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Chromatographic and physical studies of tropomyosin in aqueous–organic media at low pH[a]

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ABSTRACT

Non-cross-linked and disulfide-cross-linked two-chain molecules comprising the α and/or β chains of rabbit skeletal tropomyosin were studied by electrophoretic, chromatographic and physical methods. Elution order on C_4 reversed-phase high-performance liquid chromatography depends markedly on the number and position of the cross-links. In the C_4 reversed-phase elution medium, cross-linked and non-cross-linked species are >85% helical by circular dichroism, but the non-cross-linked elute later from high-performance size-exclusion chromatography (G4000) and have molecular mass of 31 000–41 000 dalton by equilibrium ultracentrifugation. The data suggest that in the C_4 reversed-phase high-performance liquid chromatography elution medium non-cross-linked tropomyosin exists as amphipathic single-chain α-helices.

INTRODUCTION

The native tropomyosin molecule is a two-chain α-helical coiled coil comprising two parallel, registered, and slightly super-twisted, amphipathic α-helical chains [1–4]. Rabbit skeletal tropomyosin (R_sTm) consists of genetically variant chains, α and β, in a 3–4:1 ratio [2,4], forming principally, two types of molecules, αα and αβ, in a ≈3:2 ratio [5,6]. Each 284 amino acid chain has a molecular mass of 33 000 dalton, and the two differ, usually by conservative substitution, at only 39 positions [7]. One important difference between the two chains is that the β chain has two cysteines, at positions 36 (C36) and 190 (C190), whereas the α chain has only one cysteine at C190. This allows the two adjacent chains of such Tm coiled-coil molecules to be covalently (disulphide) cross-linked [3,4,8–12].

In some studies, no ββ molecules were found in native R_sTm [5,6]. However, in

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other studies, $\beta\beta$ molecules have been observed in disulphide-cross-linked R_sTm [11]. To determine whether or not $\beta\beta$ molecules exist in native R_sTm, and, if not, whether their absence results from thermodynamic, kinetic, or biological factors, requires a sensitive analytical assay for the relative amounts of the various molecular species. The aforementioned studies employed hydroxyapatite (HA) chromatography and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), neither of which gives sufficient resolution for accurate quantitation of the molecular species. In fact, the resolution using HA chromatography is ordinarily so poor that the 4–6% $\beta\beta$ molecules that would result from random association of the α and β chains in R_sTm could easily be masked.

In this study we investigate the efficacy of 4–20% gradient SDS-PAGE and C_4 reversed-phase high-performance liquid chromatography (RP-HPLC) in separating the various cross-linked and non-cross-linked species that can be formed from the α and β chains of Tm. *In vitro* all three possible molecular species, $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$, form readily and can be selectively cross-linked [3,4,8–12] to give five different cross-linked species: C190 cross-linked α ($\alpha^{-}\alpha$); C190 cross-linked, C36 blocked $\alpha\beta$ ($\alpha^{-}\beta_{\cdot}$); C190 cross-linked, C36 blocked β ($_{\cdot}\beta^{-}\beta_{\cdot}$); C190 blocked, C36 cross-linked β ($^{\cdot}\beta_{-}\beta^{\cdot}$); and C190 and C36 cross-linked β ($\beta^{-}_{-}\beta$). [We use a superscript (subscript) dash to indicate cross-linking at C190 (C36), and a superscript (subscript) dot to indicate blocking at C190 (C36).] These are listed in Table I along with the non-cross-linked species studied here, *i.e.*, reduced and blocked α ($\alpha\alpha$, $^{\cdot}\alpha\alpha^{\cdot}$) and β ($\beta\beta$, $_{\cdot}\beta\beta_{\cdot}$).

We find that, although it is often possible to resolve $\beta^{-}_{-}\beta$ using 4–20% SDS-PAGE, that $\alpha^{-}\alpha$ and non-cross-linked molecules as well as $\beta^{-}_{-}\beta$ are easily resolved using C_4 RP-HPLC. Not only can small amounts of these species be detected by measuring the absorbance at 214 nm, their relative amounts in a given sample can determined by integration. Moreover, we find that the cross-linked species elute earlier

TABLE I

SUMMARY OF TROPOMYOSIN TWO-CHAIN MOLECULES

Species[a]	C36[b]	C190[b]	Elution order		Order of mobility	
			C_4 RP-HPLC	HPSEC	4–20%	9%[c]
$\alpha\alpha$	–	RD	6, 7, 8, 9	6, 7, 8, 9	1, 2	1, 2
$^{\cdot}\alpha\alpha^{\cdot}$	–	CAM or CM	6, 7, 8, 9	6, 7, 8, 9	1, 2	1, 2
$\beta\beta$	RD	RD	6, 7, 8, 9	6, 7, 8, 9	3, 4	3, 4
$_{\cdot}\beta\beta_{\cdot}$	CAM	CAM	6, 7, 8, 9	6, 7, 8, 9	3, 4	3, 4
$\alpha^{-}\alpha$	–	XL	2	1, 2, 3, 4	6, 7, 8	7
$\alpha^{-}\beta_{\cdot}$	CAM	XL	3, 4	1, 2, 3, 4	6, 7, 8	8
$_{\cdot}\beta^{-}\beta_{\cdot}$	CAM	XL	3, 4	1, 2, 3, 4	6, 7, 8	9
$^{\cdot}\beta_{-}\beta^{\cdot}$	XL	CAM	5	1, 2, 3, 4	5	5, 6
$\beta^{-}_{-}\beta$	XL	XL	1	5	9	5, 6

[a] We use a superscript (subscript) dash to indicate cross-linking at C190 (C36), and a superscript (subscript) dot to indicate blocking at C190 (C36).

[b] RD, XL, CAM, CM = Reduced, cross-linked, carboxyamidomethyl-blocked, carboxymethyl-blocked sulfhydryl, respectively.

[c] From ref. 11.

than the non-cross-linked contrary to expectations based on their hydrophobicities and chain lengths alone [13].

Many previous investigations have dealt with the effects of HPLC solvents and hydrophobic matrices on the secondary and quaternary structures of proteins and peptides and have attempted to elucidate the role of conformational changes and/or chain dissociation in their binding to and elution from such columns [13–17]. Whether conformational changes take place upon binding to a RP-HPLC column matrix is difficult to determine experimentally and beyond the scope of this study. However, in order to investigate whether the RP elution medium induces changes in the secondary and/or quaternary structures of Tm upon release from the column matrix, we examined circular dichroism (CD), high-performance size-exclusion chromatography (HPSEC) and equilibrium ultracentrifugation for various Tm species in a representative RP elution medium, namely, 0.0975% (v/v) trifluoroacetic acid (TFA) in 45% aqueous acetonitrile, without and with added NaCl. Using millimolarity as subscript and pH in parentheses, these media can be designated $CH_3CN_{8500}TFA_{13}NaCl_x$ (2).

