Circular Dichroism of the Adenine and 6-Mercaptopurine Nucleotide Complexes of Actin*

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ABSTRACT: G- and F-actin exhibit circular dichroic spectra in the 200- to 250-mμ region which indicate a considerable amount of order in their secondary structure. Based on the spectra of α, β, and random forms of poly-L-lysine (Greenfield, N., and Fasman, G. D. (1969), *Biochemistry 8*, 4108), it is estimated that G-actin contains 26% α-helix and 26% β-pleated-sheet structures; for F-actin these values are 29 and 23%, respectively. In the near-ultraviolet region, above 265-mμ G-actin displays a spectrum which can be resolved into two negative and two positive Gaussian bands. The positions (and molecular ellipticities, $[\theta]$, $\times 10^{-3}$ in (deg cm^2) dmole^{-1}) of these bands are 293 (−9.7), 286 (−6.0), 278 (+6.3), and 272 mμ (+11.6). The corresponding bands for F-actin are 291 (−13.0), 284 (−28.0), 277 (−17.4), and 270 mμ (−3.2). Based on the polarization spectra of tyrosine and trytophan (Weber, G. (1960a), *Biochem. J. 75*, 335), the 277- to 278-mμ band is assigned to tyrosine and the other three bands to tryptophan transitions. The greater rotational strength of F-actin suggests that these aromatic residues interact more strongly with each other in the polymer. The effect of polymerization on the bound nucleotide was measured after bound ADP was replaced by 6-mercaptopurine ribonucleoside triphosphate. Its ellipticity is unchanged by being bound to G-actin (−4000 at 321 mμ); polymerization, however, results in an increase to 11,700 (deg cm^2) dmole^{-1}, suggesting considerable purine–protein interaction in the polymer. This result was paralleled by the effects of the proteins on the ionization constant of the purine; whereas G-actin complexed with the sulfhydryl nucleotide did not alter its ionization properties, the F-actin-bound nucleotide was observed to have heat and entropy of ionization values significantly more negative than those of the free nucleotide.

Upon increasing the ionic strength of its solutions, actin undergoes polymerization from a monomeric globular (G) form to a double-stranded helical, fibrous (F) form. It is of interest to know what changes in secondary and tertiary structure accompany this change in quaternary structure. It may be that increased ionic strength simply overcomes repulsive forces and allows the monomer to associate essentially unchanged intramolecularly. Then again polymer formation might involve extensive intramolecular rearrangement. That some conformational changes do in fact accompany the G–F transition, has been inferred from ultraviolet absorption (Higashi and Oosawa, 1965), fluorescence (Vedenkina *et al.*, 1968), and spin-label (Stone *et al.*, 1970) studies. Employing the conformation-sensitive technique of circular dichroism, the present study suggests that the secondary structure of the actin monomer is little altered upon polymerization, while on the other hand, the environment of the aromatic residues is changed considerably.

Actin contains one ADP (or ATP) per monomer. Though the role of this nucleotide cofactor is unclear, except insofar as its stabilizing influence on G-actin, its value as a structural probe lies in its binding to a single, presumably localized site. Unfortunately, the light absorption properties of the bound adenine nucleotide are largely masked by those of the protein. For this reason, the adenine nucleotide was replaced by a 6-mercaptopurine nucleotide, SH-TP,[1] the absorption band of which is above 300 mμ (Murphy and Morales, 1970). In this way it has been possible by CD and spectrophotometric titration studies to show that the purine portion of the bound nucleotide interacts strongly and weakly with F- and G-actin, respectively.

