Stoichiometry of Covalent Actin–Subfragment 1 Complexes Formed on Reaction with a Zero-Length Cross-Linking Compound†

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ABSTRACT: The interaction of actin and rabbit subfragment 1 has been reexamined by using a carbodiimide cross-linking reagent. A major doublet with an apparent molecular weight of 134 000 as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis has been identified. A minor product of much higher molecular weight is also formed. Stoichiometry determinations using [^{3}H]actin and [^{14}C]subfragment 1 indicate that the major doublet is a 1:1 complex of actin and subfragment 1. This result confirms that reported by Sutoh (1983) [Sutoh, K. (1983) *Biochemistry 22*, 1579–1585].

Muscle contraction is caused by the interaction between actin and the head region of myosin, known as subfragment 1 (SF1). Cross-linking reagents facilitate specific covalent links between the two proteins (Mornet et al., 1981a; Sutoh, 1982) and hence can be used to deduce the sites of close contact between the proteins. The carbodiimide cross-linking reagent 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) forms covalent bonds between amino and carboxyl groups in proteins without introducing any other atoms (hence, the name zero-length cross-linker). The major complex formed between actin and SF1 by EDC was found by Mornet et al. (1981a) to have an apparent molecular weight of 180 000 and a stoichiometry of about 2:1. As these authors detected no complex of lower molecular weight consistent with a 1:1 ratio of actin to SF1, they suggested that each myosin head binds to two actin monomers along the actin filament. Further studies with bis(imido ester) cross-linking agents gave similar results (Labbé et al., 1982). The phenomenon is of great interest since it provides a mean of force generation during muscle contraction by moving from one attachment site to the other and is consistent with the observed position of SF1 between the actin monomers in decorated thin filaments (Amos et al., 1982).

Recently Sutoh (1983) reexamined the cross-linking of actin and SF1 with EDC. He found the same set of cross-linked species as found by Mornet et al. (1981a), but the stoichiometry determined from the use of fluorescent actin and SF1 was about 1:1; furthermore, the size of peptides produced under different proteolytic conditions was not compatible with the conclusions of Mornet et al. (1981a). He concluded that the myosin head only binds to one actin monomer. However, he still found that the apparent molecular weight of the cross-linked species was about 170 000, much greater than the 137 000 expected from a 1:1 complex of actin and the SF1 heavy chain.

In the present study, we determined the apparent molecular weight of cross-linked actin–SF1 by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with varying acrylamide concentrations and measured the stoichiometry of the cross-linked actin–SF1 by using radiolabeled proteins in an attempt to resolve the discrepancies between the two laboratories.

Materials and Methods

Chemicals were of Analar grade or equivalent. Solutions were made with double-distilled water. Iodo[1-^{14}C]acetamide ([^{14}C]IAM) and iodo[2-^{3}H]acetic acid ([^{3}H]IAA) were obtained from Amersham International. The liquid scintillants Monophase, Permafluor, and 299 were from Packard Instrument Co.

Preparation of Proteins. SF1 was prepared by the method of Weeds & Taylor (1975) from rabbit fast muscle myosin. Actin was prepared from an acetone powder of rabbit fast muscle as described by Drabikowski & Gergely (1964).

Protein Concentrations. Protein concentrations were determined from the absorbances at 280 nm by using an extinction coefficient of $E^{1\%}_{280nm} = 7.5$ cm^{-1} for SF1 (Wagner & Weeds 1977) and 11.0 cm^{-1} for actin (West et al., 1967). These were confirmed by the Folin procedure (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard.

Alkylation of SF1 and Actin. SF1 (2 mg/mL) in 25 mM tris(hydroxymethyl)aminomethane (Tris)–30 mM KCl, pH 7.6, was reacted with a 41-fold molar excess of [^{14}C]IAM at 0 °C for 2 h in the dark. Excess IAM was quenched with β-mercaptoethanol (β-MSH) and the reaction mixture dialyzed exhaustively against coupling buffer (see below). Two different labelings gave 0.46 and 0.23 mol of ^{14}C/mol of SF1 (25 and 12.5 mCi/mmol, respectively).