MATERIALS AND METHODS

Protein preparation and characterization

Rabbit skeletal tropomyosin (R_sTm) and rabbit cardiac tropomyosin (R_cTm) were prepared and characterized as previously described [18,19]. R_cTm contains only α chains [20] and was used whenever pure $\alpha\alpha$ tropomyosin was required. Pure β-tropomyosin ($\beta\beta$) was obtained by carboxymethylcellulose ion-exchange chromatography of reduced, denatured R_sTm and subsequent renaturation of the β fraction [3].

The preparation and properties of the cross-linked homodimer species: $\alpha\bar{}\alpha$; $\beta\bar{}\beta$; $\beta\bar{}\beta$.; β_β· have been described previously [8–12]. For the samples used in this study, disulphide cross-linking was carried out by oxidation with ferricyanide [10], and sulfhydryl blocking was carried out by reaction with iodoacetamide or iodoacetic acid [12,21].

The preparation and isolation of the heterodimer species, $\alpha\bar{}\beta$., was carried out as follows. R_sTm, consisting mostly of $\alpha\alpha$ and $\alpha\beta$ molecules was cross-linked at C190, then blocked at the C36 position of the β chains as previously described [12]. The resulting mixture of cross-linked dimers, mostly $\alpha\bar{}\alpha$ and $\alpha\bar{}\beta$., was denatured using 8 *M* urea and separated using carboxymethylcellulose ion-exchange chromatography [22]. The final product was characterized by SDS-PAGE using both non-reducing and reducing 9% Laemmli gels, and by sulfhydryl titration of the re-reduced material [23].

The preparation and properties of ·$\alpha\alpha$· have been described [21]. The :$\beta\beta$: used in this study was produced willy-nilly, as a byproduct in the preparation of ·β_β· and separated from it by SEC [11].

Gel electrophoresis and chromatography

Samples were prepared for SDS-PAGE as described earlier [24] and analyzed on 4–20% linear acrylamide gradient mini-gels of 1 mm thickness (Enprotech, Hyde Park, MA, U.S.A.) for 1.5 h at a constant voltage of 150 V. Normally, the sample size was 5–20 μl (2.5–10 μg of protein).

RP-HPLC was performed using a Vydac 214TP54 C_4 column (25 cm × 0.46 cm I.D.) and HPSEC using a TSK 4000SW column (60 cm × 0.75 cm I.D.) plus a guard

column (7.5 cm × 0.75 cm I.D.), all purchased from the Nest Group (Southborough, MA, U.S.A.). The sources of solvents, configuration of the chromatographic hardware, temperature control, and data acquisition procedures have been previously described [25,26].

The solvent program used to effect separation on the C_4 RP column was a linear gradient from 0 to 100% B in 60 min at 1 ml min^{-1}, with mobile phases A = 0.1% TFA and B = 0.095% TFA in 90% aqueous acetonitrile. HPSEC was performed isocratically at 0.5 ml min^{-1} using a 50:50 mixture of the RP solvents A and B described above. The chromatograms were monitored at 214 nm.

Protein samples were prepared by reconstituting each lyophilized sample to a protein concentration of ≈ 1 mg ml^{-1} with 1% acetic acid (Fisher Scientific, St. Louis, MO, U.S.A.); these were stored at 4°C until use. Usually, 25 μl (25 μg of protein) or 50 μl (50 μg of protein) of cross-linked and/or blocked sample was injected onto the column. To reduce disulphide bonds, 50 μl (50 μg) of samples prepared as described above were mixed with 140 μl of $NaPi_{100}EDTA_5$ (7.4) and 10 μl dithiothreitol $(DTT)_{1000}$. The mixture was blanketed with nitrogen and then incubated at 45°C overnight. Control samples were prepared by substituting 10 μl water for the reductant. For both the reduced and control samples, 150 μl (37.5 μg of protein) of the reaction mixture was injected on the column.

CD measurements

CD measurements were carried out using a Jasco J500A spectropolarimeter interfaced to a THE personal computer. Temperature control and measurement, data collection and analysis, and calculation of fraction helix from the ellipticity at 222 nm have all been described previously [8,11]. All denaturation curves were reversible.

Solutions for CD measurements were prepared by quantitative dilution of stock protein solutions (5–10 mg ml^{-1}) in 0.195% aqueous TFA (TFA_{26}). Each stock solution was passed through a 0.45-μm filter (Gelman, Fisher Scientific) before further manipulation. For concentration determination (and CD measurements in benign buffer) an aliquot of each stock was diluted tenfold with $NaCl_{433}HCl_{10}$ (2) and the absorbance at 275 nm of the resulting solution measured against a blank prepared by diluting TFA_{26} in the same manner. The protein concentration was then calculated using an extinction coefficient of 0.314 cm^2 mg^{-1} [27]. For CD measurements in the RP medium another aliquot of each stock was diluted twofold with 90% acetonitrile (CH_3CN_{17000}) resulting in a medium with composition $CH_3CN_{8500}TFA_{13}$ (2).

Equilibrium ultracentrifugation

A Spinco Model E analytical ultracentrifuge equipped with UV optics and a photoelectric scanner was used for sedimentation equilibrium molecular mass determinations of αα and ·αα· in $CH_3CN_{8500}TFA_{13}$ (2). Protein solutions having concentrations of 0.8 to 1.6 mg ml^{-1} were first dialysed against solvent for 8 h at room temperature (using dialysis bags that had been soaked successively in a series of solutions containing 13 m*M* TFA and 10, 20, 30, 40, 45% acetonitrile) and then centrifuged at 18 000 rpm for 24–48 h at *ca.* 20°C in an An-H rotor with 12-mm, double-sector cells.

The data for each run were plotted as $\ln A_{280}$ *vs.* r^2, where r is distance from the center of rotation. Where the data fell on a straight line, the molecular mass was calculated from:

$$d(\ln A_{280})/d(r^2) = KM \quad (1)$$

where M is the molecular mass and $K = (1 - v_\rho\rho)\omega^2/2RT$; we used $v_\rho = 0.729$ ml g^{-1} [28], and a solution density, $\rho = 0.930$ g ml^{-1}. For runs where the data showed obvious downward curvature, the data were computer-fit using a finite difference Levenberg–Marquardt algorithm (program ZXSSQ in the IMSL library of programs) to the equation:

$$r^2 = A_0 + A_1 (\ln A_{280}) + A_2(A_{280}) \quad (2)$$

The coefficients of each term in the first derivative of eqn. 2 with respect to ln A_{280} can be equated with the corresponding coefficients in the two term virial expansion of the sedimentation equilibrium equation, giving the molecular mass, $M = 1/KA_1$, and second virial coefficient, $B = K(A_2)\varepsilon l/2$, where ε is the extinction coefficient in cm^2 g^{-1} and l the optical path length in cm.

