23. We thank R. Kingsbury for technical assistance, A. Hinnebusch for providing a recombinant DNA clone of the yeast gene encoding GCN4, C. Vinson for construction of plasmid vectors containing C/EBP and GCN4 binding sites, K. LaMarco for editorial comments, W. Landschulz for helpful discussion, and C. Norman for clerical assistance. Supported by grants from NIH, American Heart Association, Johns Hopkins University Hematology Division (P.A.), Howard Hughes Medical Research Institute (S.L.M.), and Carnegie Institution of Washington (S.L.M.).

31 July 1989; accepted 10 October 1989

Assembly of the Native Heterodimer of *Rana esculenta* Tropomyosin by Chain Exchange

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***Rana esculenta* tropomyosin assembles in vivo into a coiled-coil α helix from two different subunits, α and β, which are present in about equal concentrations. Although the native composition is αβ, a mixture of equal amounts of αα and ββ is produced by refolding dissociated α and β at low temperature in vitro. Refolding kinetics showed that αα formed first and was relatively stable with regard to chain exchange below approximately 20°C. Equilibration of the homodimer mixture at 30° and 34°C for long times, however, resulted in the formation of the native αβ molecule by chain exchange. Biosynthesis of αβ from separate α and β genes is, therefore, favored thermodynamically over the formation of homodimers, and biological factors need not be invoked to explain the preferred native αβ composition.**

TROPOMYOSIN PURIFIED FROM SEVeral sources (skeletal, cardiac, and smooth muscle) is composed of two kinds of subunits, α and β, which differ slightly in amino acid sequence (*1*). In some muscles, α and β are present in about equal amounts (*2*). Assembly of the two subunits in such muscles after chain biosynthesis, or in vitro after chain dissociation, can in principle produce a mixture of equal numbers of homodimers (αα and ββ), all heterodimer (αβ), or a mixture of the three species. Attempts to determine the native composition and to find out if it is controlled by thermodynamic and kinetic parameters or by biological factors have been made for tropomyosin from rabbit skeletal muscle (*3–7*) and fowl gizzard smooth muscle (*8–12*).

Tropomyosin is a coiled-coil α helix, which dissociates into subunits (*13, 14*) in parallel with a major cooperative helix-coil transition (*13, 15, 16*). Thus, the dissociation and association processes can be studied by directly monitoring changes in helical content with circular dichroism (CD) methods. We have purified tropomyosin from *Rana esculenta* muscle, in which α and β are present in about equal amounts, and found that the native composition is predominantly αβ. The native αβ molecule and the two reconstituted homodimers, αα and ββ, had different thermal stabilities characterized by different helix thermal unfolding profiles. This allowed us to determine the relative amounts of the three species refolded in vitro from separated α and β chains.

The thermal unfolding profile of the α helix of αα (*17*) showed one main transition at 50°C and that of ββ showed a broad transition centered at 30°C, both of which were reversible on slow cooling (Fig. 1A). In contrast, the unfolding profile of αβ showed two cooperative transitions with midpoints at 36° and 50°C (Fig. 1A, curve 1) and was not reversible on refolding by cooling, as made evident by the missing 36°C transition (Fig. 1A, curve 2). A second unfolding run of the unfolded and refolded αβ sample gave data that coincided with the refolding profile (Fig. 1A, curve 3); this profile consisted of a broad transition between 20° and 40°C and a sharp transition at 50°C, suggesting that the curve originated from contributions from ββ and αα, respectively. The agreement between a curve calculated by taking equal contributions of the unfolded and refolded αβ (Fig. 1A, curve 3) showed that the heating and cooling processes resulted in a conversion of the αβ heterodimer to equal amounts of homodimers.

Samples of the three different dimers were dissociated separately in 5*M* guanidinium hydrochloride, and dimers were allowed to re-form by dialyzing the samples at 5°C. The thermal unfolding profiles of the homodimers refolded from guanidinium hydrochloride (Fig. 1B) were essentially the same as those obtained for untreated homodimers (Fig. 1A). The unfolding profile of the

