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Thermally induced chain exchange of frog $\alpha\beta$-tropomyosin

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Abstract

The thermally induced unfolding of the α-helix of *Rana esculenta* $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ tropomyosin and two tryptic fragments approximately corresponding to the N- and C-terminal halves of $\alpha\beta$ have been investigated by use of optical rotation, circular dichroism and UV difference spectroscopy. Reversible unfolding transitions of $\alpha\alpha$ and $\beta\beta$ occur around 49°C and 32°C, respectively. The helix unfolding of $\alpha\beta$ shows two major transitions at 36°C and 48°C, with only the latter being reversible. The major unfolding transitions of each of the N- and C-terminal $\alpha\beta$ peptides roughly correspond to the low and high temperature transitions of intact $\alpha\beta$, respectively. This suggests that the unfolding of $\alpha\beta$ could be due to unfolding of two independent domains in $\alpha\beta$. UV difference data, crosslinking and chromatography results show, however, that the unfolding of $\alpha\beta$ at 36°C is due to chain exchange with the formation of $\alpha\alpha$ homodimers and largely unfolded β monomers, and that the transition at 48°C is due to unfolding of $\alpha\alpha$ dimers.

Keywords: Tropomyosin; Helix-coil transition; Domain unfolding; Thermal stability; Chain exchange; Frog $\alpha\beta$-tropomyosin

1. Introduction

Tropomyosin (TM) is an important component of the contractile apparatus of skeletal and cardiac muscles, where it is involved in the regulation of the myosin–actin interaction [1]. TMs from striated and smooth muscle consist of two 284-residue peptide chains, arranged in register and parallel, forming a coiled-coil structure. Many muscle cells contain two major forms of TM, often designated $\alpha\alpha$ and $\alpha\beta$, where α and β refer to two types of peptide chains in the coiled-coil structures. The α and β chains are very similar with respect to amino acid sequence [2]. The biological significance of the presence of two TM forms is uncertain, but different cells have varying ratios of α and β which change during differentiation and development [3,4]. For many muscle cells, where the concentration ratio of the two chains is close to unity, the predominant molecular species is found to be the heterodimer, $\alpha\beta$ [5–7]. Refolding from separated chains under certain experimental conditions results in a mixture of homodimers, $\alpha\alpha + \beta\beta$ [7–9]. It is therefore unclear how cells which contain both α and β subunits regulate the relative amounts of homodimers and heterodimers.

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Coiled-coil structures are found as a stabilizing motif in many different types of proteins [10], and TMs are therefore also of interest as simple model systems for more general studies of helix stability in proteins. Conformational changes of TM have been measured by a number of techniques mostly on rabbit skeletal TM and have involved measurements of various physical properties as a function of temperature [11–20]. There is general agreement that the α-helical TM molecule unfolds and dissociates in parallel during a heating experiment [17], but the number of transitions and the nature of the putative intermediates have not been resolved. Most studies [11–21] show a complex thermal unfolding pattern indicating two or more regions of varying stability or many states of partially unfolded molecules. It is currently not clear whether unfolding of TM homodimers is better described by a fairly limited number of domains, as indicated by, for example, calorimetry [13], or by a continuum-of-states model [16]. The unfolding of heterodimers is expected to be even more complex, since not only domains or states of heterodimers are involved, but homodimer domains or states also contribute to the equilibrium state.

In a CD study of the thermal unfolding of *Rana esculenta* $\alpha\beta$ TM two major helix-coil transitions were observed [7] which could have been interpreted as the unfolding of independent domains. On reversal and reheating of the heterodimer sample, however, a new reversible unfolding pattern was observed which was in excellent agreement with the sum of unfolding curves for the $\alpha\alpha$ and $\beta\beta$ homodimers. It was therefore suggested that chain exchange takes place to form $\alpha\alpha$ and $\beta\beta$ during the first temperature transition of the initial heating cycle. Because $\beta\beta$ is largely unfolded at temperatures corresponding to the first $\alpha\beta$ transition, about half of the helix is expected to be lost, as observed. Thus, the two unfolding transitions observed for $\alpha\beta$ did not appear to be due to the unfolding of two α-helical domains of equal size with different stability, but rather to a more dramatic change involving complete separation of the two chains in the $\alpha\beta$ heterodimer and formation of new coiled-coil homodimers. In this paper we present results which more directly show that chain exchange rather than domain unfolding describes the unfolding properties of frog $\alpha\beta$ TM.

