Chromatographic analysis of tropomyosins from rabbit skeletal, chicken gizzard and earthworm muscle

Dan L. Crimmins* and Richard S. Thoma

Howard Hughes Medical Institute, Core Protein/Peptide Facility, Washington University School of Medicine, 660 S. Euclid Ave., Box 8045, St. Louis, MO 63110 (USA)

ABSTRACT

Tropomyosins from rabbit skeletal, chicken gizzard and earthworm muscle all exist as dimeric, *ca.* 100% α-helical coiled-coil species in benign media. Two major tropomyosin isoforms from each muscle source have been identified and can be conveniently designated α (fast) and β (slow) based on electrophoretic mobility under denaturing conditions. The ratio of α to β chains is *ca.* 3–4:1 for rabbit skeletal and *ca.* 1:1 for chicken gizzard and earthworm tropomyosins. Each chain from the former two muscle sources has been sequenced, thus providing a molecular basis for interpreting the *in vivo* population of homo- and hetero-dimers. The characteristics of each purified tropomyosin in weak-anion exchange, strong-cation exchange and reversed-phase high-performance liquid chromatography are described. Binding to and/or elution from the reversed-phase matrix results in dissociation into highly helical monomeric chains. This mode of chromatography separates the α and β chains of earthworm and chicken gizzard tropomyosins, but not those of the rabbit protein. Both anion- and cation-exchange chromatography use mild (benign) elution conditions under which the native, *in vivo* dimer population should be preserved. Only the rabbit protein exhibited peak separation on the anion-exchange resin, with peak assignment corresponding to the known molecular organization of homo- and hetero-dimers. In strong cation-exchange analysis, all three tropomyosins exhibit a chromatographic transition near pH 6.5, possibly the result of histidine(s) titration. Collectively, the chromatographic data confirm the present understanding of the *in vivo* mixture of dimers for tropomyosin from rabbit skeletal and chicken gizzard. It is concluded that native earthworm tropomyosin exists predominantly as an αβ hetero-dimer.

INTRODUCTION

The α-helical coiled-coil motif, once thought to be the sole province of fibrous proteins, has been found in a wide variety of biological systems, *e.g.*, "spike-projections" from bacterial cell walls (streptococcal M) and viral membranes (influenza hemagglutinin), plasma (spectrin and fibrinogen) and cell nuclei (DNA-binding proteins, *e.g.*, "leucine-zippers") [1,2]. Tropomyosin (Tm) is the prototypical protein exhibiting this molecular architecture, consisting of two amphipathic, parallel, in-register and slightly super-twisted chains whose amino acid sequence follows a pseudo-heptad repeat [3–5]. The physical forces responsible for maintaining chain association have been the subject of extensive experimental and theoretical studies and a consensus picture has emerged that invokes hydrophobe–hy-drophobe and ionic ("salt-bridge") interactions as the predominant contributors [1,2].

Tm has been isolated and partially characterized from several sources of vertebrate skeletal, cardiac and smooth muscle, and also from a few invertebrate species [6]. The protein from rabbit skeletal muscle [Tm(R)] is the best studied Tm to date. It consists of two distinct protein isoforms differing slightly (39 substitutions/284 total, mostly conservative) in primary structure [7]. These are designated α (fast) and β (slow), respectively, based on electrophoretic mobility on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and exist in a *ca.* 3–4:1 α:β ratio [4,8,9]. Each chain has a cysteine located at sequence position 190 and the β chain has an additional cysteine at position 36 [7], hence parallel dimer molecules, having adjacent sulfhydryls, can be disulfide cross-linked [3,4,10,11].

In vitro all three dimeric coiled-coils, $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$, can be produced and these have been studied [10–13] in an effort to explain the observed *in vivo* dimer population of *ca.* 92–96% $\alpha\alpha$ + $\alpha\beta$ and *ca.* 4–8% $\beta\beta$ [14,15].

Tm from chicken gizzard [Tm(C)] has also been sequenced [16,17] and in this instance the two isoforms, designated in this paper as α and β, are present at a *ca.* 1:1 ratio, differ by 72/284 residues, and the α chain has a cysteine at position 36 whereas the β chain has a cysteine at position 190 [16,17]. Hence, only Tm(C) homo-dimers can be disulfide cross-linked. *In vivo,* the $\alpha\beta$ hetero-dimer of Tm(C) is the predominant molecular species [18–24] and this has led to the suggestion that tissue sources containing nearly equal amounts of the two genetic variants will preferentially assemble as $\alpha\beta$ hetero-dimers [25]. In support of this hypothesis are the observations reported for a variety of tissues from fetal and adult rabbit muscle [25] and frog muscle [26].

Earthworm Tm [Tm(W)], on the other hand, has not yet been sequenced and few data on the properties of this invertebrate Tm have appeared [6,27], nor has information appeared regarding the composition of the *in vivo* dimer mixture in terms of the individual, herein designated α and β, chains. Data from a previous study have shown that native Tm (W) cannot be disulfide cross-linked [6].

In this investigation, purified preparations of Tm (R), Tm(C) and Tm(W) were subjected to C_4 reversed-phase (RP) high-performance liquid chromatography (HPLC), weak anion-exchange (WAX) HPLC and strong cation-exchange (SCX) HPLC to compare the chromatographic characteristics of these structurally similar proteins. For all three protein sources, the native, reduced dimer(s) dissociate into individual subunits during binding to and/or elution from the C_4-RP column, as has been previously observed for Tm(R) [14]. Only the cross-linked Tm(R) preparation exhibits a different chromatographic elution profile when compared with its reduced counterpart. WAX-HPLC indicates that only the Tm(R) coiled coils gives a chromatographic separation, with subsequent peak assignment consistent with the known dimer population [14,15]. SCX-HPLC illustrates that all three Tms display a chromatographic transition near pH 6.5, possibly the result of histidine(s) titration. As

both ion-exchange systems use non-denaturing elution conditions, the associative dimeric assemblies are expected to be preserved during this mode of chromatography. Therefore, the collective chromatographic data confirm the *in vivo* dimer population for Tm(R) and Tm(C) and suggest that the predominant coiled-coil species for native Tm(W) is the $\alpha\beta$ hetero-dimer.

EXPERIMENTAL

Protein preparation, chemicals and reagents

Tm(R) was purified from rabbit skeletal muscle [28], Tm(W) was purified from earthworm body wall muscle [6] and Tm(C) was purchased from Sigma (St. Louis, MO, USA; T-3026 Lot 66F-9675). Each protein was judged to be satisfactorily pure by SDS-PAGE and UV spectrophotometry, and gave the expected circular dichroism (CD) at low temperature in benign buffer. The lyophilized protein, previously stored desiccated at $-20°C$, was generally resuspended to a nominal concentration of 3 mg/ml with 1% (v/v) acetic acid (Fisher Scientific, St. Louis, MO, USA) [14]; the resulting solution was then stored at 4°C until use. This low-pH acetic acid reconstitution procedure offers several advantages, including rapid protein solubility, solvent volatility, inhibition of cysteine oxidation and apparent absence of bacterial growth, but suffers from high UV background absorption. The sources of HPLC solvents, amino acid reagents, water purification and additional materials have been described [29,30].