F-Actin was alkylated in one of two ways, (i) F-Actin (4 mg/mL) in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes)–0.1 M KCl, pH 7.0, was reacted with a 25-fold molar excess of [^{3}H]IAA at 25 °C in the dark; the reaction was quenched and dialyzed as described above. This gave 0.07 mol of ^{3}H/mol of actin (7 mCi/mmol). (ii) F-Actin (13 mg/mL) in 25 mM K_2HPO_4–0.1 mM dithiothreitol, pH 8, was flushed with N_2 for 20 min. A 7-fold molar excess of [^{3}H]IAA was added, left at 25 °C for 1.5 h in the dark, and dialyzed as described above. This gave 0.42 mol of ^{3}H/mol of actin (44.6 mCi/mmol).

Cross-Linking of F-Actin and SF1. F-Actin (1 mg/mL) and SF1 (1 mg/mL) were cross-linked in 10 mM imidazole, 2 mM $MgCl_2$, and 100 mM NaCl, pH 7.0, with 2 mM EDC for 2 h at 25 °C as described by Sutoh (1982). The cross-linking reaction was terminated by boiling the samples in SDS–PAGE sample buffer. Three different cross-linking reactions were performed with radiolabeled proteins; reactions 1A and 1B were performed with 25 mCi/mmol SF1 and 7 mCi/mmol actin; reaction 2 was performed with 12.5 mCi/mmol SF1 and 44.6 mCi/mmol actin.

SDS–PAGE. SDS–PAGE was performed on slab gels (18 × 15 × 0.15 cm) by using the sodium phosphate buffer system described by Weber & Osborne (1969). Acrylamide concentrations varied between 5 and 10%; bis(acrylamide) was 1.5% of the total. Apparent molecular weights were estimated

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Table I: Apparent Molecular Weights of the Three Cross-Linked Species Illustrated in Figure 1 As Deduced from SDS–PAGE at Various Acrylamide Concentrations[a]

acrylamide concn (%)	M_r of bands (X10³) 1a	1b	2	limits of accepted gel markers (X10³)
4	126		165	67–220
5	135		176	60–220
6	136		186	36–220
7	140	143	199	14–95
8	137	141	220	14–95
9	140	157	>220	14–95
10	145	168	>220	14–95

[a] A regression line was calculated by using those molecular weight markers which appeared to obey a log linear molecular weight vs. mobility relation for the particular gel concentration (last column). Interpolated molecular weight values are calculated directly therefrom. When the band lay above the linear range, extrapolation from this line was by a hand-fitted curve to the higher molecular weight marker (ferritin).

by using the following molecular weight markers: ferritin half-unit, M_r 220 000; phosphorylase *b*, M_r 94 000; albumin, M_r 67 000; catalase, M_r 60 000; ovalbumin, M_r 43 000; lactate dehydrogenase, M_r 36 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r 20 100; ferritin subunit, M_r 18 500; and α-lactalbumin, M_r 14 400.

Densitometry. Gels were scanned with an LKB 2202 ultrascan.

Isotope Counting. Protein-containing gel bands were cut out of SDS–polyacrylamide gels by hand and solubilized in 30% w/v hydrogen peroxide at 50 °C for 16 h. Twenty milliliters of scintillant 299 was then added; vials were left at least 18 h in the dark before counting. Alternatively, gel bands were burnt in a sample oxidizer (Packard 306) with carrier cellulose and 0.4 mL of oxidant (Combustaid) for 1.4 min; ^3H_2O and $^{14}CO_2$ were separated by passage through Carbosorb and counted in Monophase and Permafluor, respectively.

All counting was done on a Beckman LS 7000. ^{14}C counts appearing in the ^3H channel were subtracted by using ^{14}C-labeled SF1 standards. Quench curves were constructed by using an external standard.

Results

Cross-linked preparations of actin and SF1 showed two major additional protein bands on 5% polyacrylamide gels run in the presence of SDS; we term these bands 1 and 2 (Figure 1a). Samples cross-linked for varying lengths of time, with different protein or EDC concentrations and from different protein preparations, all showed bands of identical mobility; no other major new components were seen. At polyacrylamide concentrations exceeding 7%, band 1 divided into two separate bands (Figure 1b). Band 1 represented up to 15% of the total protein present as judged by the degree of Coomassie staining. The relative intensities of bands 1a and 1b were difficult to assess, but band 1a appeared slightly stronger. Band 2 was always much weaker than band 1, measuring up to 2% of total protein; it was not always detected. The molecular weight markers for SDS–PAGE showed an apparent linearity (of log M_r vs. mobility) over a range which varied with the polyacrylamide concentration (last column, Table I). This allowed direct calculation of the apparent molecular weight of bands 1 and 2 in the 4, 5, and 6% gels, and only slight extrapolation in the 7% gel; for 8–10% gels, the extrapolation was larger and the subjective error communsurately greater.