2. Materials and methods

2.1 Protein purification

Tropomyosin was extracted from *R. esculenta* leg muscles, and the $\alpha\beta$ and $\alpha\alpha$ forms were separated by use of hydroxyapatite chromatography, as described elsewhere for another frog type [22]. Since $\beta\beta$TM was not found in the frog muscle, it was prepared from $\alpha\beta$ using carboxymethyl-cellulose ion exchange chromatography in 6 *M* urea [23]. Fractions containing separated β-chains were pooled and refolded by adjusting the pH to 7.0 followed by dialysis for 48 h against 0.6 *M* NaCl, 20 m*M* Na-phosphate (pH 7.0), 0.5 m*M* EDTA, 0.2 m*M* NaN_3, and 0.5 m*M* dithiothreitol (DTT). This solvent will be referred to as Buffer M. The sample was finally dialyzed against water and lyophilized.

The N-terminal ($\alpha\beta$T1) and C-terminal ($\alpha\beta$T2) peptides of $\alpha\beta$ were obtained by digesting purified $\alpha\beta$TM at 2 mg/ml with trypsin at 20 μg/ml for 1 h at 0°C and quenching the reaction with soybean trypsin inhibitor. Digestion at low temperatures results in more specific cleavage, leading to fewer peptides, in agreement with results for rabbit TM [14]. The pH was lowered to 4.6, where undigested $\alpha\beta$ and $\alpha\beta$T2 precipitate. The supernatant contained $\alpha\beta$T1, which was precipitated by addition of 3 volumes of ethanol. The pellet was dissolved in and dialyzed against Buffer M. Both $\alpha\beta$T2 and $\alpha\beta$T1 were finally purified by size exclusion chromatography in Buffer M on a 90 cm long and 2.8 cm diameter column of Sephacryl S-300 (Pharmacia). Absorbance at 277 nm was used to monitor the elution, and fractions were analyzed by gel electrophoresis. The purified $\alpha\beta$T1 fragment and $\alpha\beta$ each give rise to two sets of bands on gels with different mobilities, but very similar staining intensities. $\alpha\beta$T2, however, migrates as one band in this gel system. $\alpha\beta$T1 has a larger hydrodynamic size than $\alpha\beta$T2, since it elutes earlier on the Sephacryl size exclusion

column. Fractions were pooled, dialyzed against water, and lyophilized. All lyophilized samples were stored at −18°C dissolved and dialyzed against Buffer M for 48 h before any measurements were made.

2.2 Concentration determination

Protein concentrations were determined from UV absorbance at 277 and 320 nm, with $C(\mathrm{mg/ml}) = (A_{277} - A_{320})/\varepsilon$. Extinction coefficients ε were determined, as described elsewhere [24], on a flow system with UV and refractive index detectors, based on the assumption of a differential refractive index increment of 0.183 ml/g. The extinction coefficients at 277 nm were found to be 0.29 for $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$; 0.13 for $\alpha\beta$T1; 0.48 for $\alpha\beta$T2, in units of l/(g cm).

2.3 Optical rotation (OR) and circular dichroism (CD)

A Cary 60 instrument with a 1-cm thermostated cell was used for OR measurements, as described elsewhere [22,25]. OR at 231.4 nm was measured as a function of temperature in steps of 1 to 3°C with 15 minute equilibration times at each temperature. An Aviv 60DS CD spectropolarimeter operated at 222 nm with a Hewlett-Packard 8910A temperature controller and 1-cm cells was used for the CD measurements [7].

2.4 UV difference spectroscopy

The thermally induced absorbance changes of TM solutions (approximately 2 mg/ml in 1-cm cuvettes) were measured at 285 nm in a Beckman ratio-recording spectrophotometer. The same solution was used in both the reference cell, which was maintained at a constant temperature of 22.0 ± 0.1°C, and in the sample cell. The temperature of the sample cell was controlled by a circulating thermostated bath, and the temperatures in both cells were measured in the cuvettes (±0.1°C) by use of thermistors. The temperature was changed between 5 and 80°C in steps, as described above for OR.