RESULTS AND DISCUSSION

SDS-PAGE and C_4 RP-HPLC analysis of Tm species

Results of a 4–20% SDS-PAGE experiment with the various cross-linked species used in this study are shown in Fig. 1 and Table I (column 6). Lanes 1–10 of the gel show, respectively, $\alpha^-\beta_.$, $\beta^-\beta$, $\alpha^-\alpha$, $\cdot\beta\_\beta\cdot$ and $:\beta\beta:$, $_.\beta^-\beta_.$, $\beta^-\beta$, $\alpha^-\beta_.$, $\alpha^-\alpha$, blank and markers. The order of electrophoretic mobility for all species is: $\alpha\alpha \approx \cdot\alpha\alpha\cdot > \beta\beta \approx :\beta\beta: \gg \cdot\beta\_\beta\cdot > \alpha^-\alpha \approx \alpha^-\beta_. \approx {}_.\beta^-\beta_. > \beta^-\beta$. The band of lowest mobility, $\beta^-\beta$, is often well enough resolved on scans to allow determination of its fraction in a mixture containing other cross-linked species. For comparison, column 7 of Table I gives the order of electrophoretic mobility of the various species on 9% Laemmli SDS-PAGE [11]. On that system it is often possible to resolve and quantitate the band consisting of both $\cdot\beta\_\beta\cdot$ and $\beta^-\beta$.

Nevertheless, because the resolution obtained using SDS-PAGE is variable and the time required for staining, destaining, and quantitation is long, we investigated the use of C_4 RP-HPLC as a possible fast and direct analytical assay for the various Tm species. The results are shown in Figs. 2 and 3 as abscissa expansions of the 30–40 min region of each chromatogram and are summarized in Table I (column 4). The peaks were assigned by comparison to those obtained for each of the individual species in separate runs made under identical conditions. Except for the unretained solvent peak no peaks other than those shown appear in the complete chromatograms.

Fig. 2A–D shows expanded chromatograms of a mixture of $\beta^-\beta$, $\alpha^-\alpha$, and $\alpha^-\beta_.$ as a function of temperature. The expanded chromatogram of a mixture of $\beta^-\beta$, $\alpha^-\alpha$, $\alpha^-\beta_.$, and ${}_.\beta^-\beta_.$ at 28°C is shown in Fig. 3A, and that of a mixture of $\beta^-\beta$, ${}_.\beta^-\beta_.$, $\cdot\beta\_\beta\cdot$, and $:\beta\beta:$ at 37°C in Fig. 3B.

The variations in retention times and peak resolutions for $\beta^-\beta$, $\alpha^-\alpha$, and $\alpha^-\beta_.$ as a function of temperature are shown graphically in Fig. 2E and F as the capacity factor, k', and the resolution between pairs of samples, R_s, respectively. As the temperature increases, the retention times and k' values for all species first decrease then increase again (Fig. 2E). The resolution of the $\beta^-\beta$ and $\alpha^-\alpha$ peaks (open dels) first increases then decreases, whereas that between the $\alpha^-\alpha$ and $\alpha^-\beta_.$ peaks (open diamonds) steadily decreases up to 46°C whereupon the two peaks coalesce (Fig. 2F).

In all cases, $\beta^-\beta$ has the smallest retention time and, except at 55°C (Fig. 2D), is

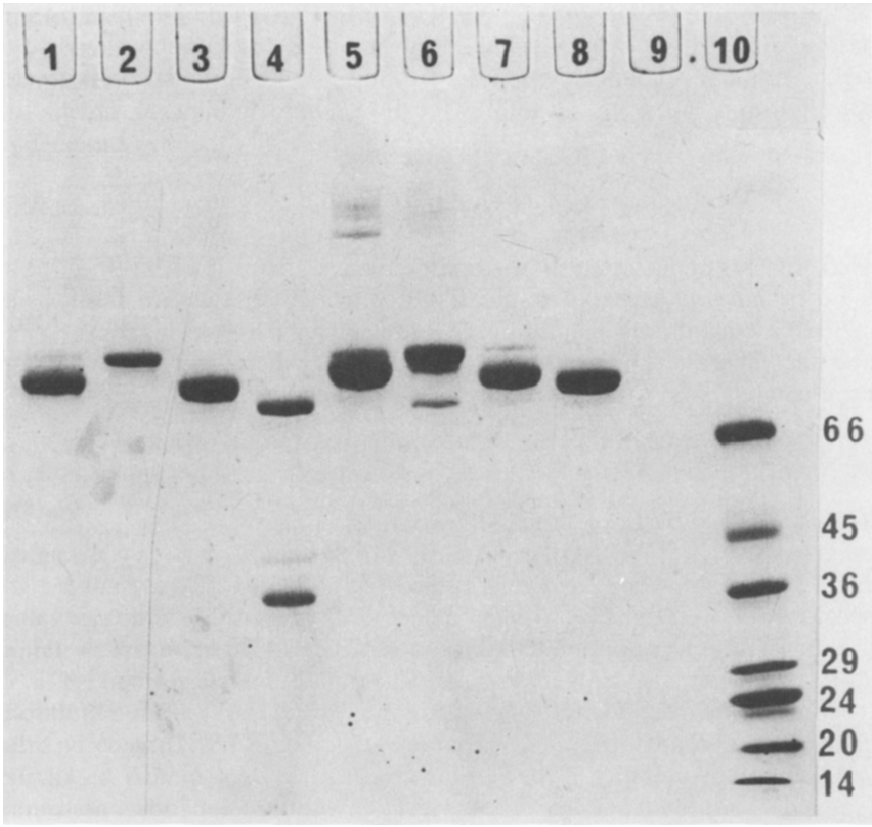

Fig. 1. Photo of 4–20% SDS-PAGE for various samples. Electrophoresis is from top to bottom. Lanes are: 1 = $\alpha^{-}\beta$.; 2 = β_β; 3 = $\alpha^{-}\alpha$; 4 = .$\beta^{-}\beta$. plus ·$\beta\beta$:; 5 = .$\beta^{-}\beta$.; 6 = β_β; 7 = $\alpha^{-}\beta$.; 8 = $\alpha^{-}\alpha$; 9 = blank; 10 = protein standard mixture with indicated molecular mass (in kilodalton). The electrophoretic mobilities of the cross-linked Tm species (66 000 dalton) are lower than that of the 66 000-dalton marker on this gel system.

well separated from the other species. The retention times for the C190 singly cross-linked species are somewhat longer, and at 28°C the $\alpha^{-}\alpha$ peak is almost baseline-resolved from that of $\alpha^{-}\beta$. (Fig. 2A). On the other hand, the retention times of $\alpha^{-}\beta$. and .$\beta^{-}\beta$. are almost the same, and for a mixture containing both, only one slightly broadened peak appears (Fig. 3A). The retention time for ·β_β· is even longer (Fig. 3B; the order of retention times is the same at 37°C as at 28°C). Retention times for the non-cross-linked species are longest and approximately equal for sulfhydryl-blocked and disulphide-reduced (data not shown) samples. The elution order at 28°C or 37°C is: $\beta\_\beta \ll \alpha^{-}\alpha < .\beta^{-}\beta. \approx \alpha^{-}\beta. < \cdot\beta\_\beta\cdot < \alpha\alpha = \cdot\alpha\alpha\cdot = \beta\beta = :\beta\beta:$.

