glycoprotein and/or glycolipid were significantly altered compared to that of an oligosaccharide free in solution. Clearly, further explorations of the peculiarities of the thermodynamics of protein–sugar binding at the cell surface are required.

ACKNOWLEDGMENTS

We express our gratitude to A. E. MacKenzie for helpful discussions and for providing his data on concanavalin A binding of glycopeptides and oligosaccharides prior to publication. We also thank B. D. Sykes for his comments on the binding model.

Registry No. N3L α2-3 isomer, 35890-38-1.

REFERENCES

Barker, R. (1971) *Organic Chemistry of Biological Compounds*, Prentice-Hall, Englewood Cliffs, NJ.
Clegg, R. M., Loontiens, F. G., Sharon, N., & Jovin, T. M. (1983) *Biochemistry 22*, 4797–4804.
Das, M. K., Zissis, E., & Glaudemans, C. P. J. (1979) *Carbohydr. Res. 73*, 235–244.
Edelman, G. M., & Wang, J. L. (1978) *J. Biol. Chem. 253*, 3016–3022.
Goldstein, I. J., Hughes, R. C., Monsigny, M., & Sharon, N. (1980) *Nature (London) 285*, 66.
Hardman, K. D., & Ainsworth, C. F. (1973) *Biochemistry 12*, 4442–4448.
Janin, J., & Chothia, C. (1978) *Biochemistry 17*, 2943–2947.
Kabat, E. A., Liao, J., Burzynska, M. H., Wong, T. C., Thorgersen, H., & Lemieux, R. U. (1981) *Mol. Immunol. 18*, 873–881.
Kronis, K. A. (1983) Ph.D. Thesis, University of Toronto, Toronto, Canada.
Kronis, K. A., & Carver, J. P. (1982) *Biochemistry 21*, 3050–3057.
Kronis, K. A., & Carver, J. P. (1985) *Biochemistry* (preceding paper in this issue).
Lacelle, N. (1979) Ph.D. Thesis, University of Toronto, Toronto.
Marchesi, V. T. (1972) *Methods Enzymol. 28*, 354–356.
Monsigny, M., Roche, A.-C., Sene, C., Maget-Dana, R., & Delmotte, F. (1980) *Eur. J. Biochem. 104*, 147–153.
Neurohr, K. J., Young, N. M., Smith, I. C. P., & Mantsch, H. H. (1981) *Biochemistry 20*, 3499–3504.
Neurohr, K. J., Mantsch, H. H., Young, N. M., & Bundle, D. R. (1982) *Biochemistry 21*, 498–503.
Privat, J.-P., Delmotte, F., Mialonier, G., Bouchard, P., & Monsigny, M. (1974) *Eur. J. Biochem. 47*, 5–14.
Rice, R. H., & Etzler, M. E. (1975) *Biochemistry 14*, 4093–4099.
Roberts, D. D., & Goldstein, I. J. (1982) *J. Biol. Chem. 257*, 11274–11277.
Ross, P. D., & Subramanian, S. (1981) *Biochemistry 20*, 3096–3102.
Swift, T. J., & Connick, R. E. (1962) *J. Chem. Phys. 37*, 307–320.
Van Landschoot, A., Loontiens, F. G., Clegg, R. M., Sharon, N., & De Bruyne, C. K. (1977) *Eur. J. Biochem. 79*, 275–283.
Wright, C. S. (1977) *J. Biol. 111*, 439–457.
Wright, C. S. (1980a) *J. Mol. Biol. 139*, 53–60.
Wright, C. S. (1980b) *J. Mol. Biol. 141*, 267–291.

Incorporation of 6-Carboxyfluorescein into Myosin Subfragment 1†

Dominique Mornet and Kathleen Ue*

Cardiovascular Research Institute, University of California, San Francisco, San Francisco, California 94143
Received May 31, 1984

ABSTRACT: We describe for the first time the introduction of a label into the "50K" domain of myosin subfragment 1 (S-1), and we investigate the properties of this fluorescent modification in relation to the ATPase and actin-binding activities, both residing in the myosin head. The labeling consists of a major incorporation of 6-carboxyfluorescein into the "50K" domain of S-1. Using different conditions for tryptic digestion that allowed a fragmentation of the "50K" domain with a loss of 5 kilodaltons (kDa) leading to a final product of 45 kDa, we have shown that the fluorescent dye remains in the 45-kDa final product. By studying cross-linking as a function of time, we have demonstrated that the "50K" domain and the 45-kDa fluorescent peptide are equally cross-linkable to actin. We have also investigated the K^+EDTA-, Ca^{2+}-, Mg^{2+}-, and actin-activated ATPase activities of this modified S-1 and after purification observed no enzymatic changes.