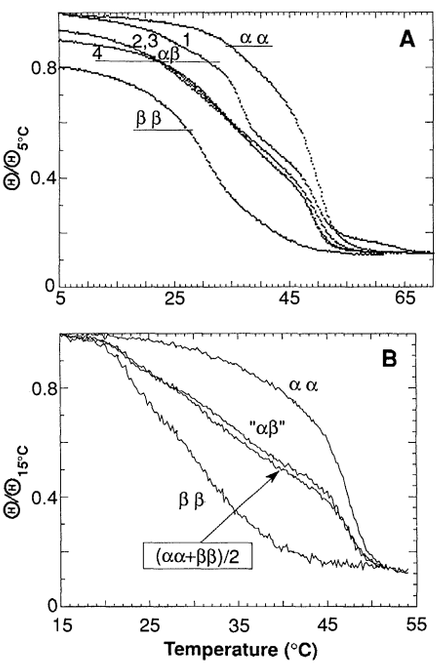

Fig. 1. Helix thermal unfolding and refolding profiles of the three species of *R. esculenta* tropomyosin. The αβ tropomyosin refolds as a 1:1 mixture of αα and ββ. (**A**) Temperature dependence of ellipticity ([θ]) at 222 nm normalized to the value for αα at 5°C. The αα and ββ profiles were reversible whereas that of αβ was not for these cooling rates. The αβ profiles are as follows: 1, on heating; 2, on cooling; 3, on reheating; and 4, calculated profile where $[\theta] = 1/2\,([\theta]_{\beta\beta} + [\theta]_{\alpha\alpha})$ at each temperature. Initial value of helicity for ββ was lower than that of αα and αβ. (**B**) Temperature dependence of ellipticity at 222 nm normalized to the αα value at 15°C for αα, ββ, and "αβ" refolded from 5*M* guanidinium hydrochloride by dialysis at 5°C. The (αα + ββ)/2 curve was calculated by taking equal contributions from the αα and ββ profiles. Ellipticity was measured on tropomyosin solutions (0.5 mg/ml) in a 1-mm cell (A), or on solutions (0.05 mg/ml) in a 1-cm cell (B), in buffer [0.5*M* NaCl, 20 m*M* sodium phosphate (*p*H 7.0), 1 m*M* EDTA, and 1 m*M* dithiothreitol] with an Aviv 60DS CD spectropolarimeter and a Hewlett-Packard 8910A temperature controller. Data were collected in steps of 0.2°C after 0.3-min dwell times with 10-s averaging, which gave a 0.4° per minute heating or cooling rate. The αβ and αα tropomyosin were purified from frog back and leg muscle by isoelectric precipitation, ammonium sulfate fractionation, and hydroxyapatite chromatography (*4*) at 0° to 5°C and were found to contain 86 and 96% αβ from leg and back muscle, respectively, and a corresponding small amount of αα (14 and 4%). The ββ dimer and also αα when needed in greater quantities were prepared by ion-exchange chromatography on urea-separated chains of αβ (*24*), and the chains were refolded by dialysis.

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tropomyosin refolded from the heterodimer was approximated by taking equal contributions of the unfolding profiles of αα and ββ. Thus, under both refolding conditions tested, homodimers, rather than the native αβ species, were preferentially formed from dissociated α and β chains.

The two thermal unfolding transitions of native αβ indicate that chain exchange probably occurs during the first transition at 36°C; the coincidence of the second unfolding transition of native αβ at 50°C with the main unfolding transition of αα suggests that αα was present before the 50°C transition. The 36°C transition is therefore apparently caused by unfolding and dissociation of αβ and reassociation of α chains, as αα remains associated up to about 45°C and β chains are largely dissociated above 35°C. Thus, in the 36°C transition, 2αβ is converted to αα and 2β, and in the 50°C transition, αα dissociates to 2α; half of the helix content is therefore lost in each transition. We obtained further evidence that dissociation and chain exchange produce the two transitions by measuring the unfolding and refolding profiles of an αβ dimer that was disulfide–cross-linked between the Cys^{190} groups of each chain to prevent dissociation (*5*). The unfolding profile showed a reversible main transition at 40°C (*18*). The shift of the main transition of αβ from 36° to 40°C after cross-linking is consistent with the stabilizing effect of an interchain disulfide (*16*). An alternative possibility, that the two transitions for αβ are the result of the unfolding of two equal domains, can be excluded by these results and by other disulfide cross-linking and chromatography data showing the direct conversion of heterodimers to homodimers in the 36°C transition (*18*).

