Intrinsic Fluorescence of Actin†

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ABSTRACT: A study has been made of the intrinsic fluorescence associated with G- and F-actin, with EDTA- and heat-denatured actin, and with actin in 8 M urea. A small decrease in the Trp and an increase in the Tyr contribution is associated with the polymerization of G- to F-actin. An appreciable red shift of the fluorescence spectrum occurs when G- or F-actin is denatured, indicating increased exposure of Trp to the aqueous environment. This change in fluorescence produced by the addition of EDTA can be used as a quick measure of the fraction of denatured species associated with a given actin preparation. Attempts to regenerate native actin from denatured actin have been unsuccessful. The fluorescence quenching caused by the binding of Cu^{2+} to G- and F-actin appears to involve Förster energy transfer from Trp to the absorption band associated with the Cu^{2+}–actin interaction. The Trp fluorescence of CuG- and CuF-actin is the same, suggesting similarity of conformation. Studies of the temperature dependence of fluorescence revealed a denaturation transition which occured at a higher temperature for F- as compared to G-actin. Decreased heat stability was also noted for CuG and CuF-actin as compared to G- and F-actin. The denaturation kinetics studied at constant temperature in the transition region showed that ATP protects F- but not G-actin from denaturation.

Actin, one of the major protein components of the myofibril involved in the process of muscle contraction, is capable of existing in different states.[1] G-actin, the monomeric native form of actin, has a tightly bound nucleotide and a tightly bound Ca^{2+} or Mg^{2+} normally found associated with it. If the nucleotide or metal ion is removed, the protein irreversibly denatures (Asakura, 1961; Maruyama and Gergely, 1961). This denatured form of actin (d-actin) appears, on the basis of optical rotatory dispersion and circular dichroism studies (Nagy and Jencks, 1962; Nagy, 1969), to be only partially unfolded compared with actin in 8 M urea (u-actin). F-actin, the fibrous form which exists in the muscle, can be formed from G-actin but not form d-actin by a reversible salt-induced polymerization. Although this reversible polymerization does not appear to have a major role in muscle contraction, localized dislocations of the actin filament may have some importance (Oosawa *et al.*, 1961; Asakura *et al.*, 1963; Szent-Györgyi and Prior, 1966).

The purpose of the present study is to characterize the intrinsic fluorescence properties of the different states of actin in order to further obtain information regarding conformational differences. We found that small differences exist between G- and F-actin with respect to the contributions of both Tyr and Trp to the intrinsic fluorescence when proper corrections for light scattering were made. Studies of the temperature dependence of the fluorescence indicate qualitative and quantitative differences between G- and F-actin in their stability toward heat denaturation. The large fluorescence spectral shift that occurs when G- or F-actin is denatured indicates an increase in average exposure of Trp side chains to the aqueous solvent. The quenching of the fluorescence of G- and F-actin due to the binding of Cu^{2+} appears to involve energy transfer to a new absorption band associated with the metal–protein interaction (Lehrer *et al.*, 1972) as had been observed for the case of transferrin (Lehrer, 1969), and also provides information regarding conformational differences between G- and F-actin. A preliminary report of this work has been presented (Lehrer and Kerwar, 1969).

Experimental Section

Materials. Highly purified samples of L-Trp (Cyclo Chemical Corp.) were used as obtained. Glycogen (oyster) and dithiothreitol were obtained from Calbiochemical, ATP from Sigma, and Ultra Pure urea from Schwarz-Mann. Distilled deionized water was used in the preparation of all the solutions.

Preparation of G-Actin. Actin was extracted from an acetone powder of rabbit skeletal muscle by the low-temperature procedure of Drabikowski and Gergely (1962) which minimizes tropomyosin contamination. The extracting solution contained 0.1 mM $CaCl_2$, 1 mM dithiothreitol, and 2–5 $\times$ 10^{-4} M ATP in 2 mM Hepes[2] buffer, pH 7.5. The presence of Ca^{2+} and ATP protects the actin against denaturation (Rees and Young, 1967), and the dithiothreitol complexes any Cu^{2+} that may be present or introduced (Lehrer *et al.*, 1972). The extract is filtered through cheesecloth and then through Whatman No. 1 filter paper on a Büchner funnel. Two cycles of salt-induced polymerization of G- to F-actin were employed for purification using 0.1 M KCl for the first cycle and 0.02 M KCl for the second cycle. The low-salt step further reduces contamination by tropomyosin (Martonosi, 1962). Depolymerization of the pellets obtained by centrifugation at 100,000*g* was performed by homogenization at 0–2° with the extracting solution. Pellets were stored for up to 2 weeks at 0–2° with 1 drop of 0.1 M KCl and 1 drop of toluene (to inhibit bacterial growth). G-Actin was prepared by dialyzing the homogenized pellets against a solution containing 2 mM Hepes, pH 7.5, for 15–36 hr. The completeness of depolymerization was indicated by the absence of birefringence when a test tube, while being

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[1] For a recent review, see Young (1969).