Materials and Methods

Actin was prepared from acetone-dried rabbit muscle powder by extraction with 0.2 mM ATP at pH 7.5 and 4° (Stone *et al.*, 1970). After a polymerization–centrifugation procedure (Carsten and Mommaerts, 1963), the F-actin pellet was homogenized at 4° in solutions of pH 7.0 containing 0.1 mM calcium, 0.5 mM dithiothreitol, and 1 mM ATP or SH-TP and clarified by centrifugation at 25,000*g* for 10 min. Polymerization was effected by adding KCl to 50 mM and allowing the solutions to stand at 25° for 45 min; centrifugation at 60,000*g* for 4 hr yielded an essentially protein-free supernatant and a transparent pellet. This procedure was repeated twice. Actin prepared in this way contains negligible amounts of nonpolymerizable actin and tropomyosin, as shown by Sephadex G-200 chromatography (D. B. Stone, personal communication; *cf.* Rees and Young, 1967). Stored at 0° for no more than 3 days, the pellets just before using were homogenized and clarified in solutions of ATP or SH-TP so that the resulting mole ratio of nucleotide: actin was 2–3:1. For studies on F-actin, polymerization was brought about by addition of KCl to 50 mM.

Affinity labeling of actin was attempted under conditions similar to those used successfully with myosin (Murphy and Morales, 1970). S·actin was prepared as above, with the exception that the final depolymerization was done with an SH-TP solution at pH 8.2 (2 mM Tris·HCl) without added dithiothreitol. One-half of this S·G-actin was polymerized by adding KCl to 100 mM. Both solutions were allowed to stand

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[1] Abbreviations used are: SH-TP, 6-mercaptopurine-9-β-D-ribofuranosylpurine 5′-triphosphate; S·actin, A·actin, the 6-mercaptopurine and adenine nucleotide complexes of actin.

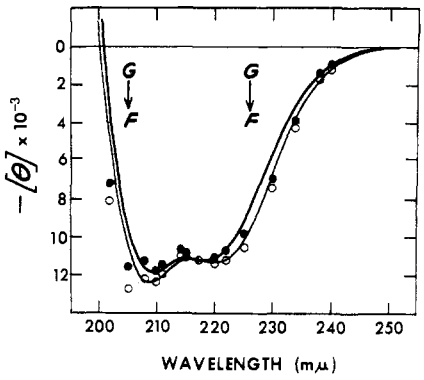

FIGURE 1: CD spectra of G- and F-actin at pH 7.0 in the far-uv region. Solid lines are the experimental curves; open (F) and closed (G) circles are best fits calculated from poly-L-lysine reference spectra, and correspond to (for F-actin) 29 and 23%; (for G-actin) 26 and 26% α-helix and β-pleated-sheet structures, respectively (*cf.* Greenfield and Fasman, 1969).

TABLE I: Calculated Per Cent α-Helix, β-Pleated-Sheet, and Random Coil Structures of G- and F-Actin.[a]

	α Helix	β-Pleated Sheet	Random Coil
G-Actin	26[b,c]	26	48
F-Actin	29[b]	22	49

[a] Estimates based on the CD spectra of the three forms of poly-L-lysine (Greenfield and Fasman, 1969); values give best fit of experimental curves (see Figure 1). [b] Based on the ellipticity of poly-L-glutamic acid at 210 mμ (Wu and Yang, 1970), these values are calculated to be 27 and 28% for G- and F-actin, respectively. [c] This percentage may be compared to a value of 29% calculated from the optical rotatory dispersion of G-actin (Nagy and Jencks, 1962).

at 0° for as long as 7 days. Aliquots taken at various times were subjected to trichloroacetic acid precipitation and the amount of nucleotide in the supernatant determined spectrophotometrically.

Nucleotide concentrations were based the following millimolar extinction coefficients: SH-TP (at 322 mμ), 23.1 (Hampton and Maguire, 1961); ATP (at 259 mμ), 15.4 (Bock *et al.*, 1956); when present, actin was removed by perchloric acid precipitation. Actin concentrations were measured by the Lowry *et al.* (1951) procedure. Its monomer molecular weight was taken to be 45,000.