2.5 Analytical hydroxyapatite (HAP) chromatography

A short 10 ml HAP (DNA grade, Bio-Rad) column packed in 0.6 *M* KCl, 10 m*M* potassium phosphate (pM 7.0), and 0.5 m*M* DTT was used to separate the TM forms. A linear phosphate gradient from 10 to 300 m*M* was used at a flow rate of 30 ml/h. 4 ml fractions were collected and their UV absorbance monitored at 277 nm. Typically 6 ml of 1 mg/ml TM solutions, with different thermal histories, as described below, were loaded on the column.

2.6 Other techniques

Amino acid compositions were determined with a Durrum Model D-500 automated analyzer, after hydrolysis in evacuated tubes containing 6 *M* HCl for 24 h at 110°C. Cysteine was lost during the hydrolysis, and no corrections were made for partial destruction of other amino acids. No tryptophan was present as determined by absorption and fluorescence spectra. Partial amino acid sequences of $\alpha\beta$T1 and $\alpha\beta$T2 were determined on a Beckman model 890C automated analyzer, as described elsewhere [26]. Disulfide crosslinked TMs and TMs with blocked sulfhydryls (by reaction with iodoacetamide) were prepared as described elsewhere [12,27].

3. Results

3.1 Characterization of TMs

In contrast to rabbit skeletal muscle, *R. esculenta* skeletal muscle contains a large fraction of β-chains as judged by SDS-PAGE of purified TM (41% β in leg muscle and 48% β in back muscle). Although the α-chain has a mobility similar to rabbit skeletal α-chain, the mobility of the β-chain is much less than that of the rabbit β-chain. Hydroxyapatite chromatography indicated that the principal molecular species is $\alpha\beta$ (> 90%) and $\beta\beta$ form was allowed separation of the $\alpha\beta$ and $\alpha\alpha$ forms. The not detected in any hydroxyapatite run, but was prepared as described above.

The low temperatures and moderate digestion times used for tryptic digestion of $\alpha\beta$ resulted in the formation of well-defined $\alpha\beta$T1 and $\alpha\beta$T2 fragments, as judged by gel electrophoresis. Partial amino acid sequences of the fragments were determined in order to identify which fragments originate from the N- and C-terminal of $\alpha\beta$. The sequence of 15 residues from the N-terminal of $\alpha\beta$T1 was found to be in perfect agreement with the published sequence of *R. temporaria* α-chain [28] starting with residue #8. The sequence of 24 residues from $\alpha\beta$T2 was in good agreement (20 out of 24) with the *R. temporaria* α-sequence starting with His-153. Amino acid #152 is a Lys in the *R. temporaria* sequence, which is consistent with a trypsin cut between Lys and His. These results show that $\alpha\beta$T1 and $\alpha\beta$T2 contain most of the N- and C-terminals of $\alpha\beta$, respectively. The amino acid compositions determined for all five systems were characteristic of tropomyosin (data not shown). The tyrosine (Tyr) content was 6/chain for all TMs, 5/chain for $\alpha\beta$T2, and 1/chain for $\alpha\beta$T1, calculated by assuming 132 amino acids/chain in $\alpha\beta$T2 and 145 in $\alpha\beta$T1 (see above). The measured extinction coefficients reflect these differences in Tyr content. The number of cysteines (Cys) per TM was determined to be 2.0, 3.0. and 4.5 for $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$, respectively, by Nbs_2 reaction, as outlined in [27]. All three TMs and $\alpha\beta$T2, but not $\alpha\beta$T1 could be disulfide crosslinked by Nbs_2, as determined on gels run in the absence of reducing agents. This suggests that the positions of the Cys residue in α and the 2 Cys in β are very likely the same as for rabbit skeletal [2], i.e., the α-chain contains Cys at #190 and the β-chain contains 2 Cys at #36 and #190.

3.2 Thermal unfolding of helix

The thermal unfolding of the α-helix, as monitored by OR at 231.4 nm, is shown in Fig. 1 for all five systems. The data for $\alpha\alpha$, $\beta\beta$, and $\alpha\beta$ are in general agreement with the previously reported CD results on the intact TMs [7]. The straight line indicates the expected OR for a completely helical system. It is seen that both $\alpha\alpha$ and $\alpha\beta$ are highly helical at low temperatures,