[2] Abbreviations used are: Hepes = *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; EDTA = sodium ethylenediaminetetraacetate.

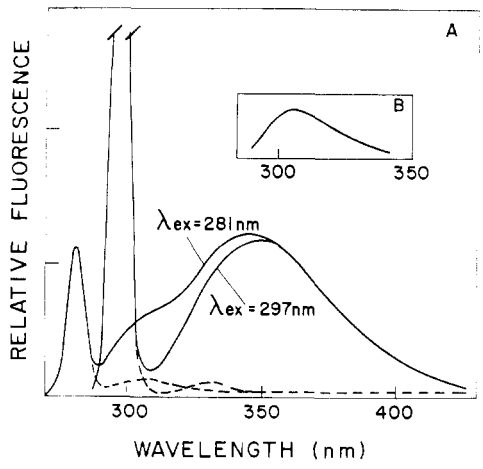

FIGURE 1: Illustration of the method of obtaining the tyrosyl fluorescence contribution. (A) Fluorescence spectra of actin in 8 M urea (u-actin) excited at 281 and 297 nm. The 297-nm spectrum was normalized instrumentally to the 281-nm spectrum at 360–370 nm. The dashed lines are buffer–urea blanks. (B) The tyrosyl contribution obtained by subtracting the 297-nm spectrum from the 281-nm spectrum. The spectrum below 305 nm was obtained by normalizing the spectrum above 305 nm to a spectrum of L-Tyr in water; 8 M urea, 2 mM Hepes, pH 7.6, 25°.

shaken, was observed between crossed Polaroid filters. The solution was put through a Millipore filter (HAWP 0.45 μ) before use. The dialysis tubing used in this work was pretreated by boiling several times with 1.5% $NaHCO_3$ and then several times with distilled water until neutrality was reached and then was stored in the presence of 1 mM EDTA.

Several criteria have indicated that the actin prepared as above contains less than 10% nonnative actin impurities. Fluorescence titrations with Cu^{2+}, which only specifically binds to native actin (Lehrer *et al.*, 1972), gave the expected 1:1 stoichiometry within 10%. Sodium dodecyl sulfate polyacrylamide electrophoresis gels usually contained only one band and occasionally a second band indicating a tropomyosin contamination of <10%. Actin prepared by the method of Spudich and Watt (1971) also gave similar ratios of peak fluorescence intensity (before to after EDTA addition).

The concentrations of the protein and ATP were determined by ultraviolet absorption measurements at 260, 290, and 320 nm using the buffer as the blank. The absorption at 320 nm, which was typically about 5–10% of the absorption at 290 nm, was subtracted from the readings at 260 and 290 nm to correct approximately for any light scattering. The actin concentration was calculated from A_{290} using ϵ_{290}^{actin} 0.63 $(mg/ml)^{-1}$ cm^{-1} obtained from the spectrum of nucleotide free actin and the ϵ_{280} value of Rees and Young (1967). This assumes that the nucleotide does not contribute any absorption at 290 nm. It appears that provided the ATP concentration is not more than 10 times the actin concentration, the actin concentration is not overestimated by more than 3–4% by neglecting the nucleotide absorption at 290 nm. At the concentrations normally used (5 mg/ml of actin and 0.5 mM of ATP) the error is even less. The nucleotide concentration is then calculated from $A_{260}^{ATP} = A_{260} - 0.92A_{290}$ and ϵ_{260}^{ATP} 15.4×10^3 M^{-1} cm^{-1}, where A_{260} and A_{290} are the absorbances corrected for light scattering as described above, $0.92A_{290}$ is the actin contribution at 260 nm obtained from the nucleotide-free spectrum, and A_{260}^{ATP} is the nucleotide contribution to the absorption at 260 nm.

Methods. Fluorescence measurements were made with an instrument that employs two Jarrell-Ash 0.25-m monochromators, an EMI 6901B photomultiplier, and an Osram 150 W high-pressure Xenon lamp having 3- and 7-nm bandwidths for excitation and emission, respectively. The fluorescence was usually measured at right angles to the excitation. The fluorescence spectra were not corrected for variations in instrumental sensitivity with wavelength since calibration procedures similar to those employed by Melhuish (1962) indicated that emission corrections were the same within 15% between 300 and 450 nm.

The solution temperature was controlled with a thermostated sample housing and measured with a calibrated, glass-enclosed thermistor and a resistance bridge.