The retention times for the various Tm species depend on the number and positions of the cross-links and thus cannot be accounted for wholly on the basis of their hydrophobicities and chain lengths as others have found for several proteins, including R_cTm [13]. The 284-residue α and β Tm chains differ at 39 positions, only 11 of which are interior hydrophobic positions; the chains almost match in the number of hydrophobic (104 *vs.* 102), basic (55 *vs.* 54), and acidic (80 *vs.* 81) residues [7]. Thus,

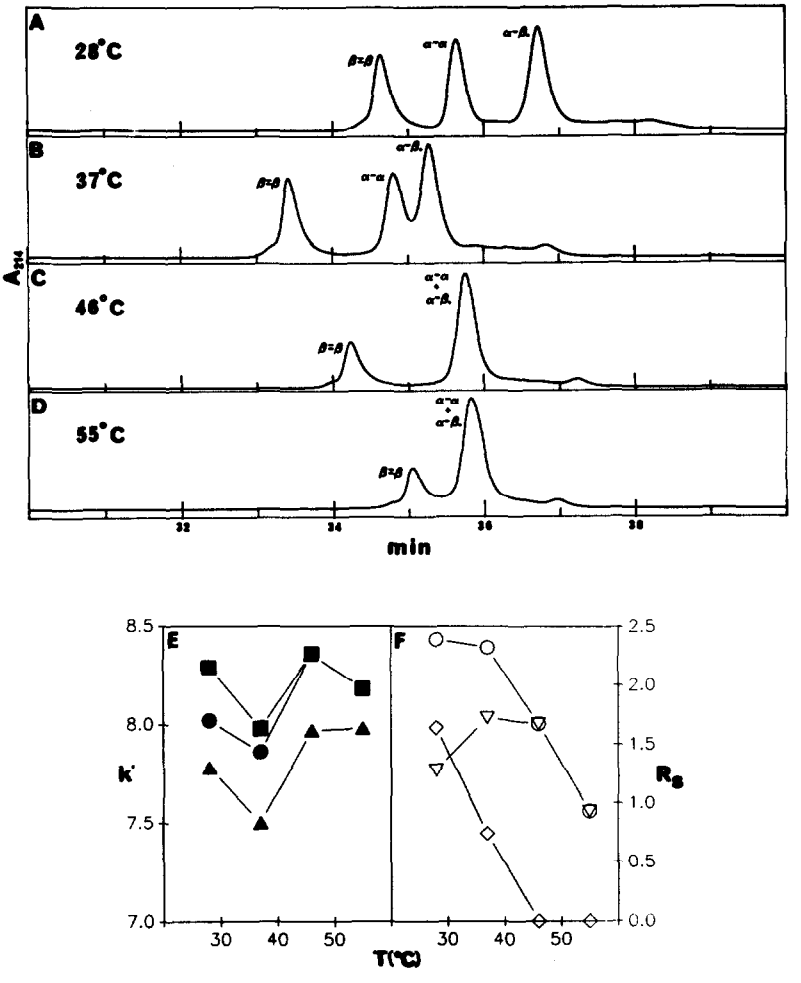

Fig. 2. Effect of temperature on the C_4 RP resolution of $\beta\text{-}\beta$, $\alpha\text{-}\alpha$ and $\alpha\text{-}\beta$,; scale = 375 mV full scale; mobile phases A = 0.1% aqueous TFA and B = 0.095% TFA in 90% aqueous acetonitrile with a linear gradient from 0 to 100% B in 60 min at 1 ml min^{-1}. (A–D) C_4 RP chromatograms at 28, 37, 46 and 55°C, respectively; (E) capacity factor, k', *vs.* temperature: ▲ = $\beta\text{-}\beta$; ● = $\alpha\text{-}\alpha$; ■ = $\alpha\text{-}\beta$,; (F) resolution, R_s, *vs.* temperature: ▽ = $\beta\text{-}\beta/\alpha\text{-}\alpha$; ○ = $\beta\text{-}\beta/\alpha\text{-}\beta$,; ◇ = $\alpha\text{-}\alpha/\alpha\text{-}\beta$,.

their overall hydrophobicities should be very similar. Indeed, we observe no separation between non-cross-linked α and β using C_4 RP-HPLC.

Since there are twice as many hydrophobic sites in a cross-linked molecule as in one of its constituent chains, the overall hydrophobicities of all cross-linked species are also very similar. All cross-linked Tm species, then, would be expected to elute at approximately the same time, but, owing to their longer chain lengths, later than the non-cross-linked species. However, we find here that the cross-linked species not only elute earlier than the non-cross-linked, but at times quite different from one another.

We also find a similar inversion of expected elution positions for the cyanogen bromide (CNBr) produced C-terminal segment of $\alpha\text{-}\alpha$. A preliminary result shows

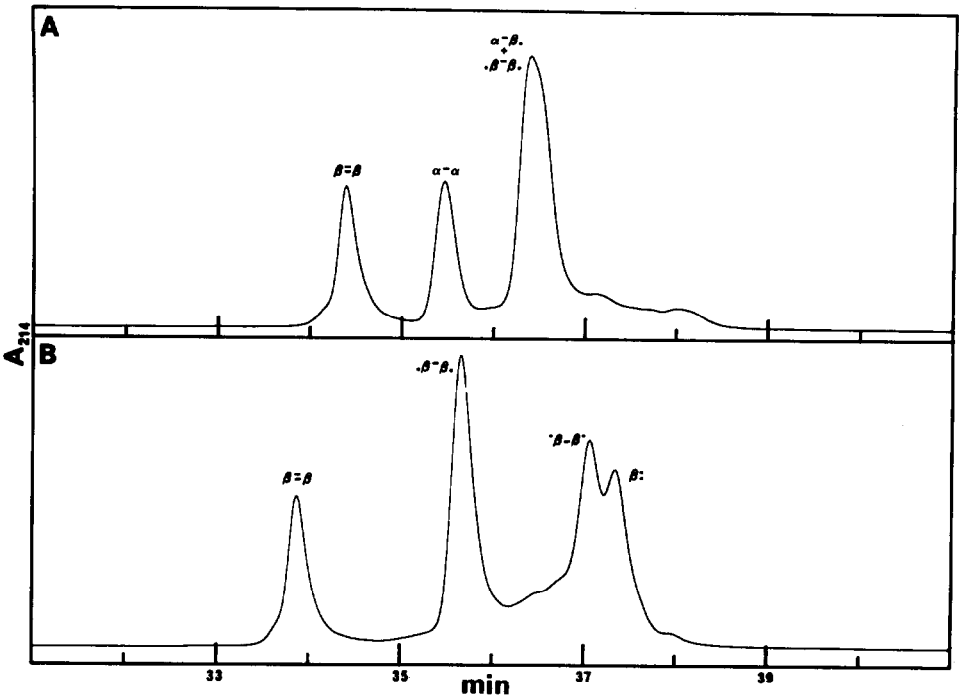

Fig. 3. (A) C_4 RP-HPLC chromatogram for $\beta\text{-}\beta$, $\alpha\text{-}\alpha$, $\alpha\text{-}\beta$ and $\beta\text{-}\beta$ at 28°C. Chromatographic conditions same as for Fig. 2, except that scale = 550 mV f.s. (B) C_4 RP-HPLC chromatogram for $\beta\text{-}\beta$, $\beta\text{-}\beta$, $\beta\text{-}\beta$, and $\beta\beta$ at 37°C. Chromatographic conditions same as for Fig. 2, except that scale = 550 mV f.s.

that when reduced, the C-terminal segment, comprising residues 142–281 ($_{142}\alpha_{281}$), elutes later than when cross-linked, and approximately at the same time as the N-terminal segment, comprising residues 11–127 ($_{11}\alpha_{127}$). The N-terminal and reduced C-terminal segments have comparable chain lengths, almost equal fractions of positive, negative and hydrophobic residues, and similar coiled-coil conformation near room temperature in benign neutral medium [29,30], so would be expected to have similar elution behavior. However, we find cross-linking $_{142}\alpha_{281}$, as in parent Tm, leads to an inversion of expected elution positions. A similar inversion, albeit accompanied by *decreased* resolution upon cross-linking, has been observed for short Tm-analogue peptides (35 residues/chain) [31].