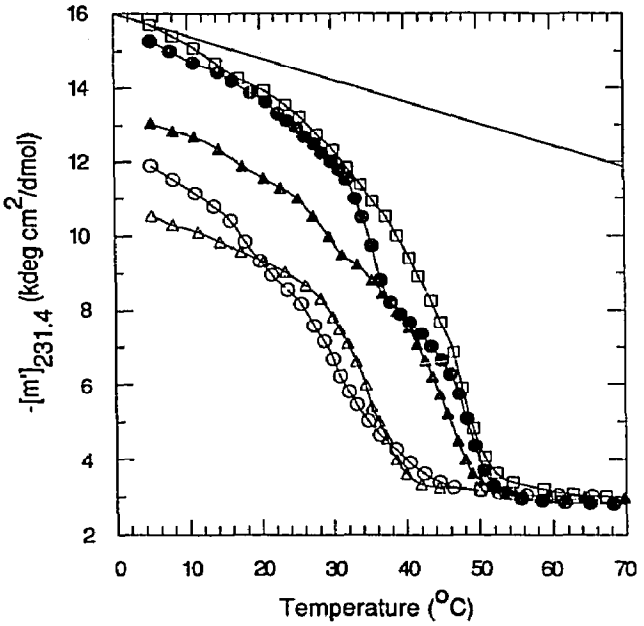

Fig. 1. Thermal unfolding of the α-helix of $\alpha\alpha$TM, $\beta\beta$TM, $\alpha\beta$TM and tryptic fragments $\alpha\beta$T1 and $\alpha\beta$T2 as monitored by optical rotation. Mean residue rotation at 231.4 nm is plotted against temperature. The protein concentrations were about 0.1 mg/ml in Buffer M in a 1-cm cell. The symbols used are: $\alpha\alpha$, (□); $\alpha\beta$, (●); $\beta\beta$, (○); $\alpha\beta$T1, (△); $\alpha\beta$T2, (▲). Straight line is the rotation expected for a completely helical system [25].

whereas $\beta\beta$, $\alpha\beta$T2, and $\alpha\beta$T1 appear to be only partly helical even at low temperatures. The helical contents at 5°C are estimated to be 75%, 84%, and 65% (±5%) from the OR data for the latter three, using the equations given in ref. [25]. It is seen that the helical contents of $\alpha\beta$T2 and especially $\alpha\beta$T1 are lower than that of the intact $\alpha\beta$ molecule. A number of other studies [11,13,15] have also shown that the stability of TM regions change when cut out of the native intact structure.

Figure 1 shows that the $\beta\beta$ helix is the least thermally stable and the $\alpha\alpha$ helix the most stable. The transition temperatures were determined as the temperature of the steepest slope and were found to be 32°C and 49°C for $\beta\beta$ and $\alpha\alpha$, respectively. Transition temperatures (±1°C) were determined by averaging at least two experiments. The $\alpha\alpha$ transition is seen to occur over a broad temperature range and significant unfold-

ing has occured at temperatures much below the transition temperature given here. The unfolding prior to the main transition may be analogous to the pretransition observed in cardiac $\alpha\alpha$TM [18]. The helical structure of $\alpha\beta$ is lost in two major transitions, with transition temperatures of 36°C and 48°C. The unfolding of $\alpha\beta$T1 and $\alpha\beta$T2 is seen to take place in the same general temperature range as the first and second transition of intact $\alpha\beta$, respectively, with transition temperatures of 34°C and 46°C. This suggests the possibility that the two transitions of intact $\alpha\beta$ are due to largely independent unfolding of the $\alpha\beta$T1 and $\alpha\beta$T2 halves. However, results presented below clearly show that the two transitions are not due to unfolding of two domains, but are a result of chain exchange. On cooling from high temperatures, the OR regained between 75 and 95% of the original value. When samples were heated a second time both $\alpha\alpha$ and $\beta\beta$ gave unfolding curves with shapes and transition temperatures similar to the initial heating cycle. When $\alpha\beta$ was re-heated the low-temperature transition was broader than observed during the first cycle, whereas the 48°C transition was unchanged, in agreement with the previous CD results [7].

In very low salt solutions the thermal unfolding of the α-helix of the three dimers was investigated with CD (Fig. 2). It is seen that the helical content of $\alpha\beta$ is lost in two transitions, as observed in high salt solutions with both OR (Fig. 1) and CD [7]. Both its transitions are shifted to lower temperatures compared to high salt solutions, and an over-shoot is apparent in the first transition, which is explained by slow kinetics of chain exchange (see discussion). The results for the homodimers also show lower transition temperatures without a change in the general features observed in high salt solutions, i.e., $\beta\beta$ least stable and $\alpha\alpha$ most stable. For the homodimers no over-shoot was observed. The decreased stability in lower salt solutions is in agreement with properties of $\alpha\alpha$TM from rabbit cardiac muscle [18].