Circular dichroism was measured on a Jasco ORD/VV-5 with a CD attachment. Cylindrical cells (1- to 50-mm path lengths) were held in a sturdy, water-jacketed block at 25°. Before spectra were recorded, solutions were allowed to stand in the instrument for 30 min (for F-actin solutions, this time was extended to 2 hr to eliminate spurious time-dependent dichroism, presumably due to ordering of the polymer as a result of being transferred to the cell; with this allowance, the spectra were time and preparation independent within ±5%). No significant effect on the spectra was observed when cells containing actin were rotated about axes perpendicular and parallel to the light beam. Ellipticities were essentially independent of protein concentration in the ranges of concentration used: 5–30 μM (G-actin) and 20–50 μM (F-actin). Typical signal-to-noise ratios were, at 208 mμ: 20:1; at 284 mμ: 24:1 (F-actin); at 293 mμ: 12:1 (G-actin). Since G-actin requires the presence of excess nucleotide to prevent denaturation (West, 1970), spectra were obtained with a 2- to 3-fold molar excess of nucleotide present. The excess ellipticities contributed by the unbound nucleotide in the near-uv region were subtracted to give the curves for the actin–nucleotide complexes. CD values in the region below 250 mμ (Figure 1) are expressed as mean residue ellipticity, based on a mean residue weight of 110, in units of (deg cm^2) dmole^{-1}. All other spectra are in terms of molecular ellipticity, *i.e.*, ellipticity per mole of actin monomer or nucleotide. From the assumed Gaussian bands the rotational strength, R, was calculated from: $R = 1.24 \times 10^{-42}[\theta]_{max}(\Delta\lambda/\lambda_{max})$, where λ_{max} is the wavelength of maximum ellipticity $[\theta]_{max}$, and $\Delta\lambda$ is one-half the bandwidth at $[\theta] = [\theta]_{max}/e$ (Moscowitz, 1960).

The ionization constant of the 6-mercaptopurine part of the nucleotide unbound and bound to G- and F-actin was obtained by spectrophotometric titration at 322 mμ (Murphy and Morales, 1970). pH and absorbance measurements were read simultaneously from a Radiometer TTT1 pH meter and Zeiss PMQII spectrophotometer which held a 2-cm pathlength optical cell of 10-ml capacity and permitted immersion of glass and calomel electrodes, thermometer, and buret. A water-jacketed cell holder with magnetic stirring assembly, connected to a constant-temperature bath, allowed temperature control to ±0.2°. Actin concentrations were 25–30 μM; SH-TP, 60–70 μM. At low and high ionic strength salt concentrations were 2 mM Tris·HCl and 50 mM KCl–2 mM Tris·HCl, respectively. The pH was changed by adding 0.1 M KOH. The heat of ionization was calculated from the slope of a plot of pK' *vs.* $1/T$ (van't Hoff plot). The entropy of ionization was then obtained from the relation $RT \ln K' = -\Delta H + T\Delta S$.

Results

G- and F-actin exhibit similar large negative ellipticities in region below 250 mμ, with troughs centered near 220 and 209 mμ (Figure 1). The shape and magnitude of the curves are similar to those of mixtures of poly-L-lysine in α-helix, β-pleated-sheet, and random coil forms, as described by Greenfield and Fasman (1969). These authors find that a reasonable estimate of the percentages of α and β structures in some proteins can be obtained by assuming that these structures in a protein give rise to CD curves similar to those of polysine. On the basis, with the aid of PDP-12 computer, least-squares fits of the experimental curves were obtained; the results are plotted at various wavelengths in Figure 1. (*cf.* Greenfield and Fasman, 1969.) The per cent α-helix, β-pleated-sheet, and random coil structures which correspond to these points are listed in Table I. The per cent α helix obtained by this method can be seen to compare favorably to calculations based on the ellipticities at 210 mμ with poly-L-glutamic acid as a model (Wu and Yang, 1970). The results suggest that there is little change in secondary structure upon polymerization of actin.