This inversion must arise from decreased interactions of the cross-linked species with the hydrophobic solid support. In the α-helical coiled-coil structure present in native parent Tm, excised Tm segments and Tm-analogue peptides, the amphipathicity of the helices provides strong inter-chain hydrophobic interactions that impart extraordinary stability to the structure under benign conditions [1,8,29–33]. These inter-chain interactions must be disrupted and the hydrophobes exposed for a coiled-coil molecule to interact with the hydrophobic solid support. Maximal interaction will occur when the chains are completely dissociated. For cross-linked molecules complete dissociation of the helices is, of course, impossible and, owing to conformational constraints imposed by one or more cross-links, complete exposure of the hydrophobic

residues is difficult. There are no such constraints for non-cross-linked molecules, resulting in increased binding to, and later elution from, the column.

Moreover, we find that the retention times for the cross-linked species themselves vary and that $\beta\text{-}\beta$ elutes before all the C190-cross-linked species, which, in turn, elute before $\beta\text{-}\beta'$. It is not surprising that two cross-links are more effective than one in decreasing the accessibility of the hydrophobic sites. It is surprising, however, that a single cross-link at C190 is more effective than a single cross-link at C36, even in the two singly cross-linked $\beta\beta$ species that are identical except for the exchanged positions of the cross-linked and blocked sulfhydryls. A preliminary result shows that this order of elution persists in excised, cross-linked CNBr-produced segments of $\beta\text{-}\beta$. That is, the C190-cross-linked C-terminal segment, comprising residues 147–265 ($_{147}\beta_{265}$) elutes before the C36-cross-linked N-terminal segment, comprising residues 11–127 ($_{11}\beta_{127}$) (C_8 stationary phase, *n*-propanol mobile phase).

The complete or near-complete resolution of some Tm species, particularly $\beta\text{-}\beta$, suggests that C_4 RP can be used as an analytical assay. We determined the relative amounts of various species in a sample of cross-linked R_sTm (data not shown) and compared the results to those obtained using 4–20% SDS-PAGE and free solution capillary electrophoresis (FSCE). The presence of $\beta\text{-}\beta$ in this sample is easily detected using C_4 RP-HPLC and the relative amounts of the principal species present (7.3% $\beta\text{-}\beta$, 55.8% $\alpha\text{-}\alpha$, 32.1% $\alpha\text{-}\beta_1$, 2.3% non-cross-linked, and 2.6% unidentified) are in agreement with those obtained from 4–20% SDS-PAGE (7.6% $\beta\text{-}\beta$, and 92.4% $\alpha\text{-}\alpha$ plus $\alpha\text{-}\beta_1$). These results are also in agreement with a preliminary result obtained using FSCE (Beckman P/ACE System 2000). In $urea_{7000}NaPi_{50}SDS_{0.07}$ (7.4) all β species in a cross-linked R_sTm sample showed the same mobility and were well resolved from $\alpha\text{-}\beta_1$, which was almost completely resolved from $\alpha\text{-}\alpha$; the relative amounts were 8.2% β (all species), 29.1% $\alpha\text{-}\beta_1$, and 62.6% $\alpha\text{-}\alpha$. Thus, we observe an amount of $\beta\beta$ molecules in R_sTm that is not inconsistent with the 4–6.25% expected for random association of α and β chains present in a 3–4:1 ratio. Since our samples were never heated (initial reduction carried out at 4°C overnight) and since chains of the native molecules do not exchange at or below room temperature [9], it is unlikely that these $\beta\beta$ species were produced through intermolecular cross-linking. However, further investigations are being carried out to exclude that possibility.

Studies of Tm species in C_4 RP-HPLC elution solvent

In order to investigate whether the elution medium itself causes secondary and/or quaternary conformational changes we carried out CD, HPSEC and equilibrium ultracentrifugation studies for several of the Tm species in $CH_3CN_{8500}TFA_{13}$ (2). The composition of this medium is very close to the average required to elute cross-linked species from the C_4 RP-HPLC solid support, and unless interaction with the solid support itself produces irreversible changes, the conformation of a given species dissolved directly in this representative RP medium should be approximately the same as it would be upon elution.

CD spectra and thermal denaturation profiles

The CD spectra at 3°C for $\beta\text{-}\beta$, $\alpha\text{-}\alpha$, and $\alpha\alpha'$ in the average RP elution medium are shown in Fig. 4 and all have the characteristic double minima at 208 and 222 nm indicative of α-helical structure. For each, the ratio of the intensity of the 222 nm to

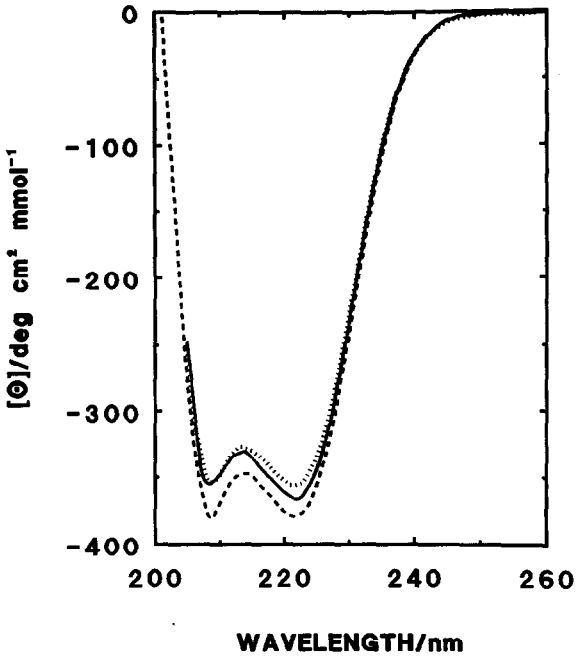

Fig. 4. CD spectra at 3°C for various Tm species in $CH_3CN_{8500}TFA_{13}$ (2). Dashed curve, 0.73 mg ml^{-1} $\alpha^{-}\alpha$; solid curve, 2.60 mg ml^{-1} ·αα·; dotted curve, 1.77 mg ml^{-1} $\beta\text{-}\beta$.

that of the 208 nm minimum is approximately unity, whereas in benign aqueous media, it is ≈1.1. Approximately equal intensities of the two minima have also been observed for Tm in trifluoroethanol (TFE) [33] and in SDS [34] solutions, and for Tm-analogue peptides in acetonitrile and in TFE solutions [31–33,35]. If the decrease in $\theta_{222}/\theta_{208}$ observed here for the Tm species upon change from aqueous benign media to the RP medium is indeed associated with a change in conformation from a two-chain coiled-coil conformation to non-interacting helices as recently predicted from theory [36], then the helices must be non-interacting in non-cross-linked and cross-linked species alike, even though the latter are physically tethered.