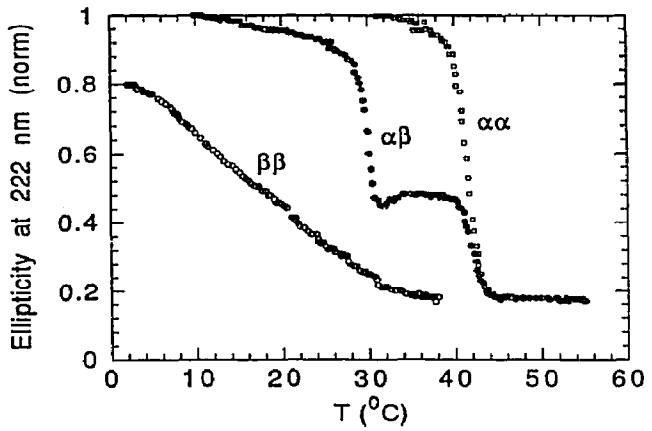

Fig. 2. Thermal unfolding of $\alpha\alpha$, $\beta\beta$ and $\alpha\beta$ in low salt, monitored by circular dichroism. Normalized ellipticity at 222 nm is plotted against temperature for 0.05 mg/ml solutions in 2 m*M* sodium phosphate (pH 7.0), 1 m*M* EDTA, 1 m*M* DTT.

3.3 Tyrosine environment changes with temperature

The UV spectrum of TM at 270–310 nm due to tyrosine absorbance, is sensitive to the environment as determined by the folding of the peptide chain [29]. The UV absorbance was therefore measured as a function of temperature in order to get information about changes of specific regions which contain tyrosine. Such localized information complements the OR and CD measurements, which probe overall structure. The temperature induced change in absorbance of tyrosine of all 3 dimers showed a maximum at 285 nm with a smaller peak at 275 nm. Absorbance changes of typically 0.07 were observed as a result of heating from 15 to 65°C for 2 mg/ml TM solutions. The initial UV absorbances were recovered on fast cooling to 15°C. Reheating experiments showed reversibility of $\alpha\alpha$, $\beta\beta$ and crosslinked $\alpha\beta$ unfolding profiles, but a broader second unfolding on heating for reduced $\alpha\beta$. The measured changes in absorbance were scaled by the values at 15°C and 60°C, i.e., $A_{285}(t) = (A(t) - A(60°C))/(A(15°C) - A(60°C))$. Plots of the temperature dependence of A_{285} are shown in the upper part of Fig. 3 for $\alpha\alpha$, $\beta\beta$ and $\alpha\beta$. It is seen that the order of stability of the three dimers agrees with the OR results. The transitions for $\alpha\alpha$, $\beta\beta$ and the second $\alpha\beta$ transition are shifted to somewhat higher temperatures compared with the OR data under the same solvent conditions.