The CD spectra of G- and F-actin in the near-uv region (Figure 2) show that polymerization results in considerable changes in the shape and magnitude of the ellipticity. G-Actin exhibits what appears to be two negative bands between 300 and 282 mμ and two positive ones between 282 and 263 mμ. The CD spectra of F-actin is negative throughout, with

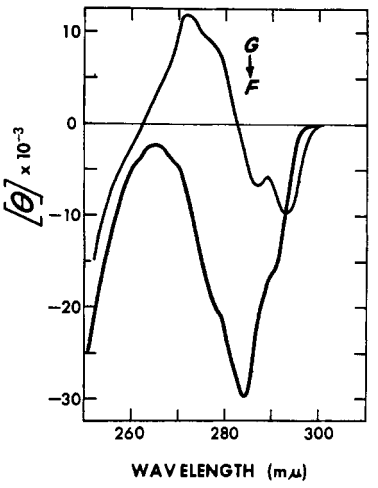

FIGURE 2: CD spectra in the near-uv region of G- and F-actin at pH 7.0.

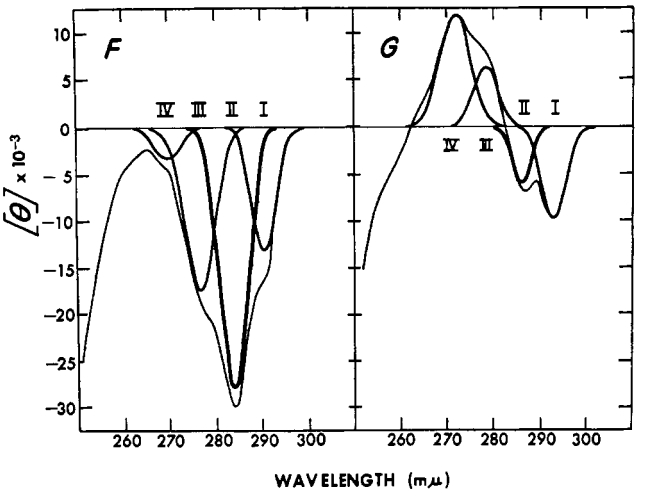

FIGURE 3: Resolution of the CD spectra of G- and F-actin into Gaussian bands.

shoulders on either side of a large band centered near 284 mμ. The spectra cross over near 292 mμ and are maximally different from 284 to 277 mμ ($\Delta[\theta] \cong 28{,}000$).

The resolution of CD curves into a minimum number of Gaussian bands is to some extent arbitrary, especially, as in the case of G-actin, where positive and negative bands lie side by side (Tinoco and Cantor, 1969). Nevertheless, this operation can be of value in defining which chromophores contribute to the observed optical activity. As shown in Figure 3, the CD spectra for G- and F-actin are closely simulated above 265 mμ by four Gaussian curves (obtained with the aid of a Dupont curve resolver). Based on the closeness of their band positions, bands I–IV of G-actin are assumed to be analogous to the same-numbered bands of F-actin. The characteristics of each band are summarized in Table II. It is noteworthy that polymerization of actin results in a blue shift of approximately 2 mμ for each band. Also, all four bands become more negative, though band I does so to a relatively small extent.

The CD spectra of ATP and SH-TP are shown in Figure 4. Like those of most unassociated purines, they resemble their absorption spectra in shape and band position. In addition to the main band at 321 mμ ($[\theta] = -3900$), SH-TP seems to show a weak band centered around 290 mμ.

To obtain CD spectra of an actin-bound nucleotide free of interference from the optical activity of the protein, the bound ADP of G-actin was replaced by SH-TP. S·Actin was free of adenine nucleotide as indicated by the absorbance of a deproteinized solution at 260 mμ, where the mercaptopurine nucleotide is almost transparent ($\epsilon_{mM} \cong 1$; Fox *et al.*, 1958). S·F-actin, freed of unbound nucleotide by treatment with Dowex 1 (Asakura, 1961), contained close to 1 mole of mercaptopurine nucleotide per mole of actin monomer. This result shows that SH-TP is bound by actin, and that the binding is noncovalent, *i.e.*, affinity labeling did not occur. The same result was obtained at pH 8.2, where affinity labeling would be more likely to take place (Murphy and Morales, 1970). Incubation at this pH of S·G- and S·F-actin for as long as 7 days caused no decrease in the amount of nucleotide found in the supernatant after trichloroacetic acid precipitation of the protein.