Calculations. The quantum yield of the intrinsic fluorescence of d-actin and u-actin was obtained by comparing the areas under the spectra with the area under the fluorescence spectrum of a solution of L-Trp, all having the same absorption at the exciting wavelength (A_{280} 0.10), and all spectra obtained under the same experimental conditions. A value of 0.13 was used as the quantum yield of the fluorescence of the standard L-Trp solution (Chen, 1967). To obtain the contributions of Tyr and Trp to the quantum yield it was necessary to calculate the relative contributions of Tyr and Trp to the total absorption at 280 nm and to separate the Tyr and Trp contributions to the intrinsic fluorescence. The former was done by assuming that actin contains 17 Tyr and 5 Trp (Elzinga, 1970) with ϵ_{280}^{Tyr} 1360 and ϵ_{280}^{Trp} 5500 and that Tyr absorption does not contribute to Trp fluorescence. The separation of the Tyr and Trp fluorescence contributions was accomplished by subtracting the normalized Trp contribution, obtained by exciting at 297 nm, from the total fluorescence obtained by exciting at 280 nm (Weber and Young, 1964). The normalization of the 297-nm spectrum to the 280-nm spectrum was done instrumentally in a wavelength region where only Trp fluorescence is expected (360–380 nm) (Figure 1). The difference spectrum is the Tyr contribution shown in the inset of Figure 1. The Tyr spectrum below 305 nm could not be directly obtained due to the interference of the light scattering but could be estimated from the values above 305 nm by assuming that the shape of the protein Tyr spectrum was the same as that of L-Tyr in water.

The quantum yield of the fluorescence of G-actin was calculated using the previously measured quantum yield of d-actin by comparing the spectrum of G-actin with the spectrum of an identical sample of EDTA-denatured actin (d-actin). Separation of the Tyr and Trp contributions was accomplished as described above. Similarly, the quantum yield of the fluorescence of F-actin was calculated by a comparison with the fluorescence of G-actin.

The energy transfer parameters, overlap integral, J, and critical transfer distance, R_0, were calculated from the absorption spectrum of Cu-actin and the fluorescence spectrum of actin as previously outlined (Lehrer, 1969).

Results

Intrinsic Fluorescence Differences. The intrinsic fluorescence spectra of G-ATP-actin, actin denatured by the action of EDTA or heat (d-actin), and actin in 8 M urea (u-actin) are shown in Figure 2, and the calculated quantum yields listed in Table I. In each case the fluorescence is mainly from Trp with lesser contributions from Tyr. The same spectrum was obtained from G-actin with ADP as the bound nucleotide and either Mg^{2+} or Ca^{2+} as the bound metal ion.

It can be seen that there is a large change in the fluorescence

TABLE I: Intrinsic Fluorescence Properties of the Different States of Actin at 25°.

State	F(Trp) λ_{max} (nm)[a]	F(Trp) Q[b]	F(Tyr) Q[b]
F-actin	328	0.21	0.025
G-	329	0.23	0.019
d-	335	0.19	0.013
u-	349	0.14	0.027
CuF-	326	0.063	0.018
CuG-	327	0.057	0.013

[a] λ_{ex} 297 nm, ±2 nm. [b] ±10%.

spectrum of G-actin upon denaturation. Due to the lability of actin and the long dialysis time often necessary for the preparation of G-actin, a variable amount of denatured actin is often present. A method has been developed to determine the degree of denaturation of a given actin preparation based on this fluorescence change. The G-actin solution obtained from the depolymerization dialysis usually contains 2–5 mg/ml and about a 5× molar excess of ATP. This solution is diluted with buffer to give an actin concentration of approximately 0.1 mg/ml and the fluorescence spectrum is recorded, usually with the excitation at 290 nm. The excess ATP protects against spontaneous denaturation during the few minutes of the experiment. Dithiothreitol to 0.5 mM is then added. An increase in fluorescence indicates the presence of Cu^{2+} (Lehrer *et al.*, 1972). EDTA to 1 mM is then added and the fluorescence is monitored until there is no further change—usually complete in a few minutes. The final fluorescence spectrum is then recorded. The ratio of the peak fluorescence intensity before to the intensity after EDTA addition varies from 1.30 ± 0.05 to 1.0 as the degree of denaturation increases from 0 to 100%.

Attempts to regenerate G-actin from either u-actin or d-actin under many different conditions have been unsuccessful. For example, when a sample of u-actin (1.5 mg/ml in 8 M urea) was slowly added at 0° to 100 volumes of a solution containing 0.5 mM ATP–0.1 mM $CaCl_2$–0.5 mM dithiothreitol–2 mM Hepes, pH 7.5, and incubated at room temperature or at 40° for 1 hr, d-actin was produced as indicated by the fluorescence spectrum. On similar dilution and incubation of a G-actin solution in buffer as a control the fluorescence spectrum of native G-actin was observed. When the urea was diluted out or dialyzed away from the sample of u-actin, a fluorescence spectrum characteristic of d-actin was always obtained. Dialysis of a sample of d-actin (2 mg/ml) *vs.* 0.5 mM ATP–0.1 mM $CaCl_2$–1 mM dithiothreitol–2 mM Hepes, pH 7.5, at 0–2° for 3 days also showed no indication of renaturation. Thus, although the transition from u- to d-actin is readily reversible, the transition from G- to d-actin is irreversible at least under the conditions defined above.