It was suggested early on (Kassab et al., 1981; Sutoh, 1982) that in the formation of the rigor complex there are contacts between actin and the "20K" region of myosin subfragment 1 (S-1)[1] and between actin and the "50K" region of S-1. This circumstance could arise if S-1 could bind simultaneously to two different actins, and indeed some structural reconstructions (Amos et al., 1982) have adopted this interpretation. However, the circumstance could also arise if, as suggested by Sutoh (1983) [see Heaphy & Tregear (1984) and Greene (1984) for recent confirmation], the same (one) actin can bind to the "20K" and "50K" regions. The electrophoretic band corre-

† This work was supported by Grant HL-16683 and a postdoctoral fellowship of the Muscular Dystrophy Association to D.M.

[1] Abbreviations: S-1, chymotryptic subfragment 1 of myosin; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; MES, 2-(*N*-morpholino)ethanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; 1,5-IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; PAGE, polyacrylamide gel electrophoresis; $NaDodSO_4$, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-0840$01.50/0

sponding to this putative "27K"–"50K"–actin–"20K" complex, however, has the unexpected molecular weight of 180K. Moreover, while it is possible to label Cys-374 of actin, and the most reactive thiol of "20K" [Takashi et al., 1975; Duke et al., 1976; see Tong & Elzinga (1983) for numeration], and to show that these fluorescences are imparted to the 180-kDa band, there has been no label with which to do the analogous experiment using the "50K" region. The inability to label the "50K" region of S-1 has also hindered various analogous investigations that have been accomplished with "20K" and "27K"[2] (Duke et al., 1976; Mornet et al., 1980).

In this paper, we describe conditions under which 6-carboxyfluorescein can be used to label S-1 in such a way that only its 95-kDa heavy chain (and not its light chains) is labeled. This modification leaves all the ATPase activities unchanged. Limited trypsinolysis reveals that the label is mainly on the "50K" region of the heavy chain. By cross-linking such a labeled and trypsinolyzed heavy chain to actin, it has been possible to show that the fluorescence of "50K" is imparted to an electrophoretic band corresponding to the union of one actin and one "50K", i.e., to 95 kDa. Previously, we had shown (Mornet et al., 1981a,b) that the fluorescence of "20K" was imparted to an electrophoretic band corresponding to the union of one actin and one "20K", i.e., to 62 kDa. In neither case was fluorescence imparted to a band corresponding to the union of actin and *both* "20K" and "50K". The "superactivated" MgATPase of the cross-linked complex of actin and trypsin-split S-1 was the same as that of actin and S-1. The 45-kDa fragment obtained by further proteolysis of "50K" retained the carboxyfluorescein label, and cross-linking a mixture of 45-kDa and "50K" peptides generated a fluorescent doublet of 95 and 90 kDa. Since the 5-kDa loss is suffered by the C-terminus of "50K"[3] (Labbé et al., 1984), we concluded that the 45-kDa peptide has an intact actin-binding site. We therefore agree with Sutoh (1983) that in the linear sequence sense the two binding sites of S-1 for actin are separated by at least 5 kDa.

In summary, we have demonstrated more clearly than was previously possible that in the linear sequence of S-1 heavy chain there are two stretches, one on "20K" and one on "50K", that bind to actin. Our work was not directed at distinguishing whether these two stretches bind to the same or to different actins; however, if it is the former, then it is probable that the actin monomer binds first to one stretch and then to the other (Chen et al., 1984), because if they could bind simultaneously we should have detected a covalent union of both "20K" and "50K" with actin.

Materials and Methods

Subfragment 1 and F-Actin Preparation. Rabbit skeletal myosin was prepared according to Offer et al. (1973). Subfragment 1 (S-1) was prepared by digestion of myosin filaments with Worthington α-chymotrypsin (Weeds & Taylor, 1975) and purified on Sephacryl S-200. Elution was in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 mM sodium azide. Rabbit skeletal muscle F-actin was prepared as in Eisenberg & Kielley (1974) and labeled with 1,5-IAEDANS as described in Takashi et al. (1976).

Tryptic Fragmentation of S-1. Limited cleavage by trypsin was performed at 25 °C in 0.05 M Tris-HCl buffer (pH 8) using a molar ratio of 1/25 for trypsin/S-1 (Mornet et al., 1981c).

In the presence of nucleotide (5 mM Mg^{2+}ATP), the molar ratio of trypsin/S-1 was increased to 1/5 as described in Hozumi (1983).

Concentrations were estimated by using $A^{1\%,1cm}_{280nm} = 7.5$ (Wagner & Weeds, 1977) for the native S-1 or split S-1 and $A^{1\%,1cm}_{280nm} = 11.0$ (West et al., 1967) for F-actin.

K^+EDTA-, Ca^{2+}-, Mg^{2+}-, or Actin-Activated ATPase Activities. These ATPase activities were measured as described in Mornet et al. (1977) at 25 °C, in 0.05 M Tris buffer at pH 8.

Polyacrylamide Gel Electrophoresis (PAGE). At different digestion times, aliquots were taken and applied to NaDodSO$_4$–PAGE, using slab gel electrophoresis (5–18% gradient acrylamide) in a 50 mM Tris/100 mM boric acid buffer system (Mornet et al., 1981b).