HPLC instrumentation and chromatography

C_4-RP-HPLC was performed at 37°C on a Vydac 214TP54 C_4 column (25 cm × 0.46 cm I.D.) purchased from the Nest Group (Southborough, MA, USA) with sample elution effected via a linear gradient from 0 to 100% B in 60 min at 1 ml/min, using mobile phases A = 0.1% trifluoroacetic acid and B = 0.095% trifluoroacetic acid in acetonitrile–water (90:10) [14,15].

WAX-HPLC was performed at 28°C on a TSK DEAE-5PW column (7.5 cm × 0.75 cm I.D.) and SCX-HPLC at 28°C on a TSK SP-5PW column (7.5 cm × 0.75 cm I.D.), each purchased from Altex (San Ramon, CA, USA). A linear gradient from 0 to 50% B in 30 min at 1 ml/min was used to elute

samples from the anion-exchange resin and a solvent program from 0 to 100% B in 30 min at 1 ml/min was used to elute samples from the cation-exchange column. For each ion-exchange system, mobile phase A = 10 mM sodium phosphate and mobile phase B = 10 mM sodium phosphate–1 M NaCl, each titrated to the desired pH. Protein samples were either exhaustively dialyzed vs. ion-exchange mobile phase A or vacuum-dried (Savant Instruments, Farmingdale, NY, USA) and resuspended in this buffer for preparative chromatography. This was necessary because the larger amounts of acetic acid co-injected in this instance substantially altered the chromatography (e.g., diminished protein binding). All chromatograms from C$_4$-RP-, WAX- and SCX-HPLC were monitored simultaneously at 214 and 280 nm with fractions collected at 0.25-min intervals (250 μl per fraction) for the preparative runs. The configuration of the chromatographic hardware, temperature control and the data acquisition procedures have been described previously [14,29,30].

SDS-PAGE

Generally, 15 μl of the appropriate ion-exchange fraction was mixed with 15 μl of 2 × loading buffer [180 mM sodium phosphate–9 mM disodium ethylenediaminetetraacetic acid–3.6% (w/v) SDS–18% (v/v) glycerol–0.036% (w/v) bromophenol blue (pH 7.5)], incubated for 30 min at 45°C and then electrophoresed for 90 min at a constant voltage of 150 V on 4–20% linear acrylamide gradient twelve-well mini-gels (1 mm thick) (Enprotech, Hyde Park, MA, USA) [14,15,31]. This gradient gel system provided better resolution for the two-chain, cross-linked dimers and was employed whenever this type of separation was desired. Otherwise, protein samples were treated as above except that 100 mM dithiothreitol was included in the 2 × loading buffer to reduce protein disulfide bonds; the resulting samples were then analyzed on 10–20% gradient mini-gels as this gradient gel system afforded better separations for the various one-chain monomeric species. The sample size was 2–20 μl per well; the gel was stained for 15 min with 0.05% (w/v) Coomassie Brilliant Blue R-250–50% (v/v) methanol–7.5% (v/v) acetic acid, de-stained with 10% (v/v) methanol–7.5% (v/v) acetic acid until the background cleared to an acceptable level and then stored in

heat-sealed plastic pouches prior to photography with a Kodak EDP print system (Fisher Scientific). C$_4$-RP fractions (15 μl) or stock Tm solutions were vacuum-dried, resuspended with 15 μl of water and then mixed with 15 μl of the desired 2 × loading buffer as described above.

Additional analytical techniques

Amino acid compositional analysis of stock Tm solutions and selected C$_4$-RP fractions was performed by manual precolumn phenylisothiocyanate derivatization of acid hydrolysates as described [29,32]. CD instrumentation, sample measurements (kindly performed by Dr. Marilyn Emerson Holtzer) and subsequent data analysis have been detailed [12,28,33].

RESULTS AND DISCUSSION

C$_4$-RP-HPLC

The individual purified proteins were subjected to C$_4$RP-HPLC and fractions collected for further analysis. Previously, it was shown that C$_4$-RP chromatography of uncross-linked Tm(R) $\alpha\alpha$ dimer resulted in chain dissociation into highly helical monomeric molecules and the individual α and β chains from uncross-linked Tm(R) $\alpha\beta$ dimer were unresolved. In addition, only the disulfide cross-linked forms of the three Tm(R) dimers ($\alpha\alpha$, $\alpha\beta$ and $\beta\beta$) could be separated both from each other and from the uncross-linked monomers [14]. These results and their implications form a basis for interpreting the data presented in this section.

Fig. 1 displays a composite chromatogram for all three of the Tms studied. The retention times for each species of Tm are similar (Fig. 1A–C), and this suggests that either the relevant contact region(s) between protein chain and column support are similar or that the overall hydrophobicity is similar for all six protein chains. Further, it is likely that the individual dissociated chains of Tm(C) and Tm(W) will possess a high degree of helicity as the Tm(R) chain counterparts [14] such that the global chain conformation and accessibility of the hydrophobic residues are expected to be comparable. Clearly, however, other more subtle factors must be present because partial chain separation is observed for Tm (C) (Fig. 1A) and the α and β chains of Tm(W) are almost baseline resolved (Fig. 1B).
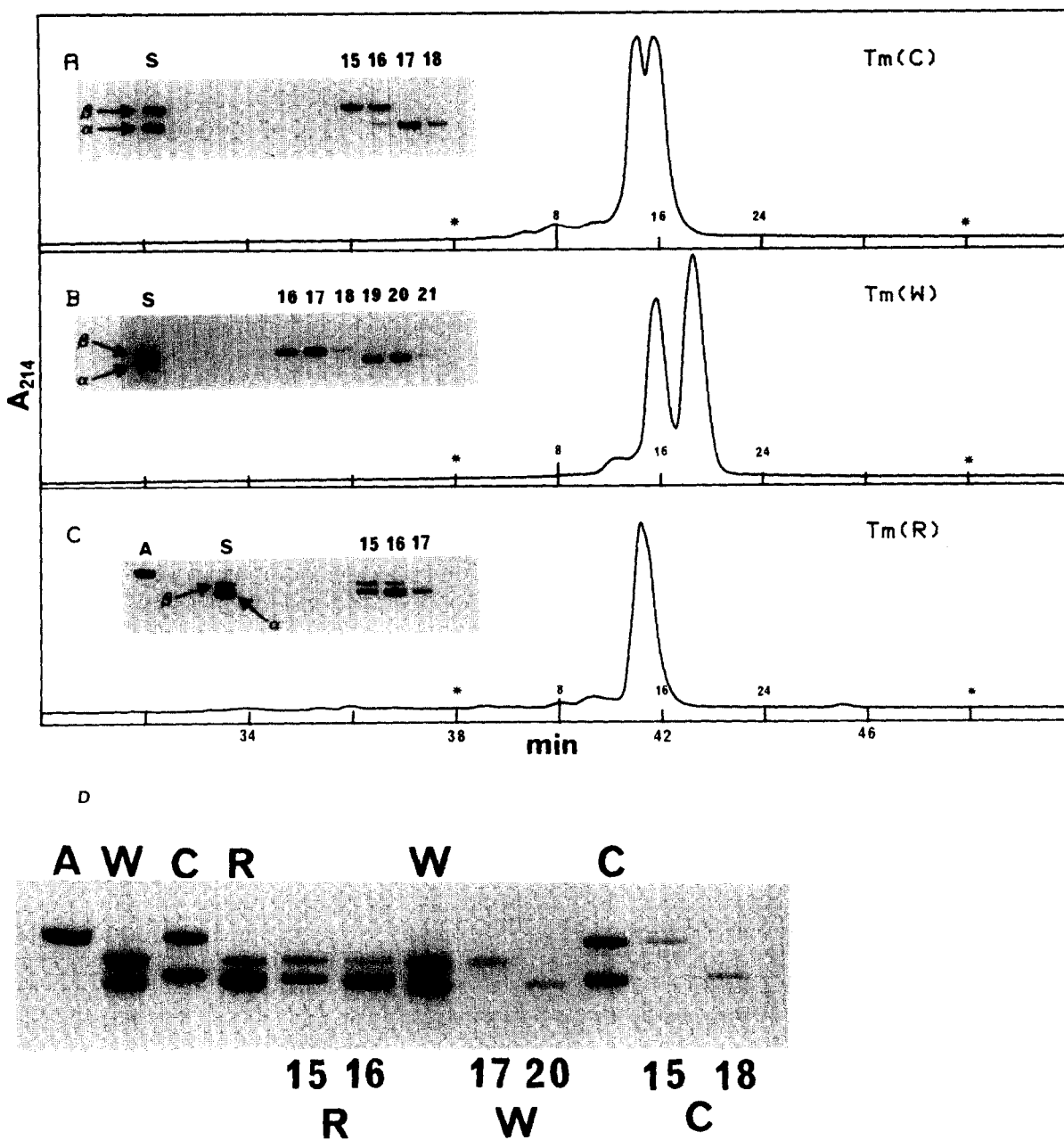
Fig. 1. C$_4$-RP-HPLC and SDS-PAGE of Tm(C), Tm(W) and Tm(R). All chromatograms were monitored at 214 nm with fractions collected at 0.25-min intervals and asterisks indicate the beginning and end of the collection period. (A) Tm(C), 650 mV full-scale. Inset shows 10–20% SDS-PAGE of indicated fractions and of the unfractionated, stock (S) protein. (B) Tm(W), 850 mV full-scale. Inset shows gel analysis of indicated fractions. (C) Tm(R), 1250 mV full-scale. SDS-PAGE of indicated fractions and an M_r marker protein rabbit actin (A) is shown in the inset. (D) Composite 10–20% SDS-PAGE of selected fractions from the individual C$_4$-RP-HPLC runs showing the relative electrophoretic mobility of the various Tm molecular species. Left to right: actin, stock Tm(W), stock Tm(C), stock Tm(R), fractions 15 and 16 from Tm(R) chromatogram C, stock Tm(W), fractions 17 and 20 from Tm(W) chromatogram B, stock Tm(C) and fractions 15 and 18 from Tm(C) chromatogram A.