A comparison of the fluorescence of G-actin and of F-actin polymerized by the addition of 0.1 M KCl to a part of the G-actin solution is shown in Figure 3 for excitation at 276 and 297 nm. Small differences in fluorescence are seen at both exciting wavelengths. In the case of F-actin, a large degree of scattering of the primary radiation is especially prominent for λ_{ex} 297 nm. The light-scattering peak overlaps the fluorescence spectrum at low wavelengths and may also

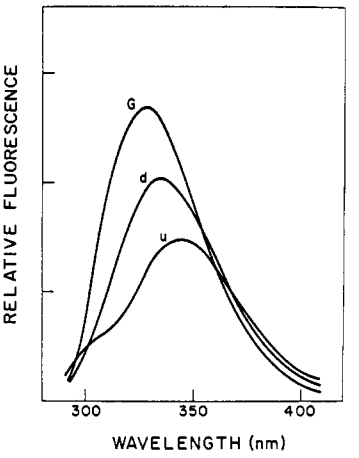

FIGURE 2: Fluorescence spectra of G-ATP or G-ADP-actin (G), EDTA- or heat-denatured actin (d), and actin in 8 M urea (u); λ_{ex} 281 nm, 25°, 2 mM Hepes, pH 7.6. The spectra of d- and u-actin were compared after dilution to 0.1 mg/ml. The spectrum of G-actin was normalized to the d-actin spectrum using the average of several determinations of relative peak height of G- and EDTA-denatured actin obtained at various concentrations.

cause a reduction of the intensity of fluorescence. In order to compensate for this effect, glycogen was added (Martonosi and Teale, 1965) to the G-actin solution to increase the light scattering, as indicated by the peak at 297 nm, to the value observed for the F-actin solutions. Equalization of the light scattering by adding glycogen to the G-actin solution was also shown by the fact that the absorption in the 320 to 400-nm region was approximately the same as that of the F-actin solution. This addition of glycogen to G-actin caused only a small decrease in G-actin fluorescence when excited at 297 nm and little or no change when excited at 276 nm (Figure 3). Comparison of the corrected tryptophan fluorescence of G-actin and F-actin, obtained by exciting at 297 nm, indicated that polymerization results in a 10% decrease in Trp fluorescence. The Trp difference fluorescence spectrum caused by

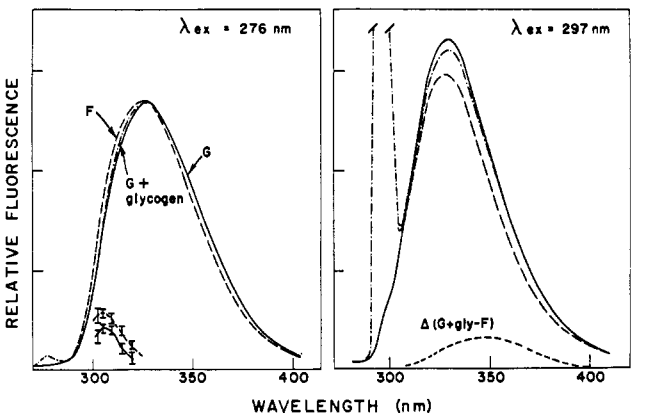

FIGURE 3: Fluorescence spectra of G- and F-actin showing influence of light scattering. F-actin, – –; G-actin, —; G-actin + glycogen, –·–. Glycogen was added to G-actin until the light-scattering peak at 297 nm (not completely shown) was the same for both F- and G-actin. Right figure, λ_{ex} 297 nm, shows the Trp fluorescence. Left figure, λ_{ex}, 276 nm, shows both Tyr and Trp contributions. The Tyr contribution is indicated by the points in the lower part of the figure (I), actin concentration = 2.0 mg/ml in 2 mM Hepes, pH 7.6, 0.2 mM ATP. F-actin was made by adding KCl to 0.1 M to G-ATP actin and waiting for 1 hr before measurements, 25°.

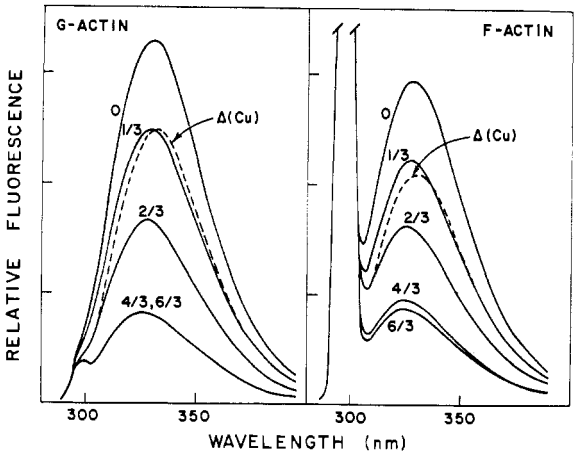

FIGURE 4: Fluorescence spectra for different molar ratios of Cu^{2+} to G- and F-actin. Δ(Cu) is the difference spectrum caused by the binding of equimolar Cu^{2+}. The curves are labled with the Cu^{2+}: actin ratio. λ_{ex} = 297 nm. Conditions as for Figure 3.

this polymerization which is plotted in Figure 3 (λ_{ex} 297 nm) shows that the slight fluorescence loss can be associated with Trp side chains whose fluorescence has a peak near 350 nm. The spectra obtained by excitation at 276 nm, where both Tyr and Trp absorb, show a small but definite increase in the Tyr contribution to the fluorescence upon polymerization. By estimating the tyrosine contribution in the manner described for u-actin (Figure 1) it appears that there is about a 30% increase in the tyrosine fluorescence when G-actin is polymerized (Table I). A small decrease is observed in the F-actin fluorescence at higher wavelengths (>350 nm) where Trp mainly contributes, in agreement with the 297-nm results. Similar differences were observed when the polymerization was brought about by the addition of 2 mM Mg^{2+} or when G-ADP-actin was polymerized, indicating that these changes are due to polymerization itself rather than some specific effect of bound ion or nucleotide.