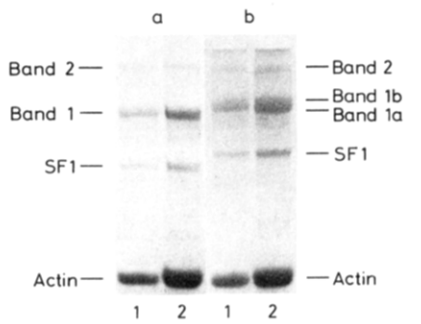

FIGURE 1: SDS–PAGE of the actin–SF1 mixture cross-linked by EDC and run on (a) 5% or (b) 10% acrylamide. Loadings were (1) 20 μg or (2) 40 μg of protein. Reaction was 1 mg/mL actin, 1 mg/mL SF1, and 2 mM EDC for 2 h at 25 °C.

The apparent molecular weight of band 1a varied only slightly with gel concentration (Table I). Band 1b, which ran in the same position as band 1a at low gel concentration, was clearly separated at a higher gel concentration. The curvature of the plots of log M_r vs. mobility in this range at a 10% polyacrylamide gel concentration caused the small differences in mobility (Figure 1) to appear as a large difference in molecular weight. The apparent molecular weight of band 2 rose steadily with gel concentration to exceed that of ferritin at 9%.

The same protein bands appeared when [^{3}H]IAA-labeled actin ([^{3}H]actin) and [^{14}C]IAM-labeled SF1 ([^{14}C]SF1) were cross-linked with EDC. More than 90% of the radioactivity applied to the SDS–polyacrylamide gels was recovered from Coomassie-stained bands on the gel. The cross-linked band 1 contained a large proportion of the activity (Table II) whereas band 2 contained relatively little; in preparation 2, it was too low to measure. The actin band contained only radioactivity associated with ^3H, but the apparent SF1 band contained some ^3H activity (3–10% of the total) as well as the expected ^{14}C activity. The cross-linked bands contained both ^3H and ^{14}C activity. The stoichiometry of actin–SF1 for band 1, calculated from the total ^3H and ^{14}C recovery from the gel lane, ranged from 0.9 to 1.5. Values were higher for the first preparation (Table II). Combustion of the gel samples gave a similar stoichiometry [for preparation 1, 1.28 ± 0.02 (4)] as did H_2O_2 treatment of gel samples [1.15 ● 0.09 (5)]. The stoichiometry values determined from actin and SF1 standards run in adjacent lanes were 20% higher than those described

Table II: Cross-Linked Bands Formed by Reaction of Iodoacetic Acid Labeled Actin ([^{3}H]Actin) and Iodoacetamide-Labeled SF1 ([^{14}C]SF1)[a]

preparation	^{14}C content (% total) in band 1	band 2	stoichiometry (mol of actin/mol of SF1) in band 1	band 2
1A	31.4 ± 0.8 (3)	3.9 ± 0.2 (3)	1.18 ± 0.08 (3)	3.8 ± 0.4 (3)
1B	32.4 ± 0.5 (6)	4.2 ± 0.4 (4)	1.22 ± 0.07 (6)	3.0 ± 0.1 (4)
2	10.7 ± 0.9 (6)		0.96 ± 0.01 (6)	

[a] Data are given as mean ± SEM (number of experiments). Cross-linking was for 2 h at 25 °C in 2 mM EDC, approximately 1 mg/mL actin, and 1 mg/mL SF1. Preparations 1 and 2 are separately prepared and labeled actin and SF1; 1A and 1B are two cross-linkings of preparation 1. Approximately 150 μg of protein was loaded on to the gel in each lane.

above for cross-links 1A and 1B but the same for cross-link 2; this was probably due to a systematic error for the protein standards of preparation 1. The apparent stoichiometry for band 2 was 3–5, with much more variability in the determinations than for band 1 (Table II). Actin cross-linked by EDC in the absence of SF1 showed three extra bands which were interpreted as dimers, trimers, and tetramers of actin; cross-linked actin species have also been found by Mornet et al. (1981a). The intensity of Coomassie staining decreased with increasing oligomeric size. [^{3}H]Actin cross-linked in the same way showed a transfer of 17–30% of the total activity to the dimer. Although the dimer migrated just faster than the SF1 heavy chain, a portion [6 ± 2% (4) of the total] spread on to the SF1 position. This could account for the ^{3}H counts appearing at the SF1 position in the actin–SF1 cross-linked preparations.