The SDS-PAGE insets from each of these chromatograms indicate that the molecular species designated as the β chain (slower gel component) elutes earlier from the C_4 column than the α chain (faster gel component) for both of these Tms. Attempted disulfide oxidation of Tm(C) and Tm(W) did not result in any retention time changes compared with those shown in Fig. 1. In contrast, Tm(R) did display such a difference, in complete agreement with our earlier results [14] (data not shown). The data for Tm(C) were expected, as the predominant native molecular species is an $\alpha\beta$ hetero-dimer and only homo-dimers can be disulfide cross-linked [16–24]. This information has not been determined for Tm(W), but the above results suggest that, here also, both an $\alpha\beta$ hetero-dimer is the predominant species and cysteines, if present, on the individual chains are not spatially proximal to participate in a disulfide bond (see also below).

A photograph of a 10–20% SDS-PAGE experiment is shown in Fig. 1D for unfractionated stock Tms, selected C_4-RP fractions from the above chromatograms and a relative molecular mass (M_r) 43 000 marker protein, rabbit skeletal actin (A). Note the electrophoretic resolution of the individual α and β chains from a given Tm. The migration order is actin $\approx \beta$-Tm(C) $< \beta$-Tm(W) $\approx \beta$-Tm(R) $< \alpha$-Tm(C) $< \alpha$-Tm(W) $\approx \alpha$-Tm(R). The α and β chains from Tm(R) and Tm(C) are 284 residue, ca. M_r 33 000 species and differ in absolute molecular mass by <200 [7,17]. Although the Tm(W) chains have not yet been sequenced, it seems plausible that similar chain characteristics will prevail such that the observed partial resolution of the six Tm chains illustrated in Fig. 1D must reside in a combined subtle effect of the amount of SDS bound to the protein and the overall conformation of this complex. This line of reasoning and cautionary note have been expressed before to rationalize the slight difference in electrophoretic mobility of the Tm(W) and Tm(R) chains when Tm(W) was first isolated [6].

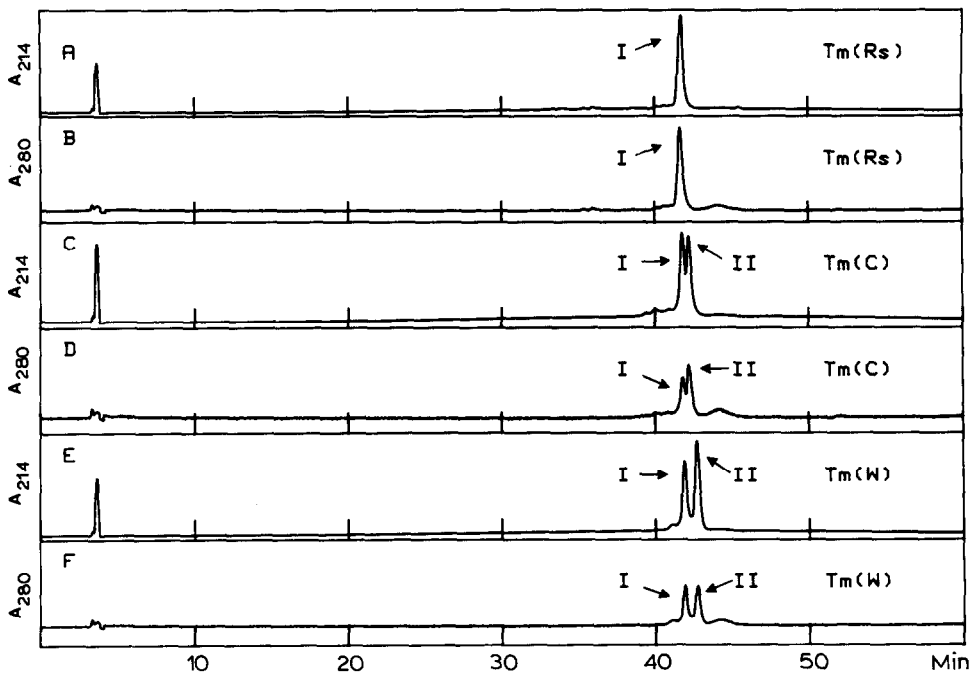The analytical C_4RP-HPLC profiles for each Tm monitored at both 214 nm and 280 nm are illustrat-

Fig. 2. Analytical C_4-RP-HPLC of Tm(R), Tm(C) and Tm(W). (A) Tm(R$_s$) = Tm(R) at 214 nm, 800 mV full-scale; (B) Tm(R$_s$) = Tm(R) at 280 nm, 25 mV full-scale; (C) Tm(C) at 214 nm, 480 mV full-scale; (D) Tm(C) at 280 nm, 15 mV full-scale; (E) Tm(W) at 214 nm, 672 mV full-scale; (F) Tm(W) at 280 nm, 21 mV full-scale. Peaks are labeled for identification purposes. See Table I for amino acid composition of Tm(R) and Tm(W) peaks and Table II for integrated absorbance area ratios of all three Tms.