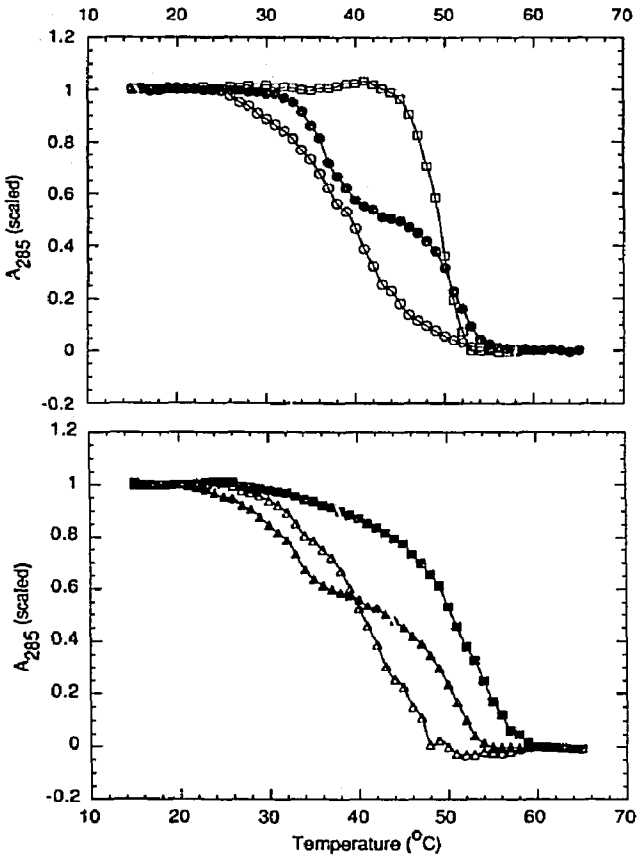

Fig. 3. Temperature dependence of tyrosine absorbance change at 285 nm for TM samples with iodoacetamide blocked sulfhydryls and disulfide crosslinked $\alpha\beta$TM. The plots show absorbances scaled to low and high temperature values against temperature. Top part: $\alpha\alpha$TM (□), $\beta\beta$TM (○), and $\alpha\beta$TM (●). Lower part: $\alpha\beta$T1 (△), $\alpha\beta$T2 (▲) and disulfide crosslinked $\alpha\beta$TM (■). Concentrations were approximately 2 mg/ml in Buffer M without DTT.

An increase in stability is expected for dissociating systems at the higher concentrations used in the absorbance studies [16]. The two $\alpha\beta$ transitions are seen to involve similar absorbance changes, which suggests that a similar number of tyrosines are affected. A covalent bond between the chains in $\alpha\beta$ prevents chain exchange and is therefore expected to drastically alter the unfolding pattern according to the chain exchange model. The unfolding of disulfide-crosslinked $\alpha\beta$ is shown in the lower part of Fig. 3, and it shows that crosslinking eliminates the first transition seen for reduced $\alpha\beta$. The unfolding of $\alpha\beta$T2 shows both a low temperature transition near 32°C and a high temperature transition near 49°C (Fig. 3) in contrast to the broader transition seen by CR in Fig. 1.

3.4 Identification of new species after thermal treatment of $\alpha\beta$

Two other techniques were used to more directly investigate whether chain exchange occurs during the 38°C transition of $\alpha\beta$TM. Evidence for chain exchange was obtained by disulfide crosslinking $\alpha\beta$ samples, which have been heated to allow the putative chain exchange to take place.

Advantage is taken of the different mobilities of disulfide crosslinked $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ TM on SDS-PAGE gels [12,27]. Only one crosslinked species is expected for both $\alpha\alpha$ and $\alpha\beta$, due to a single crosslink at Cys-190. Three $\beta\beta$ crosslinked species are possible, because the β-chain has two Cys. The crosslinking results in Fig. 4, show the expected pattern for the unheated TM samples.

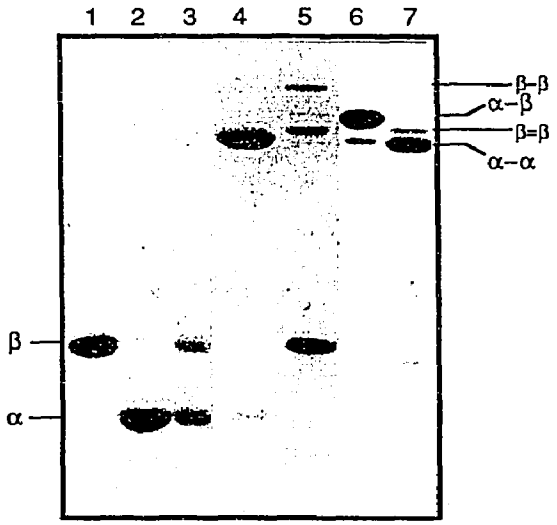

Fig. 4. Evidence for homodimer formation after heating of $\alpha\beta$TM. The figure shows a 9% polyacrylamide gel with samples before and after disulfide crosslinking at 25°C. Lanes 1–3 contain $\beta\beta$, $\alpha\alpha$ and $\alpha\beta$, respectively, before crosslinking. Lanes 4–6 contain crosslinked TMs, $\alpha-\alpha$, $\beta=\beta$ and $\beta-\beta$ and $\alpha-\beta$. Lane 7 contains $\alpha\beta$ heated to 45°C for 10 min cooled on ice and then crosslinked. Positions of a single α and β band are indicated with α and β. Single crosslinked $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ positions are shown together with double crosslinked $\beta\beta$, which is marked with $\beta=\beta$.