Discussion

All observers have found that EDC cross-links actin–SF1 mixtures to produce a major doublet cross-linked band and a minor band of considerably higher molecular weight. Our band 1 corresponds to the doublet studied by the previous groups. Mornet et al. (1981a) used 5–18% SDS–polyacrylamide gradient gels with a Tris–borate buffer system to estimate the molecular weight of the major doublet as 175 000 and 185 000, while Sutoh (1983) used 10% SDS–polyacrylamide gels buffered with sodium phosphate (Weber & Osborne 1969) and obtained molecular weights of 165 000 and 175 000. Our estimates of molecular weights from SDS–polyacrylamide gels were lower than either of the above estimates; on 10% polyacrylamide gels, where extrapolation from the log linear portion of the mobility plot was necessary, they were 145 000 and 168 000, respectively. At lower polyacrylamide concentrations where extrapolation was not required, the doublet band fused to give a single band with an apparent molecular weight of 134 000 (mean of values from 4–7% gels). This value varied little with gel concentration (Table I) and is consistent with an actin–SF1 dimer (calculated M_r 137 000). However, it is known that cross-linked proteins sometimes show anomalous mobility with respect to molecular weight on SDS–polyacrylamide gels (See et al., 1981). The mobilities of both components of the doublet are identical at low acrylamide concentrations but different at higher acrylamide concentrations. The simplest hypothesis is that band 1a runs close to an ideal manner over the acrylamide concentrations studied, whereas band 1b is anomalously retarded because of a difference in the actin–SF1 connection.

Mornet et al. (1981a) and Sutoh (1983) disagree on the stoichiometry within band 1 (both observers treat the doublet as a unit for analysis). Sutoh used fluorescently labeled actin and SF1 and determined a stoichiometry of about 1. However, there is a possible systematic error in the method in that the fluorescence of the cross-linked proteins may differ from that of the unlinked proteins. Mornet et al. (1981a) have determined the stoichiometry by using [^{3}H]actin and [^{14}C]SF1 as $actin_2$–SF1. Their data are less precise, but counting of solubilized gel slices containing $^3H/^{14}C$ is difficult (Moore, 1980). We repeated the stoichiometry determination with [^{3}H]actin and [^{14}C]SF1 and found a value of 1, in agreement with Sutoh's result. Counting methods based on either gel solubilization with H_2O_2 or total combustion to $^{14}CO_2$ and ^3H_2O gave the same result. The most obvious source of systematic error in our experiment is that an oligomeric actin species may comigrate with the cross-linked species. If this did occur, the effect would not be large as the actin content of the cross-linked species was greater than that contaminating the SF1 position and the actin trimer band seen in control actin cross-linking experiments was much weaker than the dimer. The effect of such contamination would be to raise the apparent ratio of actin:SF1, so our results should be seen as an upper limit.

Mornet et al. (1981a,b) have shown that EDC cross-links actin to two sites in the SF1 heavy chain, namely, the M_r 20 000 and 50 000 fragments produced by mild trypsin hydrolysis (Balint et al., 1978). Sutoh (1983) has shown that chemical cleavage of bands 1a and 1b produces fragments characteristic of the two actin binding sites, respectively. He has pointed out that such fragments could not be produced if two actins were bound to one SF1. On these grounds, it is likely that the principal cross-linked species formed on reaction with EDC, and probably with other cross-linking reagents (Labbé et al., 1982), are actin–SF1 dimers rather than $actin_2$–SF1 trimers. The two forms of the dimer represent alternative modes of actin–SF1 binding as explained above (Sutoh, 1983). These two modes of binding could be to the same actin monomer or to adjacent actin monomers within the actin filament. The absence of a third sub-band of molecular weight similar to that of the dimer which would correspond to an actin monomer cross-linked to both SF1 binding sites and the presence of one of higher molecular weight are prima facie evidence in favor of the latter. Neither our molecular weight determination nor our stoichiometry measurements of band 2 are sufficient to determine whether it is an $actin_2$–SF1 trimer.

Since the principal cross-linked species are actin–SF1 dimers, the obvious hypothesis is that SF1 readily contacts one or the other of two sites on actin in a thin filament but does not easily contact both at the same time.