Cu Binding to G- and F-Actin. G- and F-actin but not d-actin strongly and specifically bind 1 mole of copper per mole of actin without denaturation. This results in an intense absorption band in the near ultraviolet (Lehrer *et al.*, 1972)

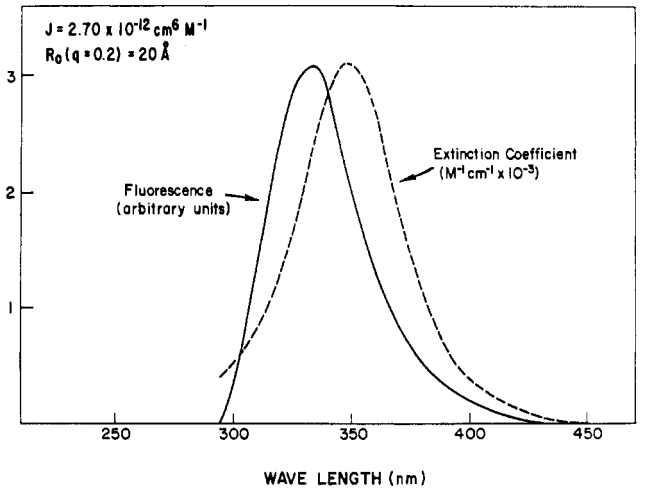

FIGURE 5: Overlap of fluorescence spectrum of G-actin and absorption spectrum of Cu-G-actin. J is the overlap integral and R_0 is the critical transfer distance. See Lehrer (1969) for details of calculation.

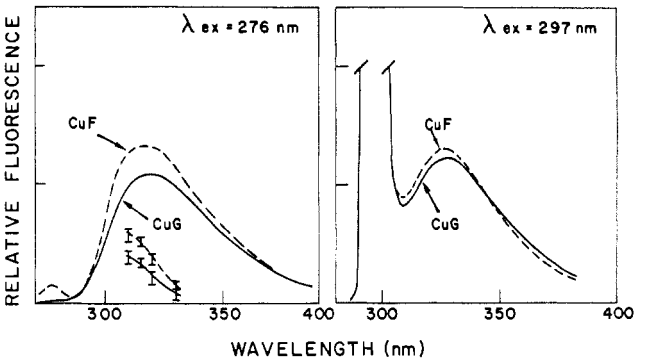

FIGURE 6: Fluorescence spectra of Cu-G- and Cu-F-actin excited at 276 nm and 297 nm, 25°. Glycogen was added to correct for light scattering as for Figure 4. The Tyr contributions are indicated by the points (I) in the lower part of the left figure.

and a quenching of fluorescence (Figure 4). In view of the location of the absorption and of the fluorescence of actin at similar energies, a long-range energy transfer mechanism seemed highly probable (Förster, 1959). In Figure 5 the overlap of the donor fluorescence and acceptor absorption is shown along with the calculated overlap integral and critical transfer distance. The magnitude of these parameters suggests that this quenching mechanism is plausible. It seemed of interest to use the bound copper as a probe for determining whether there is a conformational difference between G- and F-actin. This would appear to be a sensitive probe since the energy transfer parameters depend upon the precise distances and relative orientation of the indole fluorophor and copper-ligand transition moments. If a conformational change did occur upon polymerization of F-actin to alter these parameters, a difference in the fluorescence spectrum of Cu-F-actin and Cu-G-actin would be expected.

In Figure 4 it is seen that the spectra of Cu-G- and Cu-F-actin are very similar. A better comparison of the spectra is seen in Figure 6, where effects due to light scattering are compensated for by adding glycogen as described above. Within experimental error the same spectrum is obtained, suggesting close conformational similarity of the Trp side chains of Cu-G- and Cu-F-actin which are within the critical transfer distance of the Cu^{2+} binding site.

In contrast to the similar tryptophyl fluorescence of Cu-G- and Cu-F-actin there are differences in the magnitude of the tryosyl fluorescence as indicated in Figure 6 and Table I. As in the case of G- and F-actin without copper, there is a larger tyrosyl contribution to the intrinsic fluorescence of Cu-F-actin. The differences are more obvious, however, with copper present since much of the overlapping tryptophyl fluorescence is absent due to quenching. Some of the tyrosyl fluorescence was also quenched by the bound copper for both G- and F-actin. This may be due to direct energy transfer from tyrosine to the metal site since there is a small degree of spectral overlap of tyrosyl fluorescence with the 350-nm absorption band of the Cu protein. Alternatively, this could be due to indirect quenching of some tyrosyl fluorescence transferred to tryptophan due to the spectral overlap of tyrosyl fluorescence and tryptophyl absorption (Badley and Teale, 1969). In any case, the fractional loss of Tyr fluorescence was about the same for both G- and F-actin, again indicating little conformational difference.