TABLE I

AMINO ACID COMPOSITION OF Tm(R) AND Tm(W) C_4-RP-HPLC FRACTIONS

Amino acid	Concentration (mol%)						
	Tm(R)			Tm(W)			
	Peak I	Stock	Lit.[a]	Peak I	Peak II	Stock	Lit.[b]
Asx	9.8	10.5	10.3	9.3	11.1	11.6	11.6
Glx	25.3	24.0	25.1	23.7	26.0	25.3	26.4
Ser	4.8	4.8	5.0	5.8	4.6	4.9	5.2
Gly	1.7	2.4	1.2	4.5	3.5	3.4	4.1
His	0.7	0.9	0.6	0.8	0.5	0.6	0.6
Arg	5.3	5.3	4.9	6.0	5.0	5.3	5.0
Thr	2.8	2.7	2.8	6.0	6.8	5.9	6.0
Ala	13.2	12.2	12.5	11.3	9.9	10.8	10.6
Pro	0	0	0	0	0	0	0
Tyr	2.0	2.1	2.1	1.5	1.2	1.3	1.4
Val	3.2	3.6	3.4	3.9	4.5	3.9	4.2
Met	1.9	2.3	2.3	1.9	1.5	1.9	1.4
Ile	3.7	3.6	4.1	2.9	3.3	2.4	3.2
Leu	11.1	11.0	11.2	10.9	10.6	11.0	10.1
Phe	0.4	0.4	0.4	1.3	1.4	1.4	1.5
Lys	14.0	14.3	13.7	10.3	10.1	10.3	8.6
Trp	n.d.[c]	n.d.	0	n.d.	n.d.	n.d.	0
Cys	n.d.	n.d.	0.4	n.d.	n.d.	n.d.	0

[a] Literature values taken from ref. 7.
[b] Literature values taken from ref. 6.
[c] n.d. = Not determined.

ed in Fig. 2. Peaks are labeled I and II for identification purposes and amino acid analysis was performed on Tm(R) peak I (Fig. 2A) and Tm(W) peaks I and II (Fig. 2E). These results are listed in Table I, where it is evident that the compositional analysis of the stock proteins and Tm(R) peak I compares very favorably with literature values and that Tm(W) peak fractions I and II have nearly identical residue numbers. The minor differences observed for these Tm(W) fractions (found in replicate analyses, data not shown) must await sequence analysis of the individual chains for confirmation.

The chromatograms monitored at 280 nm (Fig. 2B, D and F) should only report on the tyrosine content of each chain because no tryptophan residues are found in any of these Tm species [6,7,17] and the phenylalanine contribution at this wavelength is expected to be minimal. Visual inspection of the peptide bond absorption at 214 nm for Tm(C) (Fig.

2C) shows peaks I and II to be virtually identical, in contrast to the apparent tyrosine content (Fig. 2D) as peak II exhibits a greater 280 nm intensity than peak I. The converse of these observations is illustrated for the Tm(W) chromatograms monitored at 214 nm (Fig. 2E) and 280 nm (Fig. 2F). In this instance, the displayed 214-nm profile indicates that peak II has a greater absorbance at 214 nm than peak I, whereas the corresponding 280-nm chromatogram shows essentially identical peak intensities. As discussed below, this implies that the tyrosine content of Tm(C) peak I is less than that of Tm(C) peak II, and for Tm(W) the opposite result holds, i.e., Tm(W) peak I has a greater tyrosine content than Tm(W) peak II.

Table II summarizes the integrated peak areas at 214 and 280 nm as the ratio 214:280 nm for each of the Tms. The number reported for the tyrosine (Tyr) content of the Tm(C) and Tm(W) chains was calculated using the observed 214:280 nm ratio for Tm(R) and a value of six Tyr for both the α and β chains of Tm(R) [7]. It is important to point out that the numbers given in parentheses in Table II were determined with an independent HPLC system. These values are consonant with those obtained with the other HPLC system and so should increase confidence in the validity of this analysis. Peak I (β) from Tm(C) gave a calculated Tyr content of 3.2 compared with the sequence value of 3 (given in brackets) while peak II (α) yielded values of 4.3 and 4 Tyr, respectively [17]. These determina-

TABLE II

ABSORBANCE RATION (214/280 nm) FROM C_4-RP-HPLC AND TYROSINE DETERMINATION FOR Tm(R), Tm(C) AND Tm(W)

Protein	214/280 nm		No. of Tyr	
	Peak I	Peak II	Peak I	Peak II
Tm(R)	36.7/1	–	6	–
	(36.5/1)[a]	–	(6)	–
Tm(C)	67.8/1	50.8/1	3.2[3][b]	4.3[4]
Tm(W)	54.8/1	68.1/1	4.0	3.2
	(52.1/1)	(66.4/1)	(4.2)	(3.3)

[a] Numbers in parentheses were independently determined on a separate HPLC system.
[b] Numbers in brackets are literature values taken form ref. 17.

tions lend further support to the identification and designation of the Tm(C) chains presented above.

A similar set of calculations for the Tm(W) C_4-RP peak fractions gave the opposite analysis for the α (peak II) and β (peak I) species of *ca.* 3 Tyr and *ca.* 4 Tyr, respectively. One can use the averaged numbers for these individual peaks (chains) and the *ca.* 1:1 ratio of chains to calculate a stock protein Tyr value of *ca.* 3.68 for Tm(W). This calculated Tyr content differs by less than 6% from an analysis performed 11 years prior using cruder methodology on unfractionated Tm(W) chains [6]. Additionally, the Tyr mol% for these fractions, 1.5 and 1.2, respectively, and the value of 2.0% measured for the Tm(R) fraction (Table I) can also be used to calculate the Tyr content of Tm(W) α and β chains if one assumes the same degree of polymerization for the Tm(W) and Tm(R) chains, *i.e.,* 284 residues. Performing such an analysis indicates experimentally 5.68 Tyr (compared with the sequence value of 6 [7]) for Tm(R), giving 4.26 Tyr for Tm(W) peak I (β) and 3.41 Tyr for Tm(W) peak II (α). Hence the compositional Tyr analysis (Table I) and the chromatographic peak-area ratio calculations (Table II) provide a consistent set of data for the Tyr content of the α and β chains of Tm(W).