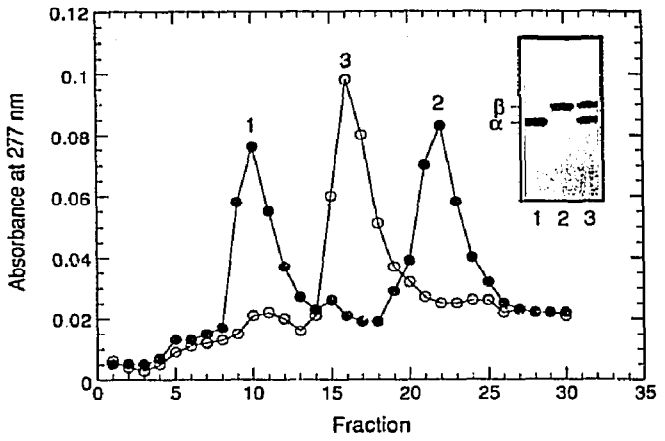

Fig. 5. Hydroxyapatite chromatograms of $\alpha\beta$TM at 5°C for an unheated sample (open circles) and the same sample heated to 38°C for 30 min and then cooled on ice (filled circles). UV absorbances at 277 nm of column fractions are plotted against fraction number. Six mg $\alpha\beta$TM samples were loaded on the column and eluted with a linear phosphate gradient, as described in the experimental section. The insert shows a polyacrylamide gel with elution peak fractions marked 1–3.

It is seen that $\beta\beta$ is not completely crosslinked, presumably due to the relatively high degree of unfolding at 25°C [7]. After crosslinking $\alpha\beta$ with Nbs_2 at 25°C, it is seen that a major band of crosslinked species (denoted α–β) was observed with different mobility than α–α, β–β or doubly crosslinked β=β. The $\alpha\beta$ sample is also seen to contain small amounts of contaminating $\alpha\alpha$. After incubating $\alpha\beta$ at 45°C the resulting crosslinked species are different from unheated $\alpha\beta$. The pattern of the crosslinked incubated $\alpha\beta$ (lane 7) is seen to consist of a band corresponding to crosslinked $\alpha\alpha$ together with bands corresponding to crosslinked $\beta\beta$ forms, and some uncrosslinked β-chains, clearly indicating the conversion of $\alpha\beta$ to the homodimer forms.

Unheated $\alpha\beta$ and purified solutions of $\alpha\alpha$ and $\beta\beta$ elute as single peaks at different phosphate concentrations on a HAP column at 5°C. The elution profile of an unheated $\alpha\beta$ sample is shown in Fig. 5. A sample from the same $\alpha\beta$ stock solution was heated to 38°C for 30 minutes, then cooled on ice and loaded on the column. The heated sample eluted as two peaks at the elution volumes of purified $\alpha\alpha$ and $\beta\beta$. Gels of samples from the two elution peak fractions (see insert in Fig. 5) confirmed that the first peak contained only α-chains and the second only β-chains. These results confirm chain exchange.

4. Discussion

In a recent paper [7] we suggested that the 36°C and 50°C transitions observed in the thermal unfolding of $\alpha\beta$ can be explained by a chain exchange reaction at 36°C in which $2\alpha\beta \rightarrow \alpha\alpha +$ largely unfolded $\beta\beta$, leading to loss of about half of the helical content followed by an unfolding of the formed $\alpha\alpha$ in the 50°C transition, resulting in the loss of the other half of the helical content. This suggestion was based primarily on the CD observation that the unfolding curves of $\alpha\beta$ were very similar to the sum of unfolding curves of $\alpha\alpha$ and $\beta\beta$ when the $\alpha\beta$ had already been heated once. The other possible explanation is that the two transitions of $\alpha\beta$ are produced by the independent unfolding of two nearly equal size domains of $\alpha\beta$. Apparent support for the domain unfolding mechanism is that the main unfolding transitions of $\alpha\beta$T1 and $\alpha\beta$T2, the N- and C-terminal halves of $\alpha\beta$, respectively, roughly correspond to the first and second $\alpha\beta$ unfolding transitions, respectively.