Temperature Dependence. The temperature dependence of the fluorescence intensity at 310 nm and of the wavelength at

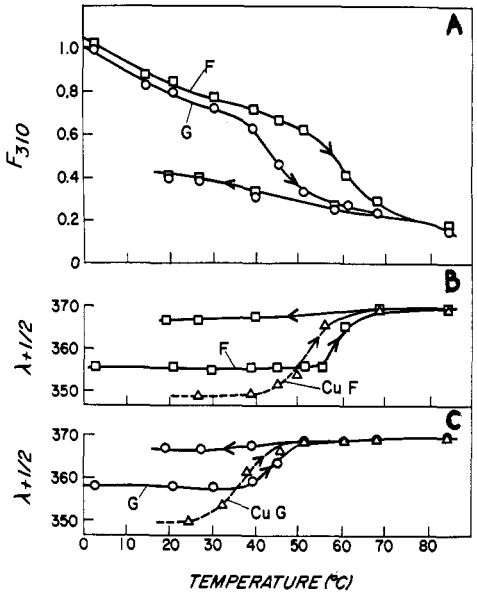

FIGURE 7: Temperature dependence of the fluorescence of G- and F-actin and of CuG- and CuF-actin. Arrows show the sequence of measurements. (A) Fluorescence intensity of F- and G-actin at 310 nm *vs.* *T*. (B) Wavelength at half-peak intensity for F- and Cu-F actin *vs.* *T*. (C) Wavelength at half-peak intensity *vs.* *T* for G- and Cu-G actin, λ_{ex} 281 nm. The cooling curves coincided for Cu-G and G and Cu-F and F. Concentration = 1 mg/ml in 2 mM Hepes (pH 7.6)–0.2 mM ATP.

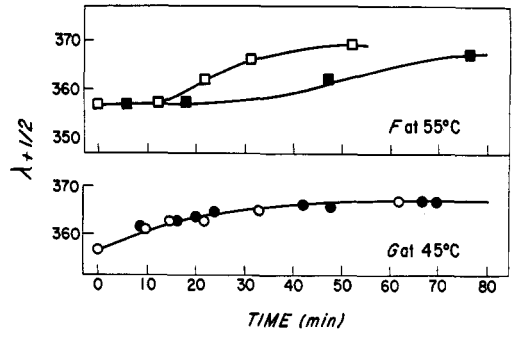

FIGURE 8: Kinetics of the spectral change associated with denaturation of F- and G-actin in each transition temperature range. □,■, F-actin; ○,●, G-actin; □,○, (ATP/actin) = 2; ■,●, (ATP/actin) = 9. Actin concentration = 1 mg/ml.

half-maximum intensity on the high wavelength side, $\lambda_{+1/2}$, is shown in Figure 7 for G- and F-actin for data taken within a few minutes after the temperature was raised. Below 40° for G- and below 55° for F-actin, the temperature dependence of the fluorescence is similar to that observed for other proteins (Gally and Edelman, 1964). Above these temperatures there is an irreversible conformational change as judged by the fluorescence changes. Since the fluorescence change in the transition region was time dependent, it is not possible to define an exact transition temperature. When the denatured solutions were cooled, the fluorescence followed the path indicated in Figure 7A. The spectrum of this heat-denatured actin did not change upon addition of EDTA and appeared identical with the fluorescence spectrum of EDTA-denatured actin. Also, the temperature dependence of the fluorescence intensity of EDTA-denatured actin showed no transition and was very similar to the lower curve of Figure 7A.

The wavelength parameter, $\lambda_{+1/2}$ was also used to follow the denaturation since it is independent of temperature unless a spectral change occurs. In Figure 7B and C it is seen that $\lambda_{+1/2}$ is indeed constant above and below the transition region and it is the same for both G- and F-actin after the denaturation is complete. Thus, it appears that heat denaturation of G- or F-actin or EDTA denaturation of G-actin results in the same state of actin (d-actin). The higher transition region for F-actin as compared to G-actin indicates that under these experimental conditions F-actin is more stable than G-actin. Figure 7B and C also indicate that a lower stability toward heat denaturation results upon the binding of Cu^{2+} to both G- and F-actin.

Since the fluorescence in the transition range is time dependent the kinetics of the denaturation were also studied. Actin samples at room temperature were quickly brought to a temperature in the transition range by placing them in a thermostated sample holder. Even though the samples were stirred to facilitate heat exchange it took a few minutes for temperature equilibration to occur. The parameter $\lambda_{+1/2}$ was plotted *vs.* time, since no changes are expected until denaturation occurs, in contrast to intensity. A comparison of the denaturation rate of F-actin at 55° and G-actin at 45° is shown in Figure 8 at two different ATP concentrations. It is seen that F-actin at 55° denatures at a much slower rate than G-actin at 45°. In addition, there is a lag period associated with F-actin denaturation, which is dependent upon the ATP concentration, in contrast to the case of G-actin. Further kinetic studies have corroborated these differences (Lehrer, 1971a).