WAX-HPLC

The three purified Tms were subjected to analytical WAX-HPLC over the pH range 8.4–5.0 (Fig. 3). One would then expect as a first approximation that as the pH is lowered from slightly basic (pH 8.4) to mildly acidic (pH 5.0) the retention time of the protein should decrease. This can be rationalized as a concomitant decrease in the net negative charge of the protein (*e.g.,* lysine and histidine protonation). Although net charge is clearly an important factor in determining the extent of protein binding to ion-exchange resins, the three-dimensional protein structure and accessibility of the appropriately signed charged residues to interact with the matrix are equally important [34]. Sample loading and elution for this mode of chromatography are performed under mild solvent conditions such that the protein chains are expected to remain associated as dimers and the native dimer population should be preserved. Interestingly, the Tms appear to be ideal model candidates to test the "contact residue" hypothesis [34], as these molecules exhibit
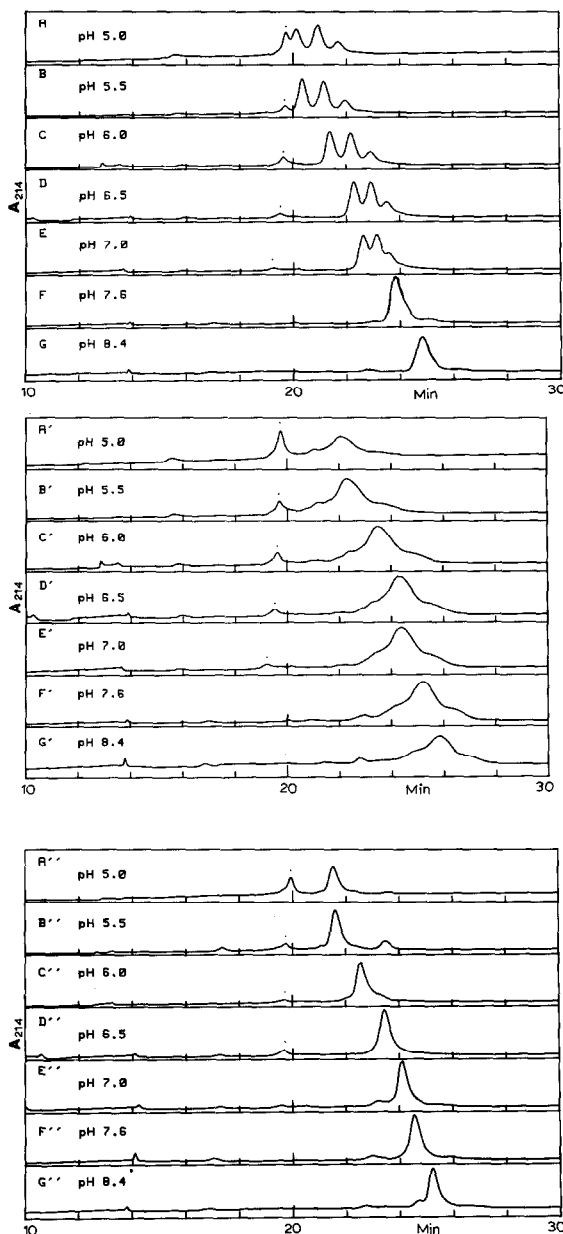

Fig. 3. Analytical WAX-HPLC at different pH values for Tm(R), Tm(W) and Tm(C). Top, unprimed panels: Tm(R$_s$) = Tm(R) monitored at 214 nm; A–G, 200, 500, 500, 500, 500, 600 and 600 mV full-scale respectively. Dot above peak at *ca.* 19–20 min indicates a gradient-associated UV artifact also observed in "blank" chromatograms. Middle, single-primed panels: Tm(W) monitored at 214 nm; A'–G', 150, 300, 300, 300, 300, 500 and 300 mV full-scale respectively. Bottom, double-primed panels: Tm(C) monitored at 214 nm; A"–G", 300, 500, 600, 600, 600, 600 and 600 mV full-scale, respectively.

a particularly simple secondary (α-helix) and quaternary (coiled-coil) structure under the experimental conditions used. Thus, potential complications arising from tertiary structure are absent in this instance such that it may be possible to pinpoint specific regions (sites) of the sequenced Tms in contact with the resin.

The Tm(R) preparation showed a progressive decrease in retention time from WAX-HPLC as the pH was lowered from 8.4 to 5.0 (Fig. 3, top, unprimed panels). Between pH 7.6 (Fig. 3F) and pH 7.0 (Fig. 3E) it is evident that peak resolution for the Tm(R) sample is occurring and that a further

decrease in pH improves this resolution. On the other hand, neither Tm(W) (Fig. 3, middle, single-primed panels) nor Tm(C) (Fig. 3, bottom, double-primed panels) displayed such peak separation, although each protein gave the same general trend of decreasing retention time with decreasing pH as Tm (R). Tm(W) chromatographed as a particularly broad peak over this entire pH range (Fig. 3, middle, single-primed panels) whereas Tm(C) eluted (Fig. 3, bottom, double-primed panels) with a peak shape similar to Tm(R) (Fig. 3, top, unprimed panels). The chromatographic profile of Tm(C) at pH 8.4 (Fig. 3G″) appears similar in detail to a previous
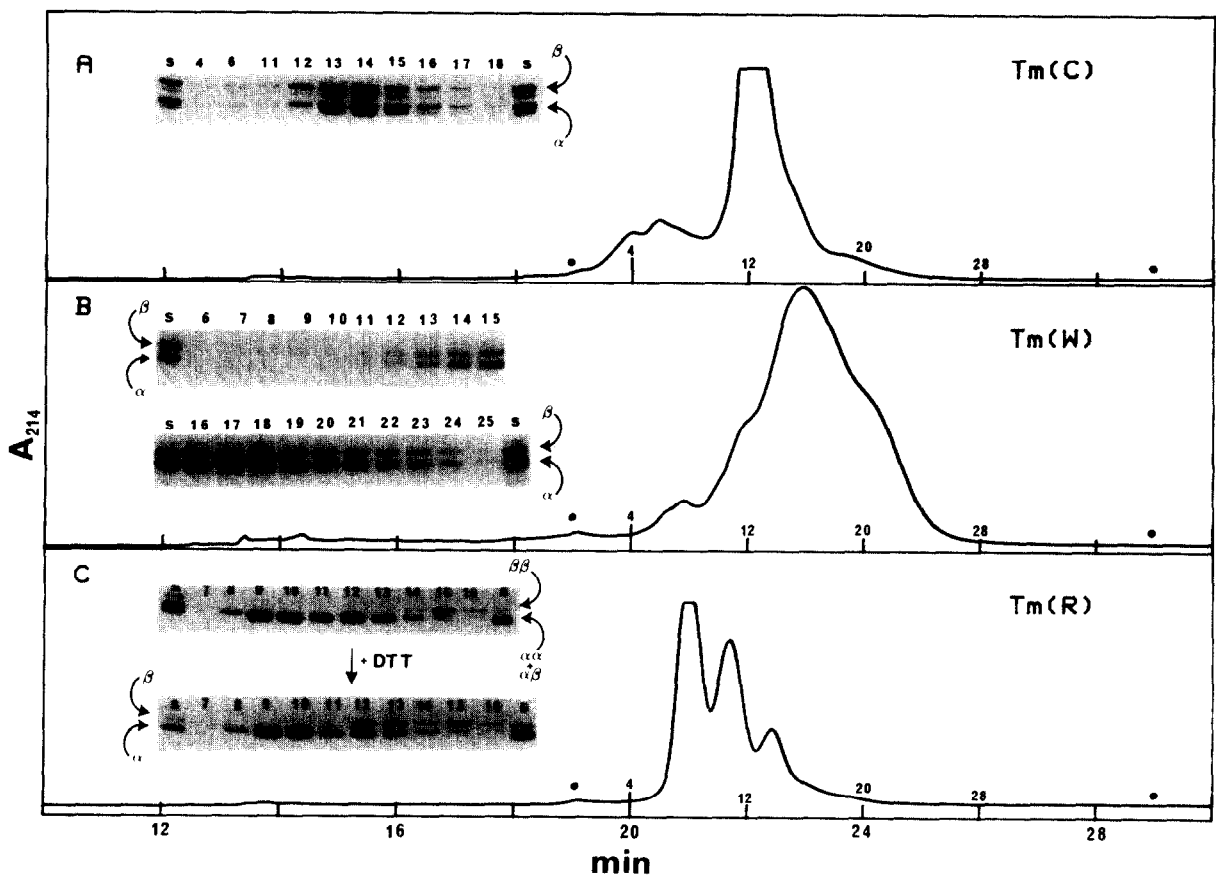