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Troponin–Tropomyosin Interactions. Fluorescence Studies of the Binding of Troponin, Troponin T, and Chymotryptic Troponin T Fragments to Specifically Labeled Tropomyosin†

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ABSTRACT: We have studied the interaction between troponin and tropomyosin by means of a fluorescent probe, *N*-(1-anilinonaphth-4-yl)maleimide (ANM), attached to the cysteine-190 residues of tropomyosin. The binding of troponin and troponin T to ANM–tropomyosin produces substantial increases in the label fluorescence. Analysis of the binding profiles indicates that both troponin and troponin T bind with a 1:1 stoichiometry. We have obtained and characterized several chymotryptic fragments of troponin T by digestion of isolated troponin T or whole troponin. An N-terminal fragment from troponin T which is slightly less than two-thirds of the whole molecule binds to tropomyosin without affecting the label fluorescence; a C-terminal fragment composed of the rest of the troponin T molecule causes a substantial enhancement of the label fluorescence. We have also isolated a complex containing the C-terminal troponin T fragment together with troponin I and troponin C from whole troponin, which also enhanced the label fluorescence. These observations indicate an elongated region of attachment between troponin T and tropomyosin.

The contraction of vertebrate skeletal muscle is regulated by changes that take place in the thin filament. These changes are initiated by the binding of Ca^{2+} ions to the troponin complex which is located at intervals of approximately 40 nm along the thin filament (Ebashi & Endo, 1968). The mechanism of regulation is believed to involve a change in the position occupied by tropomyosin in the actin long-pitch helical grooves (Haselgrove, 1972; Huxley, 1972; Parry & Squire, 1973; Wakabayashi et al., 1975; Gillis & O'Brien, 1975). The tropomyosin molecule is a coiled coil formed from two α-helical polypeptide chains 284 residues long (Stone & Smillie, 1978; Mak et al., 1980). Knowledge of the interactions between troponin and tropomyosin is necessary to understand how such changes may be brought about.

Troponin is thought to bind to tropomyosin principally through its troponin T (TnT) component (Greaser et al., 1972). There may also be an interaction between troponin I (TnI) and tropomyosin (Drabowska et al., 1976), though the evidence for this is less clear. It has been suggested that troponin attaches to a position on tropomyosin between residues 197 and 217 (McLachlan & Stewart, 1976), which corresponds to a distance of about 3 nm. However, recent evidence, obtained by electron microscopy, indicates that the bound TnT molecule extends a greater distance along the thin filament and hence along the tropomyosin molecule (Ohtsuki, 1979). In this work, Ohtsuki showed that antibodies directed against N-terminal and C-terminal chymotryptic fragments of TnT were located at positions displaced from each other by about 13 nm along the thin filament axis.

We have investigated the nature of the binding of troponin to tropomyosin by attaching a fluorescent probe, *N*-(1-anilinonaphth-4-yl)maleimide (ANM) (Kanaoka et al., 1973), to the cysteine-190 residues of tropomyosin and observing the changes in fluorescence caused by the binding of troponin, TnT, and the chymotryptic TnT fragments. ANM has previously been used as a probe of changes in tropomyosin conformation and interactions in the thin filament (Ohyashiki et al., 1976). Our results show that both troponin and TnT cause substantial increases label fluorescence when they bind and indicate that the stoichiometry of the interaction is 1:1. An N-terminal fragment of TnT binds with very little effect upon the fluorescence, indicating that it is located away from cysteine-190. A C-terminal fragment causes a substantial increase in fluorescence, indicating that it binds near cysteine-190.

Materials and Methods

Preparation of Proteins. All myofibrillar proteins used in this study were prepared from rabbit skeletal muscle. Troponin was prepared by the method of Ebashi et al. (1971) and purified by that of van Eerd & Kawasaki (1973). TnT prepared by the method of Greaser & Gergely (1973) was the generous gift of Dr. P. Leavis and E. Gowell. Actin was prepared by the method of Spudich & Watt (1971). Reduced tropomyosin was prepared as described by Lehrer (1975) with modifications described by Lehrer & Morris (1982).

Digestion of TnT and Purification of Its Fragments. Rabbit skeletal TnT was digested with chymotrypsin by using a method based on that of Ohtsuki (1979). TnT was dissolved in 0.4 M NaCl, 0.02 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and 2 mM $MgCl_2$, pH 7.5 at 5 °C, at a concentration of about 1.5 mg/mL and digested at 0 °C

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