Discussion

In view of the known sensitivity of the fluorescence spectrum of the indole fluorophor to the polarity of the environment (Van Duuren, 1961) it appears that the increasing red shift of the Trp fluorescence associated with G-, d-, and u-actin can be explained as a corresponding increase in average exposure of Trp to the aqueous environment. The location of the fluorescence maximum for u-actin near 350 nm, which is the position of the maximum fluorescence of L-Trp in water, also suggests that the Trp side chains are fully solvated in this state. These studies agree with the ORD and CD studies of Nagy (1969; Nagy and Jencks, 1962) who showed that actin in 8 M urea has optical rotation characteristics of a random coil, that G-actin has a reasonable degree of α helix and that EDTA-denatured actin has characteristics intermediate to the other states. It would be tempting to conclude that a Trp side chain is in the nucleotide binding site in view of the increased average exposure to solvent that accompanies nucleotide loss and formation of d-actin. The ORD studies, however, indicate an appreciable conformational change allowing for possible conformational changes affecting Trp far from the nucleotide binding site. It should be noted that Trp environmental changes have previously been implicated in studies of EDTA denaturation by ultraviolet difference spectral studies (West *et al.*, 1967).

The inability to even partially renature actin appears somewhat of a paradox since studies with many proteins have indicated that the amino acid sequence determines the conformation and that the most stable conformation is the active one (Epstein, 1970). If d-actin is not the most stable state, the lack of ability to renature actin may be due to a kinetic barrier caused by the masking of certain groups necessary for interaction with the nucleotide and metal ion cofactors.

Questions regarding the biosynthesis of native actin remain to be answered. It may be that the environment of the protein synthesizing compartment of the cell is such that the favored conformation is G-actin rather than d-actin, as suggested by the studies of Kasai *et al.* (1965) in which nucleotide-free native G-actin was prepared in a 50% sucrose solution. It is equally possible that the nucleotide–metal complex combines with the newly synthesized actin while still on the polysome, preventing the formation of the energetically favored denatured form.

There are small but reproducible changes in the Trp and Tyr fluorescence of actin upon polymerization. Similar changes are observed when ADP is the G-actin bound nucleotide instead of ATP, showing that the fluorescence changes are not a direct result of the dephosphorylation that accompanies the polymerization of actin in the presence of ATP. Polymerization causes a 10% decrease in Trp fluorescence and a difference spectrum that peaks near 350 nm. This suggests that the affected side chain(s) were largely exposed to solvent before polymerization. Although more difficult to detect due to the intense Trp fluorescence background, a 30% increase in Tyr fluorescence was also noted. For Cu-actin in which 75% of the Trp fluorescence is lost so that the Tyr component is more easily observable, an increase in Tyr fluorescence of similar degree was also observed. Independent studies of Vendenkina *et al.* (1968) have shown a similar decrease in Trp fluorescence but no change in Tyr fluorescence upon polymerization. By analogy with studies of fluorescence changes produced on binding detergents in which increases in Tyr fluorescence (Cowgill, 1968a) and blue shifts in spectra of Trp fluorescence (Cowgill, 1968b; Konev, 1967) were observed, it may well be possible to suggest that some Tyr and Trp are in the polymerization site of actin. Environmental changes of aromatic side chains due to polymerization to F-actin have also been suggested in studies of ultraviolet difference spectra (Higashi and Oosawa, 1965; West, 1970) and CD spectra (Murphy, 1971). Under the conditions of our measurements an absorption change at the exciting wavelength could contribute to a fluorescence change. Small absorption changes have been observed at 297 nm due to polymerization of F-actin, but they were in the wrong direction. At 280 nm, however, where Tyr also absorbs, a small absorption increase occurs which may contribute to the increase in Trp fluorescence observed. The increase only amounts to a few per cent, however, if the total change at 280 nm is assumed to be due to Tyr. Thus, the changes in fluorescence observed in actin upon polymerization appear to be due to environmental affects on the fluorescence efficiencies of Tyr and Trp.

When equimolar Cu^{2+} is bound to G or F-actin about 75% of the Trp fluorescence is quenched. From the magnitude of the parameters calculated above, the mechanism appears to involve long-range energy transfer from Trp to the Cu-actin binding site. Considering the approximate shape of the actin monomer in F-actin and the critical transfer distance of 20 Å, about 0.25 of the volume of the actin monomer lies within the critical transfer distance. It is impossible to decide how many of the five Trp residues of actin lie within the critical transfer distance. From the magnitude of the quenching and the quantum yield of actin, however, it can be calculated that the fluorescence of more than one Trp is affected. Table I and Figure 6 show that the Trp quantum yield and spectrum for Cu-G-actin and Cu-F-actin are the same within experimental error. Thus it appears that there is little backbone conformational difference between G- and F-actin. This is in agreement with recent CD studies (Murphy, 1971). It cannot be ruled out, however, that the quenching by bound copper is not sensitive to small conformational changes, a situation that may result if the Trp involved were located very close to the Cu binding site. The observation that the difference spectrum produced by the quenching by Cu^{2+} is shifted by 5 nm from the final spectrum (Figure 4) suggests that one class of Trp is strongly quenched as has been observed in many other systems (Lehrer, 1971b).