Fig. 4 Preparative WAX-HPLC at pH 6.0 and SDS-PAGE of Tm(C), Tm(W) and Tm(R). All chromatograms were monitored at 214 nm with fractions collected at 0.25-min intervals and dots indicate the beginning and end of the collection period. (A) Tm(C), 2500 mV full-scale. Major peak is off-scale; see Fig. 3C″ for analytical profile. Inset shows 10–20% SDS-PAGE of the indicated fractions and of the unfractionated, stock (S) protein. (B) Tm(W), 2000 mV full-scale. Inset shows gel analysis of indicated fractions. (C) Tm(R), 2500 mV full-scale. Major peak is off-scale; see Fig. 3C for analytical profile. Upper inset shows 4–20% SDS-PAGE of indicated fractions and lower inset shows 10–20% SDS-PAGE of the same samples reduced with dithiothreitol (DTT) prior to electrophoresis.

analysis [21] of the native dimer population of Tm (C), where the main peak was identified as the $\alpha\beta$ hetero-dimer and a small preceding peak was as-singed to an $\alpha\alpha$ dimer. Therefore the integrity of the native dimer population in this mode of chromatography is maintained for Tm(C) and Tm(R) (see below).

Prepatative amounts of the three Tms were in-jected and fractions collected on the WAX resin at pH 6.0 (Fig. 4). The pH was chosen for the prepara-tive runs because the analytical profile for Tm(R) (Fig. 3C) gave acceptable peak resolution without interference from a gradient-associated artifact (dot above peak at *ca.* 19 min) and little change was observed for the other two Tms over the studied pH range. The gel inset for Tm(C) (Fig. 4A) indicates approximately equal intensity and distribution of the α and β chains across the peak fractions, *i.e.*, the majority of the native dimer population of Tm(C) is an $\alpha\beta$ hetero-dimer [18–24]. Some slight chain deg-radation of the stock sample is more apparent at these higher sample loads, as indicated by minor bands of faster electrophoretic mobility than the major α and β bands.

Tm(W) (Fig. 4B) chromatographed as a broad peak with both leading and trailing shoulders, al-though the corresponding gel inset showed only the expected two major isoforms of this Tm in roughly equal amounts. The origin of this chromatographic heterogeneity is not readily apparent but may resi-due in a small population of post-translationally modified chains, *e.g.*, phosphorylated or deamidat-ed, or may represent the partial separation of minor contaminants seen as the slower and faster migrat-ing components relative to the major β and α chains, respectively (see gel inset, Fig. 1B).

The gel analysis of the chromatographic elution profile for Tm(R) (Fig. 4C) showed that the starting sample prepared by dialysis into mobile phase of pH 6.0 was virtually all oxidized, *i.e.*, disulfide cross-linked (upper gel inset, lane S). This was un-expected because sulfhydryl oxidation via molecu-lar oxygen tends to be more efficient at neutral to slightly basic pH. The same UV distribution was observed for an uncross-linked Tm(R) sample (data not shown), suggesting that the oxidation state of the cysteines have no observable effect on WAX-HPLC. Neither Tm(C) nor Tm(W) became disul-fide cross-linked after dialysis into pH 6.0 buffer

(data not shown). Importantly, the elution order of the three major peaks is $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$, as assessed from the gel analysis. The first-eluting peak con-tains α chains (+DTT gel inset, lanes 8–10), the next peak contains approximately equal amounts of α and β chains (+DDT gel inset, lanes 12–14), and the third peak is clearly enriched in the β chain com-ponent (+DDT gel inset, lanes 15 and 16). Similar conclusions regarding the peak assignments are possible from the upper gel inset for this chromato-gram. The elution order at pH 6.0 follows the net negative charge of the Tm(R) dimers: $\alpha\alpha(-50)$, $\alpha\beta(-52)$ and $\beta\beta(-54)$ and is analogous in charge content distribution to the WAX chromatograms for *in vitro* produced homo- and hetero-dimers of Tm(C) [21]. It was not possible to determine the percentage of each dimer species for the Tm(R) preparation owing to insufficient peak resolution, but qualitatively it is obvious that all three Tm(R) dimer species are present in native Tm(R). In addi-tion to the previous C_4-RP-HPLC, SDS-PAGE and capillary electrophoresis data [14,15], the WAX-HPLC data presented here represent the fourth in-dependent analytical technique that unequivocally demonstrates the existence of $\beta\beta$ homo-dimers in native Tm(R).

SCX-HPLC

A set of experiments comparable to those report-ed above for WAX-HPLC were performed on the three Tms chromatographed on an SCX column. The pH in the instance was varied from 8.0 to 6.0 (Fig. 5). If the "contact residue" hypothesis [34] is correct then, as the pH is lowered from 8.0 to 6.0, the Tm retention time should increase. The compos-ite analytical chromatograms shown in Fig. 5 sup-port this assertion and one possibility is that the protonation of histidine(s) and/or protonation of abnormally high titrating acidic residues may be re-sponsible for these observations. The fact that both Tm(R) (Fig. 5, top, unprimed panels) and Tm(C) (Fig. 5, bottom, double-primed panels) are highly net negatively charged at these pHs (from their known sequence) yet still bind to the cation-ex-change column provides further experimental evi-dence supporting the "contact-residue" hypothesis [34]. All three Tms were tightly bound to the cation-exchange matrix at pH 5.5–4.5 such that no protein eluted using the normal gradient program (data not shown).
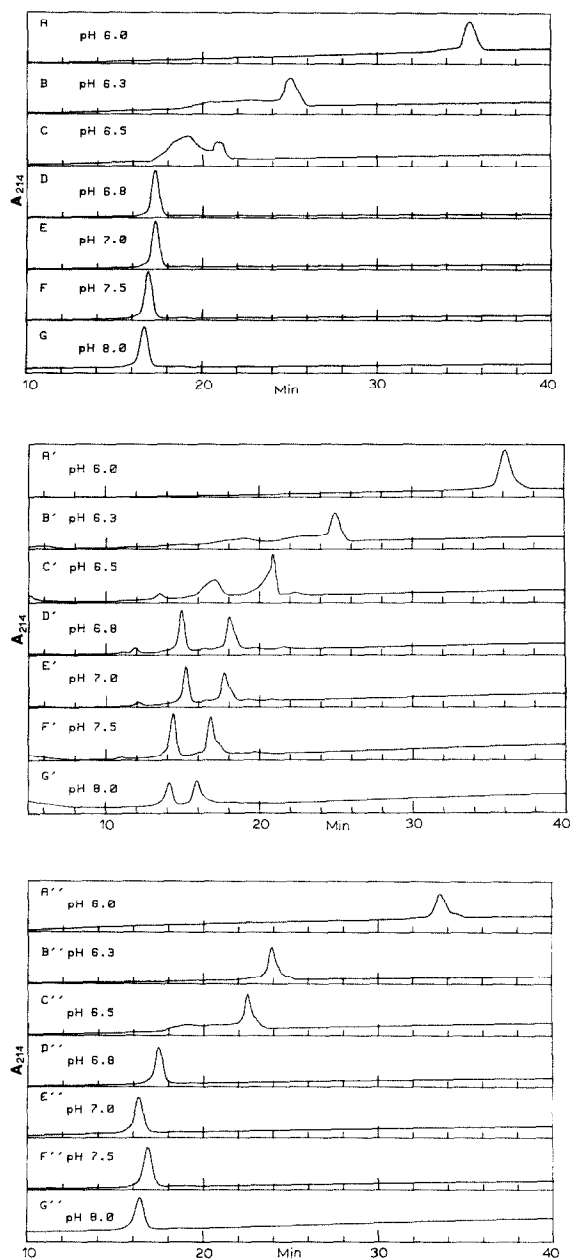
Fig. 5. Analytical SCX-HPLC at different pH values for Tm(R), Tm(W) and Tm(C). Top, unprimed panels: Tm(R$_s$) = Tm(R) monitored at 214 nm; A–G 350, 300, 300, 900, 800, 600 and 600 mV full-scale, respectively. Middle, single-primed panels: Tm(W) monitored at 214 nm; A'–G', 600, 300, 300, 300, 300, 200 and 200 mV full-scale, respectively. Bottom, double-primed panels: Tm(C) monitored at 214 nm; A"–G", 300, 500, 500, 400, 400, 400 and 200 mV full-scale, respectively. Note the broad elution profile near pH 6.5 for all three proteins.