Two thermal transitions of $\alpha\beta$ with similar losses in helical content (with OR and CD) and unfolding enthalpies (with preliminary DSC measurements) can be explained both by the exchange and domain models. However, the observed nearly equal changes in absorbance for $\alpha\beta$ TM during its two transitions (Fig. 3) can be explained by chain exchange, but not by two independent domains in view of the low tyrosine content in the T1 half of $\alpha\beta$ (1/subunit). In addition, the interchain disulfide crosslinked $\alpha\beta$ sample does not show any sign of a major low temperature transition, either with CD (data not shown) or with absorbance (Fig. 3) and only a slightly broader transition in the 45°C to 50°C region, consistent with lack of exchange and the known effect of a disulfide crosslink on the unfolding of TM [12]. The crosslink is only formed in the T2 part of $\alpha\beta$ because only the β-chain has a Cys at #36 and there is no Cys at the

complementary position 36 of the α-chain. If the T1 part was an independent domain, its unfolding properties should be independent of crosslinking in the T2 domain and a low temperature transition corresponding to the unfolding transition of $\alpha\beta$T1 should have been observed also in the crosslinked $\alpha\beta$. It is therefore clear that T1 is not an independent domain with low thermal stability in the $\alpha\beta$ structure. The lack of complete amino acid sequences of the α- and β-chains makes it difficult to explain the observed differences in thermal stability of homo- and heterodimers in terms of molecular structures.

Direct evidence for homodimer formation by incubation of $\alpha\beta$ at temperatures above 36°C, is shown by disulfide crosslinking and HAP chromatography (Figs. 4 and 5). In both cases, one species corresponding to $\alpha\beta$ before heating converts to two species corresponding to $\alpha\alpha$ and $\beta\beta$ after heating. The fact that we are able to maintain homodimers on cooling to room temperature and below indicates that the reverse of the $2\alpha\beta = \alpha\alpha + \beta\beta$ chain exchange equilibrium is very slow at low temperature as observed in our initial CD study [7]. A similar conclusion about very slow kinetics has recently been reached also for rabbit TM [30]. The thermal unfolding results in low salt solutions, presented above, provide further information about chain exchange and kinetics in the low temperature transition of $\alpha\beta$. The distorted first transition (Fig. 2) with the over-shoot can be explained by slow reassociation rate of the $\alpha\alpha$ homodimer from separated α and β chains, causing an apparent small reversal of the unfolding in the transition. A similar over-shoot has recently been observed for gizzard TM in high salt solutions [31].

At first glance it is difficult to understand how the main unfolding of the $\alpha\beta$T2 peptide could occur at 46°C, i.e., at a higher temperature than the first transition of the parent $\alpha\beta$ molecule. On closer examination of the unfolding profile of $\alpha\beta$T2 in Fig. 1, it is seen that there is another transition of lower cooperativity at about 30°C corresponding to about half of the loss of helix. It thus appears that $\alpha\beta$T2 unfolds/dissociates in the 30°C transition to form $\alpha\alpha$T2 and largely unfolded $\beta\beta$T2, and the formed $\alpha\alpha$T2 then unfolds in a more cooperative 46°C transition. In this view chain exchange also occurs for $\alpha\beta$T2, and the main transition at 46°C is due to the unfolding of $\alpha\alpha$T2 not $\alpha\beta$T2, explaining the higher transition temperature. The $\alpha\beta$T2 absorbance data in Fig. 3 supports this interpretation, since this technique detects the two transitions. The reason for the sharp transition in absorbance and more gradual transition in OR, can be explained by a gradual non-cooperative unfolding in $\alpha\beta$T2 regions which lack tyrosines. The presence of only one unfolding transition for $\alpha\beta$T1 in both OR and UV means that chain exchange does not occur for this system, presumably because the homodimer species is of similar or less stability compared to the parent heterodimer peptide. Such a situation appears to apply to $\alpha\beta$TM from gizzard muscle where only one transition was observed [9]. Recent calculations based on thermodynamic values obtained from the unfolding profiles of the three dimers from gizzard TM showed that $\alpha\beta$ preferentially assembles and unfolds without appreciable formation of homodimers, because $\Delta G^{\circ}_{\alpha\beta} \gg \frac{1}{2}(\Delta G^{\circ}_{\alpha\alpha} + \Delta G^{\circ}_{\beta\beta})$, where the ΔG°'s are the free energies of dissociation of the respective dimers [32]. Similar preliminary calculations on frog TM indicate that thermodynamic considerations can explain the preferential formation of $\alpha\beta$ and its remarkable unfolding/chain exchange properties.

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