The ellipticity of SH-TP is little changed by its being bound to G-actin (Figure 5). The wavelength of maximal negative ellipticity appears to be red shifted by 1–2 mμ and a small positive band, possibly attributable to the nucleotide, is present at 300 mμ. The main band, however, displays essentially the same ellipticity (−4000; $R \cong 2.2 \times 10^{-40}$ cgs unit) as does the free nucleotide (Figure 4). That the nucleotide has an effect on the protein can be seen by a comparison of the CD spectra of A·G-actin and S·G-actin (Figures 2 and 5). Thus, for example, for S·G-actin the negative band at 293 mμ is smaller than the one at 286 mμ, while the opposite is true for A·G-actin.

As Figure 5 illustrates, actin in the polymerized state exerts a considerable effect on the circular dichroism of its bound

TABLE II: Features of the Resolved Gaussian Bands of G- and F-Actin.

	λ_{max} (mμ)		$[\theta]_{max} \times 10^{-3}$		$R \times 10^{40}$ (cgs units)	
Band	G	F	G	F	G	F
I	292.8	290.5	−9.7	−13.0	−1.5	−2.1
II	286.2	283.8	−6.0	−28.0	−0.7	−5.2
III	278.5	276.8	+6.3	−17.4	+1.0	−3.6
IV	271.9	269.8	+11.6	−3.2	+2.5	−0.6

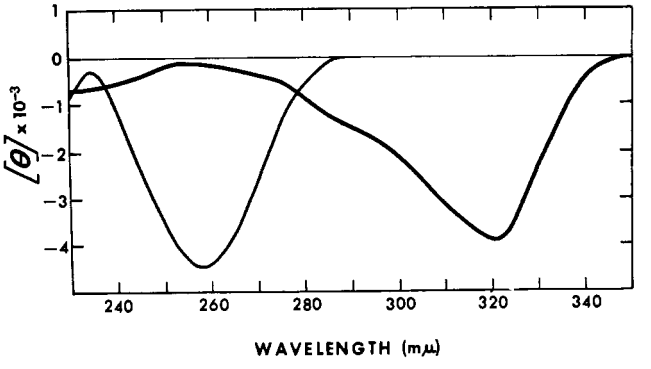

FIGURE 4: CD spectra of SH-TP and ATP at pH 7.0. SH-TP, curve with minimum at 321 mμ; ATP, curve with minimum at 258 mμ.

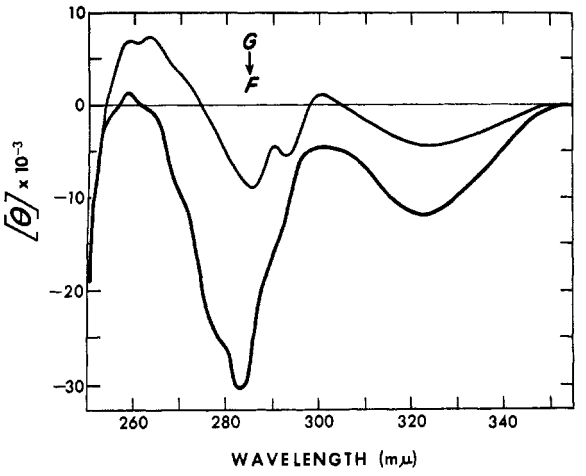

FIGURE 5: CD spectra in near-uv region of S·G- and S·F-actin at pH 7.0.

nucleotide. The maximum ellipticity is some three times greater ($-12{,}000$; $R \cong -6.8 \times 10^{-40}$ cgs unit) than that of S·G-actin. The portion of the spectrum ascribable to the protein (below 300 mμ) is similar to that of A·F-actin, the main difference, in the region below 265 mμ, is probably a reflection of the presence or absence of the adenine nucleotide.

To obtain further information on the nature of the nucleotide–actin interaction, the perturbing effect of the protein on the spectrophotometrically observable proton dissociation of the mercaptopurine moiety was measured (Fox *et al.*, 1958; Murphy and Morales, 1970). The results of determining at three temperatures the ionization constant of the nucleotide free of and bound to G- and F-actin are listed in Table III. The values obtained in the absence of protein are seen to be markedly dependent on the ionic strength, there being a decrease of almost 0.4 pH unit upon increasing the salt concentration by 50 mM. This effect is observed in general for dissociations involving production of charged species from uncharged ones (Edsall and Wyman, 1958). Presumably increased shielding by salts lessens the decrease in entropy which results from electrostriction of the solvating water molecules.