SCX-HPLC of Tm(R) at pH 6.5 resulted in a broad, poorly resolved set of peaks (Fig. 5C). These peaks eventually coalesced into one peak at pH 6.0 (Fig. 5A) whose width, however, was slightly greater than in the chromatograms at pH 6.8–8.0 (Fig. 5, D–G). A set of standard proteins (carbonic anhydrase, ribonuclease A and lysozyme) chromatographed under identical conditions exhibited increased retention times as the pH varied from 8.0 to 6.0 but did not show peak broadening or splitting (data not shown), suggesting that the observed chromatographic transition is Tm specific.

It seems likely that the sites involved in this transition near pH 6.5 (Fig. 5C) are Tm histidine residues, for the following reasons. First. the observed transition occurs in a pH range normally entailing histidine residue titration [35]. Second, extant NMR data on purified $\alpha\alpha$ and $\beta\beta$ Tm(R) indicate that the two α-chain histidines, his-153 and his-276, titrate at different pHs with his-153 exhibiting micro-pK_a states; the β chain has only his-153, which displays a similar titration profile identical with his-153 from the α chain [36] {Tm(R) β chain residue 276 is asparagine [7]}. Further, at pHs outside the transition regon, the NMR resonances could be superimposed [36], an observation compatible with the coalescence of the SCX-HPLC peaks observed at pH 6.0 (Fig. 5A) and pH 8.0 (Fig. 5G). Third, additional NMR experiments have demonstrated that the micro-titration states of his-153 are the result of different conformational states of Tm which have different pK_a values for this histidine residue and are in slow exchange with one another near room temperature [37].

Caution is necessary, however, in merging the NMR and SCX-HPLC results into a unified structural interpretation at these sites on Tm(R) because the experimental parameters prevailing in these distinct analytical techniques are sufficiently different. The obvious differences were in protein concentration, 10–20 mg/ml in NMR vs. 0.5–3 mg/ml in SCX-HPLC, and in salt concentration, 1 M for NMR where Tm is unaggregated vs. 10 mM for SCX-HPLC where Tm could be appreciably aggregated. It is also unclear whether both Tm binding to the cation matrix resulting in a high local concentration of protein and Tm elution from the column with high salt would actually bring the two sets of experimental parameters into closer agreement. In any

event, even if the chromatographic transition near pH 6.5 (Fig. 5C) is due to histidine titration, it is far from certain if this derives from micro-pK_a states of his-153 or multiple conformational states, or simply to titration differences between the two histidines of the α chain.

Analytical SCX-HPLC of Tm(C) shows essentially the same chromatographic trend (Fig. 5, A"–G") as for Tm(R) (Fig. 5, A–G). Specifically, this includes an increased retention time as the pH is decreased from 8.0 to 6.0, a slightly wider peak at pH 6.0 (Fig. 5A") than pH 8.0 (Fig. 5G") and a transition region near pH 6.5 (Fig. 5C"). For Tm (C), this chromatographic transition is different in

absolute appearance than Tm(R), yet it seems reasonable to expect the two proteins to behave similarly. NMR titration data have not appeared for the histidine residues of Tm(C) and it would be informative to compare the NMR and SCX-HPLC data for both Tm(R) and Tm(C).

The chromatographic profiles displayed for Tm (W) (Fig. 5, middle, single-primed panels) were at first disturbing because of the two distinct peaks observed over the majority of the pH range. There are three possible explanations. First Tm(W), unlike Tm(R) and Tm(C), may be highly susceptible to dissociation into the corresponding α and β chains during binding to and/or elution from the
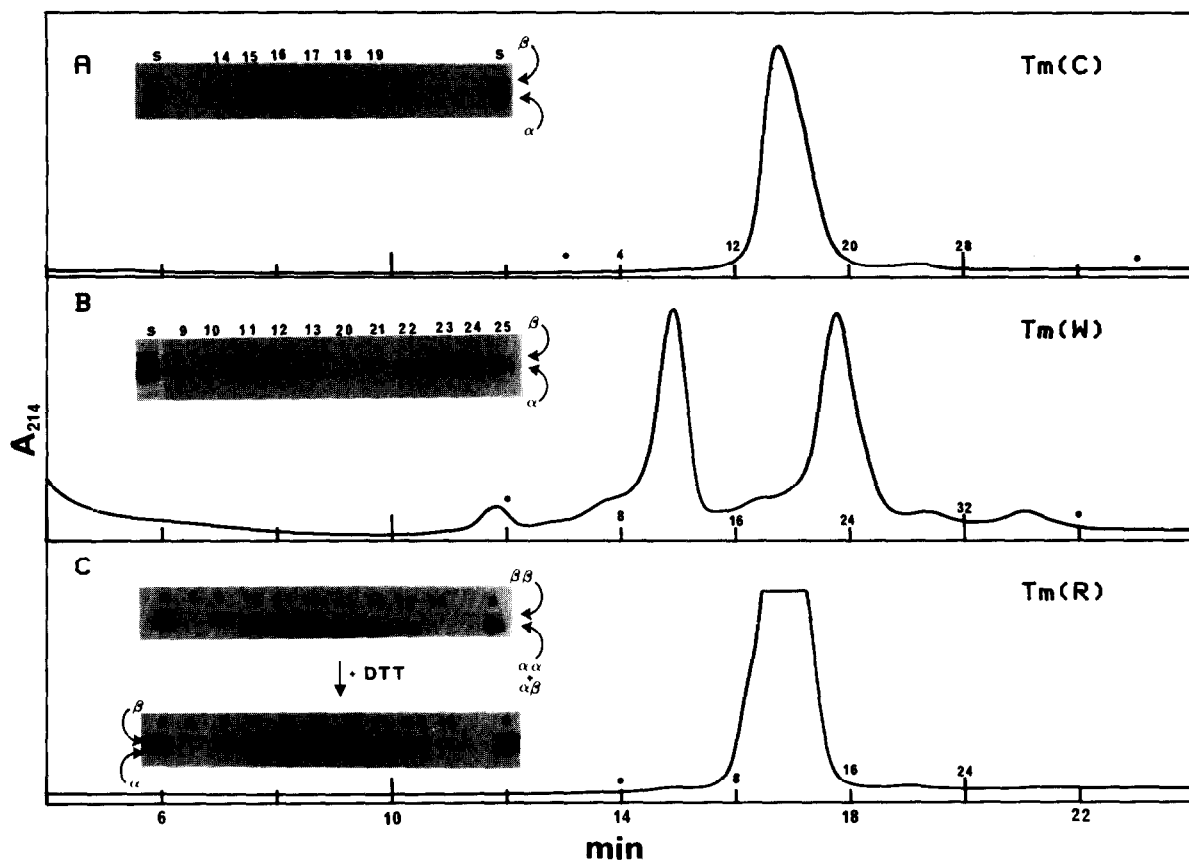