Fraction helix, [Φ] (from CD at 222), is plotted *vs.* temperature in Fig. 5A for $\alpha^{-}\alpha$, αα, and ·αα·, and in Fig. 5B for $\beta\text{-}\beta$. Thermal profiles are shown for benign aqueous as well as RP acidic media. Up to ≈40°C, both $\alpha^{-}\alpha$ (Fig. 5A) and $\beta\text{-}\beta$ (Fig. 5B) are almost as helical in the RP medium (≈90% at 25°C and ≈80% at 40°C) as in benign aqueous acidic solution, wheras ·αα· (< 1.6 mg ml^{-1}) is slightly less stable in the RP elution medium (≈85% at 25°C and 75% at 40°C; Fig. 5A). Nevertheless, in the medium used here for C_4 RP-HPLC all species are highly helical and, therefore, none of them undergo major changes in secondary structure. In previous studies of the conformations of proteins both in free solution and when bound to an RP support it was also found that the helical forms of those proteins constitute the eluted forms and that those proteins were less helical when bound to the RP-HPLC support [14,15].

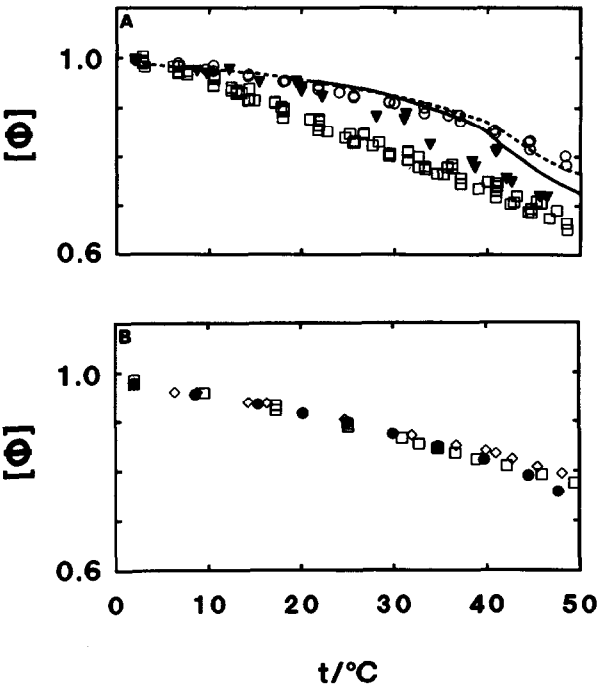

Fig. 5. Fraction helix, [Φ], (from CD at 222) *vs.* temperature. (A) αα species: dashed (solid) curve is spline curve through data for αα (α⁻α) in $NaCl_{390-590}HCl_{10}$ (2) [37]. ○ = ·αα·, 0.59 mg ml^{-1} in $NaCl_{390}HCl_{10}$ (2); □ = ·αα·, 0.017–1.6 mg ml^{-1} in $CH_3CN_{8500}TFA_{13}$ (2); ▼ = α⁻α, 0.0126, 0.73 and 0.90 mg ml^{-1} in $CH_3CN_{8500}TFA_{13}$ (2). (B) β⁻β solutions: □ = 0.315 mg ml^{-1} in $NaCl_{390}HCl_{10}$ (2); ◇ = 1.77 mg ml^{-1} in $CH_3CN_{8500}TFA_{13}$ (2); ● = 0.133 mg ml^{-1} in $CH_3CN_{9440}TFA_{4.1}NaCl_{146}HCl_5$ (2).

Size-exclusion chromatography

To determine whether the RP elution medium causes quaternary structural changes we carried out HPSEC for the Tm species in the RP medium at 25°C. The elution profiles for α⁻α, ·αα· and a mixture thereof (Fig. 6A), and those for β⁻β, .β⁻β., ·β_β·, and :ββ: (Fig. 6B) show a sharp peak for each of the principal species. The elution order is: α⁻α = α⁻β. (data not shown) = .β⁻β. = ·β_β· ≈ β⁻β < αα (data not shown) = ·αα· = ββ (data not shown) = :ββ: and is given in column 5 of Table I. Samples of α⁻β., ·β_β·, ·αα·, and :ββ: that had been subjected to RP-HPLC were collected and then subjected to HPSEC (data not shown). The results are the same as those for samples that had not previously been subjected to RP-HPLC. Thus, interaction with the support during C_4 RP-HPLC produces no irreversible changes in these species that affect their HPSEC behavior.

We find here that in the RP medium, the cross-linked species elute before the non-cross-linked, indicating that the former have larger Stokes' radii than the latter. Since both have similar, highly helical, secondary structures in the RP medium, the differences in HPSEC elution times must be caused by diferences in the quaternary structure, *i.e.*, by further association of the cross-linked molecules to higher aggregates or by dissociation of the non-cross-linked molecules into single-chain α-helices. The former is highly unlikely; the presence or absence of a cross-link should have little