Based on the differences in the ΔH, ΔS, and pK's values between free and bound SH-TP, it would appear that G-actin has little effect on the purine part of the nucleotide. By contrast, these parameters for the mercaptopurine nucleotide are altered by being bound to F-actin. The differences, while small, confirm the conclusion from the CD data that there is considerable purine–protein interaction in F-actin.

Discussion

The small difference between the circular dichroic spectra below 250 mμ of G- and F-actin implies that the protein has a secondary structure which is independent of its degree of association. It suggests that the overall spherical shape of G-actin, deduced from light scattering (Sakakibara and Yagi, 1970) and fluorescence polarization (Cheung *et al.*, 1971) measurements, is retained in the polymer. That the monomer units of F-actin are roughly spherical has been inferred from electron microscopy pictures (Hanson and Lowy, 1963).

Actin would appear to contain a considerable amount of α-helical structure. Using the CD spectra of poly-L-lysine as a basis, it is estimated that 26 and 29% of the residues of G- and F-actin are contained in helices. The present results agree

TABLE III: Thermodynamic Parameters of the Ionization of the Purine of SH-TP and Its Complexes with G- and F-Actin.

	pK'			ΔH (kcal mole^{-1})	$-\Delta S$ (cal deg^{-1} mole^{-1})
	12°	25°	40°		
SH-TP (2 mM Tris)	8.53	8.37	8.18	5.1	21
S·G-actin (2 mM Tris)	8.58	8.37	8.20	5.6	20
SH-TP (2 mM Tris–50 mM KCl)	8.20	7.99	7.74	6.9	13
S·F-actin (2 mM Tris–50 mM KCl)	8.25	8.10	7.95	4.5	22

well with previously reported value for G-actin of 29%, based on optical rotatory dispersion measurements (Nagy and Jencks, 1963).

Estimations of the amounts of α and β structures by the method used here are based in the main on two assumptions: first, that the circular dichroic spectra of the α, β, and random forms of poly-L-lysine are the same as those of similar structures in proteins; and second, that the ellipticities of other chromophores in the region below 250 mμ are insignificant compared to those of the peptide linkage (for a further discussion of the limitations of the method, see Greenfield and Fasman, 1969). Nevertheless, the method was seen to be valid for those proteins which contain a considerable amount of ordered structure and which display shoulderless, smoothly increasing ellipticities as the wavelength is lowered from 250 mμ. Since the actin spectra obtained here fulfill these criteria, the estimation of the β-pleated-sheet content of 26 and 22% for G- and F-actin seems justified. At any rate, it is probably reasonable to state that actin contains 25–30% α-helix and 20–30% β-pleated-sheet structures (the amount of β structure is less certain because of its smaller rotatory strength). In addition, the small changes seen upon polymerization do not warrant the conclusion that there is an increase of α-helix at the expense of β structure during the G–F transformation. The presence of β structure in actin was proposed some time ago by Standaert and Laki (1962). Though based on optical rotatory dispersion data which varied from one protein preparation to another, and on the localization of the β-structure-forming seryl residues to about 40% of the actin peptides, the suggestion is nevertheless confirmed here. These facts, plus the inherent potential for intramolecular β structures to re-form intermolecularly prompt the intriguing suggestion that the monomers of F-actin are in part held together by intermolecular β-pleated sheets.