Fig. 6. Preparative SCX-HPLC at pH 7.5 and SDS-PAGE of Tm(C), Tm(W) and Tm(R). All chromatograms were monitored at 214 nm with fractions collected at 0.25-min intervals and dots indicate the beginning and end of the collection period. (A) Tm(C), 1800 mV full-scale. Inset shows 10–20% SDS-PAGE of indicated fractions and of the unfractionated, stock (S) protein. (B) Tm(W), 1200 mV full-scale. Inset shows gel analysis of indicated fractions. (C) Tm(R), 2500 mV full-scale. Major peak is off-scale; see Fig. 5F for analytical profile. Upper inset shows 4–20% SDS-PAGE of the indicated fractions and lower inset shows 10–20% SDS-PAGE of the same samples reduced with DTT prior to electrophoresis.

SCX resin. Second, chain exchange may occur during the chromatographic process. Third, the cation-exchange resin, in contrast to reversed-phase and anion-exchange supports, may actually separate according to subtle differences in the dimer isoforms. It will be seen next that preparative SCX-HPLC of Tm(W) at pH 7.5 points to a variation of the latter possibility as the correct one.

Fig. 6 illustrates preparative SCX-HPLC for all three Tms. The chromatography was performed at pH 7.5 to address the origin of the two peaks observed for Tm(W) in the analytical run (Fig. 5F') and little relevant chromatographic change was evident for Tm(R) and Tm(C) from pH 8.0 to 6.8. No unexpected results occurred for either Tm(C) (Fig. 6A), which gave only the $\alpha\beta$ hetero-dimer band, or Tm(R) (Fig. 6C), where a hint of $\beta\beta$ enrichment as the leading shoulder of the main off-scale peak was observed (upper and lower gel insets, lane 9). The starting Tm(R) sample, again, became almost exclusively oxidized, i.e., disulfide cross-linked, by dialysis into mobile phase A pH 7.5 buffer, but an uncross-linked sample yielded the same UV profile (data not shown) as is shown in Fig. 6C.

Returning to the Tm(W) preparative run (Fig. 6B), we see from the gel inset that the early eluting peak at ca. 15 min comprised approximately equal amounts of α and β chains (lanes 10–13) in addition to an upper band of slower electrophoretic mobility originally seen as a minor contaminant in the unfractionated, stock protein (lane S). The second major peak at ca. 18 min is exclusively approximately equal amounts of Tm(W) α and β chains (lanes 20–25). Apparently, this minor contaminant becomes highly enriched and is able to interact with Tm(W) α and β chains during SCX chromatography. A similar occurrence has been reported for anion-exchange chromatography of a rabbit actin–Tm(W) complex as binding of actin to Tm(W) dimer resulted in a concomitant decrease in the original Tm(W) peak and the appearance of a new peak representing the actin–Tm(W) complex [27]. If the early-eluting peak in SCX-HPLC really represents a worm actin–Tm(W) complex, then this chromatographic system could provide a general means of assaying such an interaction. Current efforts are focused on identifing this upper band by comparative peptide mapping and immunological studies.

CONCLUSIONS

This study compared the chromatographic characteristics of Tms from two vertebrate [Tm(R) and Tm(C)] and one invertebrate species [Tm(W)]. Three modes of HPLC (C$_4$RP, WAX and SCX) were used and several conclusions can be drawn from the results. C$_4$RP-HPLC of all three unoxidized proteins results in chain dissociation into individual α and β subunits. Separation of these isoforms was accomplished for Tm(W) and Tm(C) but not Tm(R). Only Tm(R) could be disulfide cross-linked, and as a consquence gave a distinct C$_4$RP chromatogram. This confirmed that the predominant native molecular species of Tm(C) is an $\alpha\beta$ hetero-dimer, as each chain has no adjacent cysteine partner to produce as disulfide cross-link. The inability to cross-link Tm(W) suggests that it also exists in vivo as the $\alpha\beta$ hetero-dimer and that if cysteines are present here they occur at distinct sequence positions in the α and β chains and are not sufficiently close to form disulfide cross-links. A preliminary denaturation–renaturation experiment [38] on Tm(W) followed by an attempted cross-linking reaction showed identical C$_4$RP chromatograms for both the native control and experimental test sample. Further, each chain probably contains a single cysteine residue [6]. Hence, under these experimental conditions, the resulting Tm(W) dimer population reached in vitro is identical with the native protein, i.e., all $\alpha\beta$.

WAX-HPLC experiments indicate that the retention times for all three Tms progressively decrease as the pH decreases from 8.4 to 5.0. Tm(R) is partially resolved into three peaks corresponding to the known mixture of dimer species found in vivo. Gel analysis of the Tm(C) and Tm(W) peaks, on the other hand, indicated that the single Tm(C) peak is exclusively composed of approximately equal amounts of α and β chains, as was the analogous Tm(W) peak which, however, exhibited apparent heterogeneity.

Similar studies using SCX-HPLC showed that all three Tms increased in retention time as the pH was lowered from 8.0 to 6.0, even though at pH 6.0 both Tm(R) and Tm(C) are highly negatively charged. Clearly, these negatively charged molecules are able to bind to a cation-exchange resin supporting the notion that net charge at a given pH is not the sole

determinant in protein–ion-exchange interaction. Near pH 6.5 all three Tms displayed a broad chromatographic transition, possibly as a result of individual histidine residue protonation. Tm(W) produced an anomalous chromatogram with the first-eluting peak containing approximately equal amounts of α and β chains yet highly enriched in a minor contaminant observed in the original protein preparation.

The chromatographic data in their entirety confirm the known *in vivo* dimer population of Tm(R) and Tm(C) and indicate, in addition, that the native *in vivo* dimer population of Tm(W) is an $\alpha\beta$ heterodimer.

ACKNOWLEDGEMENTS

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