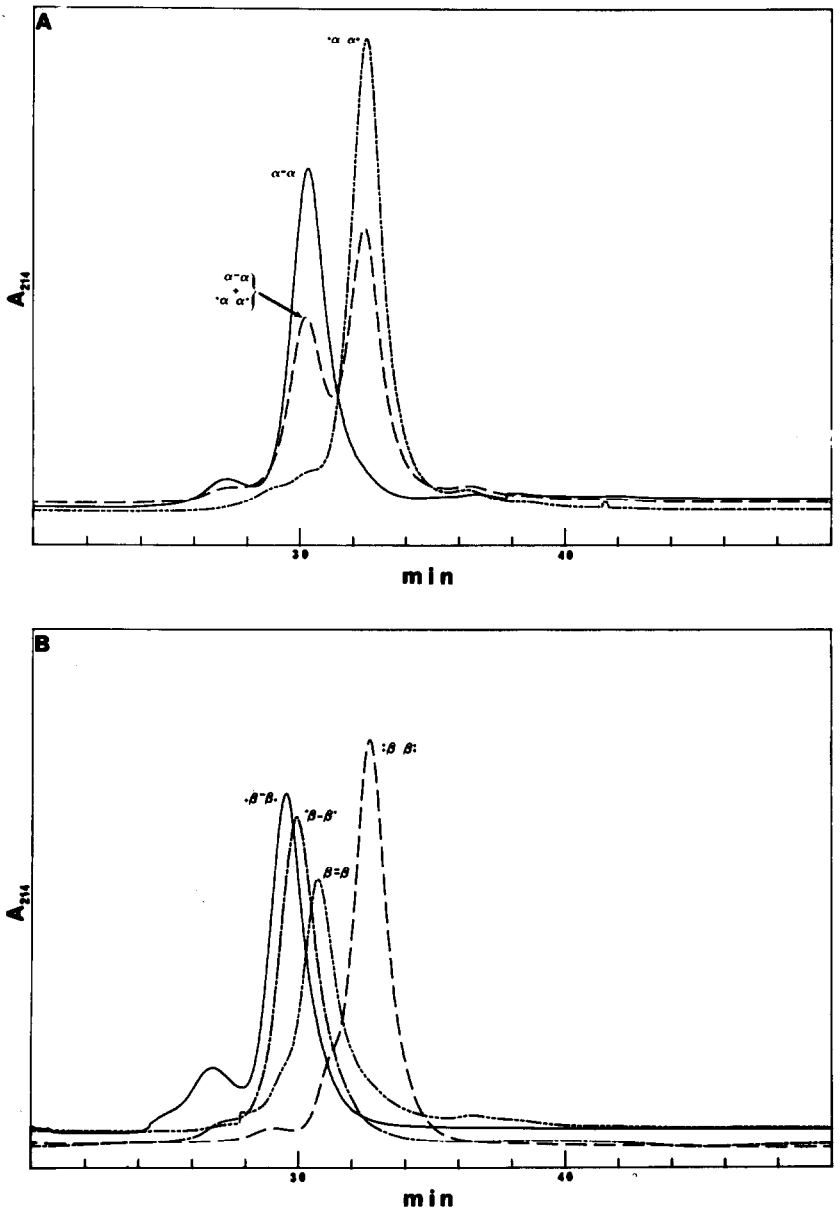

Fig. 6. HPSEC chromatograms of Tm species. Mobile phase is $CH_3CN_{8500}TFA_{13}$ (2) for all. (A) Overlay of three separate chromatograms for $\alpha^{-}\alpha$ (solid curve), $\cdot\alpha\alpha\cdot$ (long-short-short dashed curve), and a mixture thereof (long dashed curve); scale = 500 mV f.s. (B) Overlay of four separate chromatograms for $\beta^{-}\beta$ (solid curve), $\cdot\beta_{-}\beta\cdot$ (long-short dashed curve), $\beta_{-}\beta$ (long-short-short dashed curve), and $\cdot\beta\beta\cdot$ (long dashed curve); scale = 700, 450, 300 and 450 mV f.s., respectively.

affect on the formation of higher aggregates. In order to investigate whether the chains, indeed, dissociate in the RP medium we carried out additional HPSEC experiments of Tm species in the presence of added salt. The addition of NaCl should increase the helix–helix hydrophobic interactions as well as decrease the chain dipole–chain dipole repulsions [38], and at high enough salt concentration cause the chains to reassociate.

Additional HPSEC experiments were carried out on ·αα·, αα, and α¯α in $NaCl_{390}HCl_{10}$ (2) and in the average RP elution medium containing 50 and 100 m*M* NaCl; the elution profiles are shown in Fig. 7. In $NaCl_{390}HCl_{10}$ (2) (Fig. 7A) all three species show the same elution profile: one main peak flanked by two considerably smaller peaks. The two small flanking peaks are probably aggregated and monomeric Tm, or some unrelated impurities. In any case their amounts are small. In benign acidic aqueous medium αα has the same intrinsic viscosity [34] as R_sTm which has been shown to be a two-chain α-helical molecule [27]. Thus, it seems reasonable to conclude that in benign media ·αα·, αα, and α¯α all exist predominantly as two-chain coiled coils.

The effect of added salt in the RP elution medium is shown in Fig. 7B, which gives elution profiles of α¯α, αα and ·αα· in $CH_3CN_{8500}TFA_{13}NaCl_{100}$ (2). The profile for α¯α is similar to that observed in benign acidic aqueous solution (Fig. 7A), except that elution times for corresponding peaks are shorter. The profile for ·αα·, however, shows two broad overlapping peaks, a larger one that elutes at the same position as α¯α (≈38 min; peak I) and a smaller one that elutes later (≈44 min; peak II). The eluent from each peak was collected and reinjected onto the column. The results are also shown in Fig. 7B. The elution profile of reinjected peak I is similar to the original; reinjected peak I elutes as two peaks —a larger one with elution time ≈38 min and a much smaller one with elution time ≈44 min. Reinjected peak II elutes as a single peak with elution time ≈44 min.

Fig. 7C shows ·αα· and α¯α in $CH_3CN_{8500}TFA_{13}NaCl_{50}$ (2). Again, the elution behavior of α¯α is similar to that observed in benign acidic solution (Fig. 7A) or in $CH_3CN_{8500}TFA_{13}NaCl_{100}$ (2) (Fig. 7B), except that the elution times for corresponding peaks are shorter. As in 100 m*M* NaCl, the profile for ·αα· shows two broad overlapping peaks, but the peak that elutes at the same time as α¯α is considerably diminished. We also find that the elution time for a given species and a given mobile phase is independent of the medium of the injected sample, indicating that equilibrium with the column mobile phase is achieved in a much shorter time than is required for elution, *i.e.*, ≪30–40 min. For example, the elution time of ·αα· is the same whether the sample was in $NaCl_{390}HCl_{10}$ (2) or $CH_3CN_{8500}TFA_{13}NaCl_{50}$ (2) prior to injection onto a column with mobile phase $CH_3CN_{8500}TFA_{13}NaCl_{50}$ (2) (Fig. 7C).

The data are consistent with the existence of an equilibrium between two-chain α-helical ·αα· species and single-chain α-helical α· species in the RP elution medium containing added salt. Increasing the salt concentration favors two-chain species and decreasing it favors single-chain species, and in the absence of added salt the single-chain species are overwhelmingly favored.

Equilibrium ultracentrifugation

Fig. 8 shows sedimentation equilibrium data for αα and for ·αα· in $CH_3CN_{8500}TFA_{13}$ (2) plotted as ln A_{280} *vs.* r^2. The values for αα fall on a straight line (correlation coefficient = 0.9997), and give an apparent molecular mass of 31 000 dal-