We subjected the same sample of αβ to several heating and cooling cycles in the CD instrument. First, the ellipticity of the native αβ was monitored through the 36°C transition to 40°C (Fig. 2A, curve 1). When the sample reached 40°C, it was cooled to 20°C within a few minutes to quench the postulated chain exchange. The ellipticity was then monitored from 20° to 60°C (Fig. 2A, curve 2), and it was found that almost all of the 36°C transition was lost. The 50°C transition was still apparent, further indicating that αβ was converted to αα and β in the 36°C transition. The kinetic processes occurring during refolding were monitored by quickly cooling the unfolded and dissociated αβ from 60° to 34°C (just below the 36°C transition). The temperature reached 34°C within 120 s, with a half-time of about 60 s; during this time the ellipticity changed rapidly and apparently reached a constant value (Fig. 2B). A plot of ellipticity versus temperature obtained from these refolding kinetics showed that above 38°C the refolding curve coincides with the previous unfolding curve (Fig. 2A, curve 3), indicating that αα re-forms as quickly as the temperature could be changed, which was about 0.3°C per second. The lack of a 36°C transition showed that αβ did not form during this time.

Continued incubation of the same sample for 5000 s after the temperature reached 34°C indicated that equilibrium was not reached because the negative ellipticity value further increased exponentially, increasing 10% from its 120-s value (Fig. 2B, inset). After 5000 s the temperature was dropped to 10°C to quench further chain exchange, and another unfolding profile was monitored (Fig. 2A, curve 4). More than 50% of the 36°C transition was recovered, indicating that the native αβ molecule can be formed by chain exchange from the αα and ββ plus β species that are present at 34°C. When an αβ at ten times the earlier concentration was dissociated at high temperature and then incubated at 30°C, a yield of >75% αβ was obtained for a similar time of incubation (*18*). These studies indicate that αα, the most stable species, preferentially and quickly assembles during the temperature drop. At temperatures below 36°C, ββ subsequently forms because the rate of chain exchange to form αβ is much slower. However, the native heterodimer can form if sufficient time is allowed for equilibrium to be reached. The rate of formation of heterodimer from the homodimer species is dependent on temperature, which explains why the homodimer mixture is stable at room temperature and below, even though it is apparently not in equilibrium. The slow rate of chain exchange also explains why equilibrium was not reached during refolding at cooling rates of about 0.4°C per minute. The half-time of chain exchange is about 50 min at 34°C.

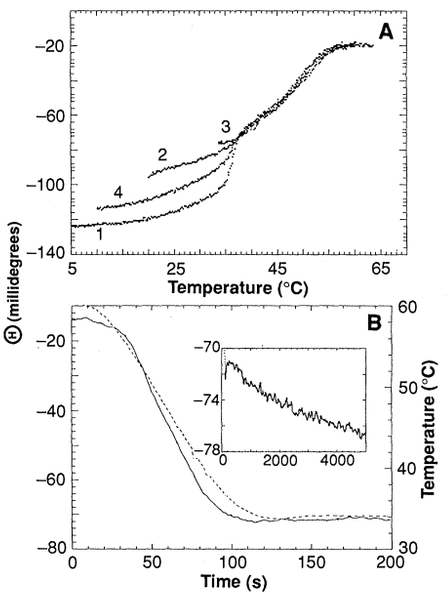

Fig. 2. Thermal unfolding and refolding of native αβ tropomyosin as measured by ellipticity at 222 nm. (**A**) The same sample of αβ was subjected to several heating and cooling cycles as follows: Curve 1, heating from 5° to 40°C; a prominent 36°C transition occurs. Curve 2, heating to 60°C after cooling from 40° to 20°C; the 36°C transition is lost and a 50°C transition is present. Curve 3, data obtained during quick cooling from 60° to 34°C [see (B)]; a 50°C transition is present but a 36°C transition is absent. Curve 4, reheating after further incubation at 34°C for 5000 s and cooling to 10°C; the 36°C transition reappears. (**B**) Kinetics of fast ellipticity (solid line) change and temperature (dotted line) change during cooling of sample from 60° to 34°C. (**Inset**) Kinetics of slow ellipticity change during further incubation at 34°C. All data were obtained on the same sample at a concentration of 0.05 mg/ml in buffer in a 1-cm stirred cell in the CD spectropolarimeter.

The preferential formation of homodimers after dialysis from guanidinium hydrochloride solutions at low temperature also appears to be explicable in terms of the initial formation of αα with subsequent formation of ββ. Even with slow dialysis, where equilibrium would appear to be attained, the homodimers will be preferentially formed because the rate of chain exchange resulting in αβ formation is so slow below room temperature.