Actin contains no disulfides; its CD above 265 mμ can be attributed to the phenol and indole side chains of tyrosine and tryptophan. From polarization spectra, Weber (1960a) found that tryptophan has two near-uv π–π^* electronic transitions at 272 and 289 mμ, and tyrosine a single one at 275 mμ. On this basis band III may tentatively be assigned to tyrosine, band IV to the shorter, and bands I and II to the longer wavelength transitions of tryptophan. That the 289-mμ transition of tryptophan is separable into vibrational components may be seen from spectra at low temperature (Strickland *et al.*, 1969).[2] Since actin contains 5 tryptophans and 17 tyrosines per

[2] In proteins the polarization spectra due to tryptophanyl residues also show two bands in the 290-mμ region (Weber, 1960b).

monomer (Elzinga, 1970), it must be borne in mind that bands I–IV represent algebraic sums of the spectra of individual residues. The differences displayed between G- and F-actin therefore represent minimal ones, for large rotational strengths of some residues mightly well be masked if they are of opposite sign.

The simplest way to explain the greater ellipticity of F-actin might be to state that in the polymer the aromatic residues interact with each other to a greater extent. The reason for suggesting this is that, according to the coupled oscillator theory (see Tinoco and Cantor, 1969; Schellman, 1968), optical activity arises from the interaction of neighboring chromophores. The resulting mutually induced rotational strengths will depend, among other things, on the dipole strengths of the coupled transitions. Since the aromatic residues have the largest dipole strengths (most intense absorption bands), their interactions will give rise to the largest CD effects.

At the same time, polymerization results in a blue shift of about 2 mμ in each of the bands (Table II). Such shifts are generally considered to indicate transfer to more polar (not necessarily aqueous) surroundings (Wetlaufer, 1962). It may be for this reason that the tyrosines of F-actin are less reactive toward the nonpolar reagent, fluorodinitrobenzene (Gerber and Ooi, 1968).

Actin contains one nucleotide per monomer. That the nucleotides of neighboring monomers of F-actin do not interact with each other is indicated by the absence of a couplet (Schellman, 1968) in the nucleotide portion of the CD spectrum of S·F-actin (Figure 5). On the other hand, interaction with other chromophores seems likely, in view of the threefold greater rotational strength over that of the free nucleotide. A similar, though larger, enhancement of the optical activity of a nucleotide by a protein has been observed in the ADP–creatine phosphokinase complex (Kägi *et al.*, 1971). For this system, it was possible to infer that the likeliest source of the rotational strength of the nucleotide was coupling with the far-uv transitions of a tryptophan. If, for the S·F-actin system, one makes similar assumptions of stacked chromophores 3.4 Å apart with transition moment dipoles at optimal angles of 45°, the calculated rotational strengths are at least equal to 6.8×10^{-40} cgs unit for any of the transitions of tyrosine and tryptophan. It is thus possible that any of the transitions of these residues is responsible for the observed rotatory strength, though it is not possible to say which one(s).

The results of the spectrophotometric titrations confirm the CD data. Neither the optical activity nor the acidity of the purine part of SH-TP is altered by its being bound to G-actin. Apparently it is the ribosyl and phosphate portions of the nucleotide which are important to the binding and stabilization of the protein. In a similar way, the thermodynamic values for S·F-actin are consistent with optical activity results. The heat and entropy of ionization are changed by -2.4 kcal mole^{-1} and -8.6 cal deg^{-1} by binding to F-actin. A simple interpretation of the similarity of the ΔS for S·F-actin to the ΔS for SH-TP at low ionic strength is that in the complex the purine is not accessible to the shielding effect of KCl. In any case, the purine appears to be at least partially buried.

The very slow exchange of F-actin-bound ADP with unbound nucleotide (Martonosi *et al.*, 1960) indicates that the nucleotide as a whole is inaccessible. Since interaction with myosin permits exchange (Szent-Györgyi and Prior, 1966), it has been suggested that the polymer becomes loosened during contraction. A CD study of this system might settle this question, in view of the association state dependence of the optical activity of the aromatic residues and bound nucleotide.

Actin also interacts with the troponin–tropomyosin system (Ebashi and Endo, 1968). Eisenberg and Kielley (1970) have shown that tropomyosin acts mainly by altering the binding of actin to myosin, rather than by inhibiting the actomyosin ATPase. Tonomura *et al.* (1969) find that the effect of calcium binding to troponin is transmitted through tropomyosin to a spin label on actin. CD studies of relaxing protein (Staprans and Watanabe, 1970) and actin in combination are likely to be of value in clarifying the details of these interactions.