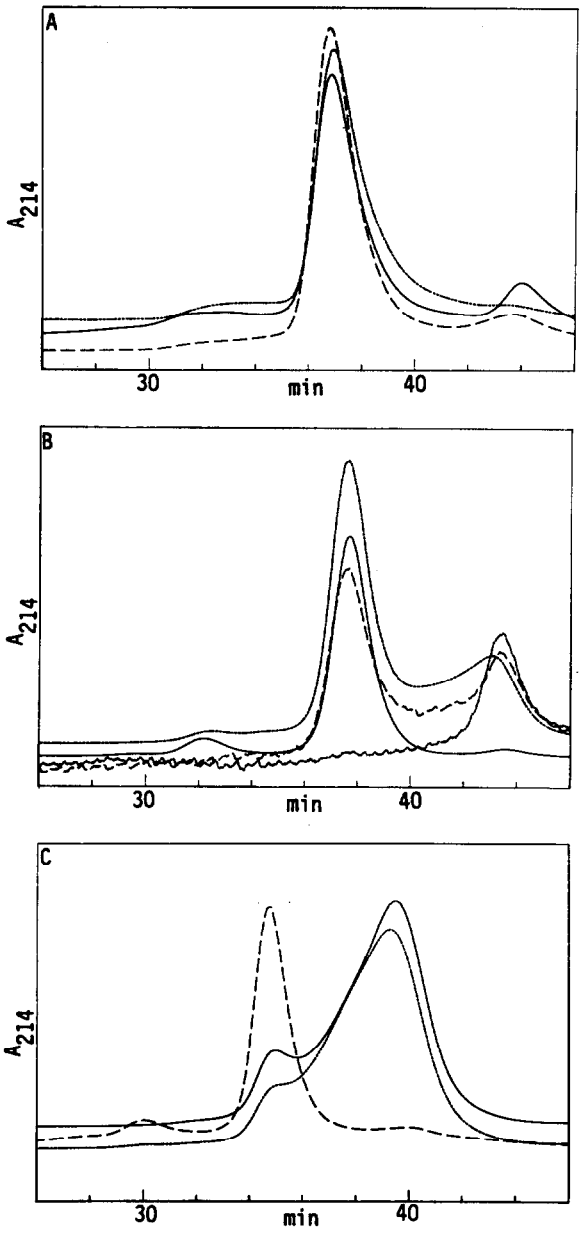

Fig. 7. HPSEC chromatograms of Tm species. (A) Mobile phase is $NaCl_{390}HCl_{10}$ (2). Overlay of three separate chromatograms for ·αα· in $NaCl_{390}HCl_{10}$ (2) (solid curve), αα in $CH_3CN_{8500}TFA_{13}$ (2) (dashed curve), and $\alpha^{-}\alpha$ in $CH_3CN_{8500}TFA_{13}NaCl_{100}$ (2) (long-short-short dashed curve); scale = 325, 500 and 100 mV f.s., respectively. (B) Mobile phase and sample buffer for all is $CH_3CN_{8500}TFA_{13}NaCl_{100}$ (2). Overlay of four separate chromatograms for ·αα· (long-short-short dashed curve), reinjected ≈38 min ·αα· peak (dashed curve), reinjected ≈44 min ·αα· peak (short-long curve), and $\alpha^{-}\alpha$ (solid curve); scale = 250, 10, 7.5 and 375 mV f.s., respectively. (C) Mobile phase is $CH_3CN_{8500}TFA_{13}NaCl_{50}$ (2). Overlay of three separate chromatograms for ·αα· in $NaCl_{390}HCl_{10}$ (2) (solid curve), and ·αα· (long-short-short dashed curve) and $\alpha^{-}\alpha$ (dashed curve) in $CH_3CN_{8500}TFA_{13}NaCl_{50}$ (2); scale = 150, 200 and 250 mV f.s., respectively.

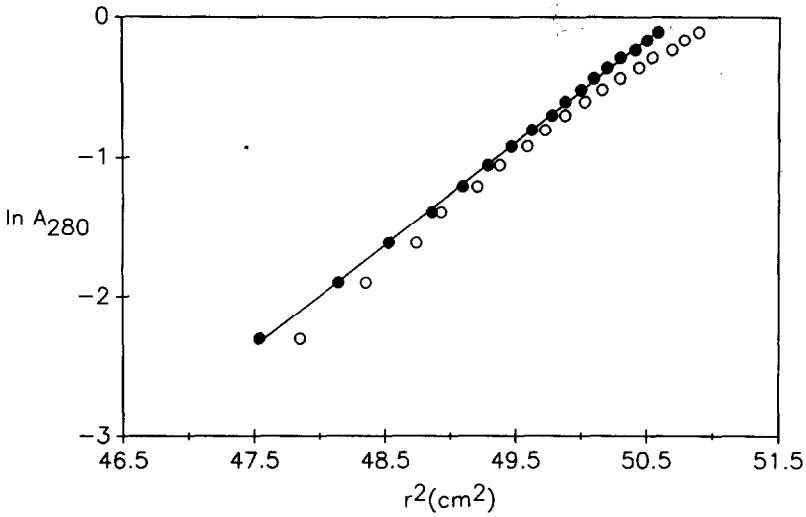

Fig. 8. Sedimentation equilibrium data plotted as the natural logarithm of A_{280} *vs.* the square of the radial distance: ● = αα, and ○ = ·αα·, in $CH_3CN_{8500}TFA_{13}$ (2). Initial concentrations, temperatures, and times for αα (·αα·) were 1.0 (0.87) mg ml^{-1}, 17.2 (19.6)°C and 24 (45 h), respectively. Rotor speed was 18 000 rpm for both.

ton (eqn. 1). Those for ·αα· fall on a slightly curved line that is concave downward. Downward curvature in such a plot indicates non-ideality, whereas heterogeneity in the system will cause upward curvature. Thus, it is possible that the straight line observed for αα results from fortuitous cancellation of the two and that the curved line for ·αα· results from predominance of the former. A straight line fit to the data for ·αα· (eqn. 1) gives an apparent molecular mass of 29 000 dalton and correlation coefficient of 0.9953, whereas a computer fit to eqn. 2 above gives a molecular mass of 41 000 dalton (infinite dilution), second virial coefficient of $4.4 \cdot 10^{-4}$ mol cm^3 g^{-2}, and root mean square (r.m.s.) residual 0.022. Using this second virial coefficient to calculate a corrected molecular mass for the αα sample gives 32 000 dalton.

For comparison we also examined the sedimentation equilibrium behavior of $\alpha^{-}\alpha$ (1.6 mg ml^{-1}; data not shown) in $CH_3CN_{8500}TFA_{13}$ (2). The data were fit using eqn. 2 above which gives molecular mass of 58 000 dalton, second virial coefficient of $28 \cdot 10^{-4}$ mol cm^3 g^{-2}, and r.m.s. residual 0.0155. Although the molecular mass of the cross-linked sample is less than the two-chain value (66 000 dalton), it is distinctly greater than those for the non-cross-linked species, indicating that the non-cross-linked species are highly dissociated in the RP medium.

CONCLUSIONS

We find here that C_4 RP-HPLC is useful as an analytical tool for detecting various non-cross-linked and disulphide-cross-linked dimers formed from the α and β chains of R_sTm, and for quantitating the relative amounts of $\beta^{-}\beta$, $\alpha^{-}\alpha$, and non-cross-linked material in a given sample. The method should be applicable to mixtures of other cross-linked coiled coils as well.

We also find that in the RP elution medium both the cross-linked and non-cross-linked species are highly α-helical, and that the non-cross-linked species are highly dissociated into single-chain α-helices, which, upon addition of salt, reassociate. Although long chain synthetic polypeptide have long been known to form single-chain α-helical structures [39] and short, synthetic amphipathic peptides have more recently been shown to form such structures [31,33,40], to our knowledge this is the first observation of a naturally occurring protein single-chain α-helix.

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