The observation that the in vivo composition of *R. esculenta* tropomyosin can be obtained in vitro by means of chain exchange indicates that biological factors are not necessary for determining the native composition. The tendency for heterodimer preference over homodimers can be determined from an equilibrium thermodynamic analysis of the reaction $2\alpha\beta \rightleftharpoons \alpha\alpha + \beta\beta$. The equilibrium constant (K_e) for this exchange reaction is equal to $(K_{\alpha\beta})^2/(K_{\alpha\alpha}K_{\beta\beta})$, where the K's are individual dissociation constants. At a given temperature, αβ will be preferred over a mixture of αα and ββ if $K_{\alpha\alpha}K_{\beta\beta} > (K_{\alpha\beta})^2$. The equilibrium dissociation constants are determined by the change in standard free energies (ΔG°) on formation of dimers from separated chains. An alternative formulation is that heterodimers will be preferentially formed if $\Delta G^\circ_{\alpha\beta} < 1/2\ (\Delta G^\circ_{\alpha\alpha} + \Delta G^\circ_{\beta\beta}) + RT \ln 2$. This will be true even if one of the homodimers is more stable than the preferred heterodimer, as is the case in this system. It thus appears that αβ forms at temperatures where ββ is relatively unstable in order to minimize the total free energy (*19*). At any temperature and total αβ concentration, the concentrations of all of the associated and dissociated species can be obtained from the temperature dependence of the dissociation

constants of αα, αβ, and ββ.

Tropomyosin from *R. esculenta* leg and back muscle, extracted and purified at low temperature where chain exchange is minimized or eliminated, consists of >90% αβ. The presence of ≈10% αα appears to be the consequence of a slightly greater relative amount of α over β in leg muscle. Thus, αβ is the preferred native composition, and excess αα appears to form after αβ. This explanation was used to explain the prevalence of αβ in several rabbit skeletal muscles in which the amounts of α and β were approximately equal (*3*).

Studies with *R. temporaria* αβ tropomyosin showed two thermal unfolding transitions (*20*). The first transition of this frog species also appears to be due to chain exchange (*18*). The heterodimer of smooth tropomyosin from fowl gizzard is the native species (*9*), but a 1:1 mixture of homodimers is produced after refolding guanidinium hydrochloride–unfolded chains (*11*). Incubation of the mixture of homodimers at physiological temperature, however, produces the αβ native composition (*12*). For rabbit skeletal tropomyosin, refolding from a 1:1 mixture of α and β chains on cooling from a high temperature gave a random mixture of dimer molecules (*6*). A tendency toward αβ formation was observed when refolding took place by dilution from denaturant into buffer at physiological temperature (7). It is possible that a greater fraction of native αβ would be produced if incubation times at the physiological temperature were long enough to allow chain exchange to reach equilibrium.

Although the assembly preference of the tropomyosin heterodimer can be understood thermodynamically, the significance of its predominance in several muscles (*3, 9*) and of the changing α/β tropomyosin ratio (which will change the dimer composition) in skeletal muscle development (*21, 22*) and in differentiating muscle cells in vitro (*23*) is not known. Frog muscle functions over a wide range of body temperatures, and it is possible that the composition and properties of the tropomyosin dimer may depend on the temperature at the time of biosynthesis, thus compensating for temperature effects on regulation. However, in preliminary studies, no significant differences were observed between the effects of *R. esculenta* αα and αβ tropomyosin on the Mg^{2+}-dependent adenosine triphosphatase activity of actomyosin subfragment 1 either at 15° or 25°C (*18*).