Acknowledgments

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Substrate Binding Site in Bovine Chymotrypsin A_γ. A Crystallographic Study Using Peptide Chloromethyl Ketones as Site-Specific Inhibitors*

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ABSTRACT: The crystallographic determination of the structure of bovine chymotrypsin A_γ at high resolution has opened the way to a study of the extended binding site of the enzyme using the difference Fourier method. Peptide chloromethyl ketones have been chosen as specific, irreversible inhibitors of the enzyme. A number of these compounds were synthesized and tested for inhibition of tetragonal crystals of A_γ at pH 5.6 in high concentrations of salt. Three compounds were selected for the preparation of inhibited crystals that were used in the collection of X-ray data at a resolution of 2.7 Å: acetyl-L-phenylalanine chloromethyl ketone (APCK), acetyl-L-alanyl-L-phenylalanine chloromethyl ketone (AAPCK), and acetyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone (AAGPCK). Although inhibition caused no changes in unit cell dimensions greater than 0.5 Å or 0.6%, the inhibited crystals were not isomorphous with crystals of the native enzyme. However, pairs of crystals inhibited with two different chloromethyl ketones were found to be isomorphous, and gave interpretable electron density different maps, when these were computed with phases for tosyl-chymotrypsin A_γ. For each of the three pairs, the highest peak in the map represented the difference in structure of the two inhibitors. Kendrew skeletal models of the inhibitor–enzyme complexes were constructed with an optical comparator, placing the inhibitor moieties in the highest electron density of the difference maps, thereby locating the ends of the chains. The C-terminal ends were fixed by a covalent bond to His-57, since it was known from amino acid analysis that the chloromethyl ketones reacted with a histidine residue. The phenyl ring was located in the hydrophobic pocket of the enzyme. The models show the peptide chain of the inhibitor bound to an extended segment of the main chain of the enzyme, consisting of Ser-214—Trp-215—Gly-216, in an anti-parallel β structure. One hydrogen bond links the C=O of Ser-214 (subsite S_1) with the NH of residue P_1 (Phe) of the inhibitor, and two hydrogen bonds link the NH and C=O of Gly-216 (subsite S_3) to the C=O and NH of residue P_3 (Ala in AAPCK) of the inhibitor. An examination of the model indicates that the enzyme should show stereospecificity for an L residue in position P_2, and that a bulky, nonpolar residue at P_2 (*e.g.*, Leu or Val) should interact with Ile-99 in subsite S_2, in accord with previous reports in the literature. It is unlikely that the enzyme interacts with the main chain of a substrate beyond three residues on the N-terminal side of the cleavage point. Without changing the position of the chain of the tripeptide inhibitor, it is possible to break the bond to His-57 and to form an ester bond between the hydroxyl group of Ser-195 and a carboxyl group placed on the phenyalanine residue of the inhibitor. This structure would correspond to the postulated acyl intermediate of enzyme catalysis.

The crystallographic determinations of the structure of chymotrypsin A_α[1] (Matthews *et al.*, 1967; Sigler *et al.*, 1968; Birktoft *et al.*, 1970) and the structure of chymotrypsin A_γ Davies *et al.*, 1969) at high resolution have opened the way to investigations of the structural basis for specificity of this serine protease. Studies carried out with A_α (Steitz *et al.*, 1969; Birktoft *et al.*, 1970; Henderson, 1970) have defined the

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[1] The forms of chymotrypsin produced by activation of bovine chymotrypsinogen A are designated by the symbols A_γ, A_α, etc. (Wilcox, 1970). A_γ and A_α correspond to the alternative designations, γ- and α-chymotrypsin, respectively. Other abbreviations used in this paper are: Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Ac, acetyl; tosyl, *p*-tolylsulfonyl; APCK, acetyl-L-phenylalanine chloromethyl ketone; AAPCK, acetyl-L-alanyl-L-phenylalanine chloromethyl ketone; AAGPCK, acetyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride.