It seems unlikely, moreover, that TM_B from *Pecten* striated is really different from that in the tonic muscle.

TABLE II

ANALYSIS OF TROPOMYOSIN A AND B OF LAMELLIBRANCH AND CEPHALOPOD MUSCLES

Type of tropomyosin	*Weight of anhydro amino acid/100 g protein*					*Residues/10^5 g protein*				
	Pinna A	*Octopus* A	*Pecten (smooth)* A	*Pecten (striated)* B	*Pecten (smooth)* B	*Pinna* A	*Octopus* A	*Pecten (smooth)* A	*Pecten (striated)* B	*Pecten (smooth)* B
Asp	13.4	11.6	13.9	14.1	12.6	117	101	121	123	109
Thr	2.35	4.2	3.1	6.0	4.9	23.5	41	31	59	49
Ser	45	5.1	5.4	2.9	3.2	52	58	62	33	37
Glu	23.5	23.0	20.4	25.6	27.2	182	179	158	198	211
Gly	0.15	1.1	1.2	0.7	0.7	13	18.5	21	12.5	12
Ala	7.6	6.0	8.3	7.4	7.7	107	88	117	104	108
Val	3.7	3.6	4.0	3.7	3.6	37.5	36	40	37	37
Met	2.15	2.5	1.8	1.5	1.1	16.5	15	13.5	11	8.5
Ileu	3.4	4.3	4.2	2.7	3.0	30	38	38	24	27
Leu	12.4	13.1	11.9	11.0	11.2	109	116	105	97	100
Tyr	2.4	1.5	2.2	1.7	2.4	15	9	13	10.5	14.5
Phe	1.3	0.8	1.5	1.9	1.6	8.5	7.5	10	13	11
NH_3-N						(116)	(107)	(112)	(117)	(99)
Lys	8.7	9.4	9.4	12.9	12.3	68	73	73	101	96
His	0.65	1.7	1.1	nil	nil	4.5	12	8	nil	nil
Arg	13.2	12.2	11.4	7.8	8.4	84	78	73	50	54
Total	100.0	100.1	99.8	99.9	99.9	867.5	870	883.5	873	874
				Calc. N (%)		18.9	18.3	18.95	17.7	17.4
				Experimental N		18.5	18.1	—	16.5	16.3
				Average res. wt.		115	115	113	114.5	114.5

Table III compares the overall analytical characteristics of TM_A and TM_B. The amounts of total base are quite uniform throughout, in spite of the fact that the lys/arg ratio is greater in TM_B than TM_A. It seems likely, for reasons which are not fully understood, that the larger amounts of arginine contribute to the lower solubility of TM_A, an effect also enhanced by the lower net charge at physiological pH. The modification of charge is achieved by slightly raising the total dicarboxylic acid content and by slightly lowering the numbers of amidized groups *i.e.* it is a directed variation brought about by changes no larger than occur in the case of other amino acids as we pass from one species to another. Since dicarboxylic acids and their amides must be considered as quite separate entities with respect to their incorporation into protein, this method of influencing net charge is not unexpected[17].

TABLE III

ANALYTICAL CHARACTERISTICS OF TROPOMYOSIN A AND B OF LAMELLIBRANCH AND CEPHALOPOD MUSCLES

The results are expressed as % of total residues.

	Tropomyosin A				*Tropomyosin B*			
	Pinna	*Pecten*	*Venus*	*Octopus*	*Pecten*	*Venus*	*Squid*	*Rabbit*
Total acid groups (a)	34.5	32	35	32	37	37	36	34
Total base groups (b)	18	17	17	19	17	17	19	18
Amide N (c)	13.5	13.5	14.0	11.8	12.9	10.3	11.0	7.3
Net anions (a—b—c)	3.0	1.5	4.0	1.2	7.1	9.7	6.0	8.7
Non-polar	37	39	38	37	35	34	33	35
Lys/arg	0.8	1.0	0.73	0.9	1.9	1.2	1.2	2.6
Glu/asp	1.6	1.3	1.5	1.8	1.8	1.9	2.1	2.4

DISCUSSION

The analytical results discussed above for tropomyosins A and B of *Pecten maximus* confirm our own tentative conclusions on somewhat limited data[3] and those of KOMINZ, SAAD AND LAKI[9], who have presented analyses for annelids (*Lumbricus*, *Arenicola*), molluscs (*Busycon*, *Venus*) and cephalopod (*Loligo*). There seems no doubt that TM_A is widely distributed in invertebrate muscles, and in the case of molluscs at least, both A and B types coexist, with the former greatly predominating in tonic muscle. It is probable that the B form, which has now been identified in all kinds of vertebrate smooth and striated muscle, is a necessary component of the actomyosin contractile system, whilst the A form has evolved to serve an ancillary purpose. The fact that the latter constitutes over 25 % of the total protein in tonic adductors and is localised in definite fibrils where it is responsible for the "paramyosin" pattern suggests that it is linked to the special mechanical properties of these muscles, particularly to their ability to resist passive stretch and to remain contracted over very long periods. A "catch" mechanism based on the idea of a simple freezing of the muscle conflicts with the findings of LOWY and coworkers[18,19] that intermittent excitatory volleys are always associated with tonic contraction. But there is no evidence which conflicts with the view that a "catch" implements the tetanic activity of the muscle, and we have recently investigated whether the normal extractives of muscle can influence the colloidal state of TM_A.

The results of these studies, to be reported in detail later, show that ATP at physiological concentration ($5 \cdot 10^{-3}$ *M*)[20,21] (a) interacts with TM_A to increase both solubility and electrophoretic mobility; (b) increases the plasticity of artificial tropomyosin fibres; (c) decreases the passive tension of a glycerinated tonic fibre in which the actomyosin system has been poisoned with Salyrgan or selectively denatured with ethanol. These and other types of study with model systems suggest that the great resistance to stretch in tonically contracted muscles is not entirely due to the active state of the contractile units themselves, but predominantly perhaps to the "set" of the paramyosin fibrils. If this is so, it must follow that during phasic contraction and also during relaxation, these latter must become plasticised, and the interaction of TM_A with ATP, if it occurs at all *in vivo*, could account for it as a purely electrostatic mechanism.

As yet, these results are no more than a basis for a suggestive hypothesis round which subsequent work may be planned. In particular, they need to be considered in relation to the fine structure of tonic muscles, and especially to the localisation of actomyosin, about which our present knowledge is extremely sketchy.

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