REFERENCES AND NOTES

1. S. T. M. Lau, C. Sanders, L. B. Smillie, *J. Biol. Chem.* **260**, 7257 (1985); C. Sanders and L. B. Smillie, *ibid.*, p. 7264.
2. P. Cummins and S. V. Perry, *Biochem. J.* **141**, 43 (1974).
3. D. D. Bronson and F. H. Schachat, *J. Biol. Chem.* **257**, 3937 (1982).
4. E. Eisenberg and W. W. Kielley, *ibid.* **249**, 4742 (1974).
5. S. S. Lehrer, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3377 (1975).
6. M. Holtzer, T. Breiner, A. Holtzer, *Biopolymers* **23**, 1811 (1984).
7. H. R. Brown and F. H. Schachat, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2359 (1985).
8. G. M. Strasburg and M. L. Greaser, *FEBS Lett.* **72**, 11 (1976).
9. C. Sanders, L. D. Burtnick, L. B. Smillie, *J. Biol. Chem.* **261**, 12774 (1986).
10. L. D. Burtnick, C. Sanders, L. B. Smillie, *Arch. Biochem. Biophys.* **266**, 622 (1988).
11. P. Graceffa, *Biochemistry* **28**, 1282 (1989).
12. S. S. Lehrer and Y. Qian, *J. Cell Biol.* **107**, 200a (1988).
13. M. J. Pont and E. F. Woods, *Int. J. Pept. Protein Res.* **3**, 177 (1971).
14. S. Yukioka, I. Noda, M. Nagasawa, M. Holtzer, A. Holtzer, *Macromolecules* **18**, 1083 (1985).
15. E. F. Woods, *Aust. J. Biol. Sci.* **29**, 405 (1976).
16. S. S. Lehrer, *J. Mol. Biol.* **118**, 209 (1978).
17. The CD spectra of the three species of *R. esculenta* tropomyosin are typical of an α helix with negative ellipticity peaks at 222 and 208 nm and a positive peak at 190 nm. The value of -3.3×10^4 cm^2 degrees per decimole for the mean residue ellipticity at 222 nm of αα and αβ at 15°C indicates >95% α helix. Values for ββ were up to 20% lower, probably because of kinetic factors affecting complete refolding during its preparation from separated chains of αβ.
18. S. Hvidt, Y. Qian, S. S. Lehrer, in preparation.
19. The preferential formation of heterodimers of leucine zipper coiled-coils from Fos and Jun was independently explained by a similar mechanism [E. K. O'Shea, R. Rutkowski, W. F. Stafford III, P. S. Kim, *Science* **245**, 646 (1989)].
20. S. Hvidt, *Biophys. Chem.* **24**, 211 (1986).
21. R. K. Roy, F. A. Sreter, S. Sarkar, *Dev. Biol.* **69**, 15 (1979).
22. D. H. Heeley, G. K. Dhoot, S. V. Perry, *Biochem. J.* **226**, 461 (1985).
23. D. Montarras, M. Y. Fiszman, F. Gros, *J. Biol. Chem.* **257**, 545 (1982).
24. P. Cummins and S. V. Perry, *Biochem. J.* **133**, 765 (1973).

25. Supported by NIH grant HL22461 and the Danish Technical Research Council.

23 June 1989; accepted 18 September 1989

Effect of Carboxylic Acid Side Chains on the Absorption Maximum of Visual Pigments

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The proposal that the absorption maximum of the visual pigments is governed by interaction of the 11-*cis*-retinal chromophore with charged carboxylic acid side chains in the membrane-embedded regions of the proteins has been tested by mutating five Asp and Glu residues thought to be buried in rhodopsin. Changing Glu^{113} to Gln causes a dramatic shift in the absorption maximum from 500 nanometers to 380 nanometers, a decrease in the pK_a (acidity constant) of the protonated Schiff base of the chromophore to about 6, and a greatly increased reactivity with hydroxylamine. Thus Glu^{113} appears to be the counterion to the protonated Schiff base. Wavelength modulation in visual pigments apparently is not governed by electrostatic interaction with carboxylate residues, other than the counterion.

RHODOPSIN CONTAINS AN 11-*CIS*-retinal chromophore covalently attached to the apoprotein opsin through a protonated Schiff-base linkage to the ε-amino group of Lys^{296} (*1, 2*). The absorption maximum for rhodopsin is at 500 nm, whereas that for a protonated Schiff base of 11-*cis*-retinal free in methanol solution is at 440 nm. This 60-nm (2700 cm^{-1}) bathochromic shift or "opsin shift" (*3*) is caused by interaction of the protonated chromophore with the protein. The difference in absorption maximum then between the unprotonated chromophore free in solution and the protonated form bound to the protein (8100 cm^{-1}) is thought to result from protonation of the Schiff base nitrogen (5400 cm^{-1}) and from the opsin shift.

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Several theories have been proposed for the mechanism of the opsin shift in rhodopsin (*4*). The same mechanism probably also accounts for wavelength modulation in the color pigments. Kropf and Hubbard (*5*) originally proposed that the photoexcited state of rhodopsin could be stabilized by an electrostatic interaction of a negative charge from an amino acid side chain with the delocalized positive charge from the protonated Schiff base in the polyene chain of the chromophore. Honig *et al.* (*6*) in their "external point-charge model" (Fig. 1A) proposed on the basis of semiempirical calculations and experiments with dihydro-retinal analogs that 11-*cis*-retinal interacts with two carboxylate residues: (i) a counterion for the protonated Schiff base nitrogen atom and (ii) a point charge located near carbon atoms 12 and 14 of the chromophore.

We have undertaken identification of the amino acid residues that function as coun-