The lack of any significant difference in Trp fluorescence between Cu-G- and Cu-F-actin provides some further information regarding the location of the Cu^{2+} binding site. That is, since no further loss in fluorescence occurs upon polymerizing Cu-G-actin the Cu^{2+} binding site is probably located at least 20 Å (the critical transfer distance) away from Trp side chains of neighboring monomers of F-actin. It should be noted that the mobility of a spin label attached to the rapidly reacting SH group of actin was observed to change upon polymerization (Stone *et al.*, 1970). This SH group is presumably the same one that binds Cu^{2+} (Lehrer *et al.*, 1972). It may be that this SH group is near the polymerization site of actin or that, as the authors suggest, the spin label senses a conformational change associated with polymerization.

Fluorescence normally decreases with temperature due to thermally activated quenching mechanisms (Gally and Edelman, 1964) sensitive to the immediate fluorophor environment. The similarity of the fluorescence temperature dependence of G- and F-actin from 0 to 40°, therefore, further indicates similarity of conformation. Above 40° an irreversible transition to the denatured state (d-actin) occurs. This has also been observed with ORD and CD techniques (B. Nagy, personal communication, 1971) for G-actin. The higher transition temperature for F-actin indicates increased stability. The binding of equimolar Cu^{2+} decreased the stability of both G- and F-actin. A preliminary study of the kinetics of the irreversible transition indicated that whereas the presence of excess ATP did not protect G-actin from denaturation, it did protect F-actin. These results are in agreement with studies of the ATPase activity and viscosity of F-actin in the temperature range 55–65° by Asai and Tawada (1966). These authors observed the ATPase activity of actin at high temperature and suggested that it is due to a localized thermally activated interruption of the F-actin structure which allows the exchange of the bound ADP with free ATP in the solution by analogy with the similar effects of an ultrasonic field (Nakaoka and Kasai, 1969). The newly bound ATP is then hydrolyzed, allowing local repolymerization. Thus, ATP protects against denaturation of F-actin, the ATP becoming hydrolyzed in the process. This suggests that as long as a reasonable excess of ATP is present, F-actin will be protected depending upon the relative rates of denaturation and ATP hydrolysis. The results in Figure 8, which show that the lag period depends upon the ATP concentration, support this view. The lack of a protective effect of ATP on G-actin suggests a different mechanism of denaturation. Further studies of the kinetics of this denaturation are in progress.

Acknowledgments

We thank Dr. J. Gergely for critically reading the manuscript and Miss M. Ho for expert technical assistance.

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Characterization and Quantitation of the Apolipoproteins from Human Chyle Chylomicrons*

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ABSTRACT: The composition of human chyle chylomicrons was studied. For this purpose, thoracic duct chylomicrons from a female subject were isolated, delipidized, and the lipid and protein part investigated separately. For the characterization of the protein part, polyacrylamide gel electrophoresis, immunoelectrophoresis, and immunodiffusion were performed. Pure peptides isolated from human serum lipoproteins were used as reference substances. Monospecific antibodies were used to check the identity of chylomicron peptides. In order to quantitate the amount of different constituents, a densitometric scan after separation in polyacrylamide gels and staining with Amido-Schwarz and coomassie blue were performed. In other experiments, the peptides were isolated using gel permeation and ion-exchange chromatography and amino acid analysis of the fractions. The quantitation of the peptides in these experiments was performed gravimetrically. The results indicate that all the peptides of human serum very low density lipoproteins were present in chyle chylomicrons too. The major part consists of apolipoprotein C (R-Val, R-Glu, R-Ala_{1+2}). About 20% apolipoprotein B were found and the amount of apolipoprotein A peptides was about 15%. From the presence of apolipoprotein AI and apolipoprotein AII in almost equal concentration it was concluded, that the apolipoprotein A content is not due to an adsorption of lipoprotein A on to the chylomicron particle from the lipoproteins of the blood.

Chylomicrons are triglyceride-rich lipoproteins of diameter >800 Å, found in intestinal lymph after absorption of dietary fat (Havel, 1970). There is evidence that all classes of lipoproteins can be synthesized in the mucosal cells of the small intestine (Alaupovic *et al.*, 1967; Hatch, 1966; Roheim *et al.*, 1966). Little is known of the structure and composition of human lymph lipoproteins with the exception of the chylomicrons. Since chylomicrons contain a small and variable amount of protein (less than 2%), investigations have concentrated upon the lipid composition. There is not complete agreement about amount, kind, and function of the protein

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