Cross-Linking of the F-Actin-Split S-1 Complex. The zero-length cross-link promoter water-soluble carbodiimide (EDC) was added as a solid, to a final concentration of 15 mM, to various mixtures of split S-1 and actin. A 0.1 M MES buffer (pH 6) was used, and the condensation reactions were followed during the first 20 min at 25 °C. A F-actin/split S-1 molar ratio of 2/1 was always used.

Excited-State Lifetime Measurements. These measurements were obtained on a pulsed single-photon counting instrument, with a nanosecond flashlamp, optical activity, and photomultiplier tube supplied by PRA, Inc. Data collection electronics were essentially as described by Mendelson et al. (1975).

Deconvolution from the finite-width lamp pulse was accomplished by using an iterative reconvolution nonlinear least-square analysis; the computer programs used were a gift from Prof. L. Brand.

Quenching Experiments. All experiments were at 4 °C. For each data point, S-1 at 80 μM was brought to the desired acrylamide concentration by adding a concentrated stock solution. We then measured the steady-state intensity and then the intensity decay curve as described in Torgerson (1984) and in Ando et al. (1982).

Chemicals. ECD was from Sigma, TPCK–trypsin and soybean trypsin inhibitor (STI) were from Worthington, Sephacryl S-200 was from Pharmacia (Uppsala), and 6-carboxyfluorescein was from Eastman (Kodak). All other chemicals were analytical grade.

Results

Subfragment 1 Labeling Conditions. Because, like Yamamoto & Sekine (1983), we found that under certain conditions there is very little EDC cross-linking of light chain 1 and heavy chain (to form a 120-kDa band), we used these conditions for labeling S-1 with carboxyfluorescein: A 100-fold molar excess of 6-carboxyfluorescein in 0.1 M MES buffer (pH 6.0) is added to 50 μM S-1. Labeled S-1 is obtained only after addition of EDC as a powder (1 mg/mL) and exposure for 15 min (without EDC, even after overnight contact, the mixture of S-1 and 6-carboxyfluorescein does not give labeled S-1). We stopped the labeling reaction by adding an excess of β-mercaptoethanol. Free carboxyfluorescein was first removed by specifically precipitating the S-1 with saturated

[2] "20K", "27K", and "50K" refer to the molecular weights of peptides obtained by trypsinolysis of S-1. Recently, Tong & Elzinga (1983), on the basis of sequence analysis, have reported a molecular weight of 23K for the peptide corresponding to "27K". However, this peptide is easily degraded, and at this writing, it is not clear that the sequenced peptide would have had a molecular weight of 27K. For this reason, we continue to use the operational designations "20K", "50K", and "27K".

[3] Labbé et al. studied the N-terminal sequence of the 45-kDa peptide, viz., Met-Gln-Gly-Thr-Leu..., and found that it agreed (for the first 20 residues) with the N-terminal sequence of the 50-kDa peptide previously described by Chaussepied et al. (1983). From this, it follows that the 50-kDa peptide must be degraded from its C-terminus.

ammonium sulfate to 60% and adjusting the pH to 7.0. The resulting pellet was easily resuspended in 50 mM Tris-HCl buffer, pH 8.0, thus attaining an S-1 concentration of 10 mg/mL. Finally, the solution was passed through a Sephadex G-25 column equilibrated in the same buffer. This modified S-1 was dialyzed overnight in the same buffer containing 0.1 mM sodium azide; it was then centrifuged at 15000 rpm for 15 min in a Sorvall centrifuge. Finally, the solution was filtered through a S-200 Sephacryl column. The labeled S-1 (yellow) was concentrated to 10 mg/mL on an XM50 Amicon membrane.

Fluorescence Properties of 6-Carboxyfluorescein Attached to S-1. The absorption spectrum of bound 6-carboxyfluorescein has a maximum at 498 nm, and the emission spectrum has a maximum at 520 nm as expected from fluorescein derivatives in general (Mercola et al., 1972; Babcock & Kramp, 1983). The fluorescence intensity of the 6-carboxyfluorescein attached to S-1 doubles as the pH is increased between pH 6.0 and 9.0; for this reason, we have measured the fluorescence of 6-carboxyfluorescein at pH 8.5 or pH 9.0, as recommended for a different fluorescein derivative of S-1 [IAF on the "20K" domain; see Takashi (1979)]. The amount of dye introduced into S-1 was estimated from the absorption spectrum (Babcock & Kramp, 1983) of the labeled S-1 in 4.8 M guanidinium chloride (pH 9.0); it was $n = 1.5 \pm 0.2$. Measuring the stoichiometry does not tell whether we have labeled one specific residue preferentially or are averaging over several comparably labeled residues. The excited-state lifetimes of the modified S-1 were 4.41 ns for S-1 alone and 4.38 ns when it was complexed with actin; i.e., they are the same within experimental error. We have also prepared a covalent acto–S-1 complex using EDC as the cross-linking agent. After the reaction was stopped with an excess of β-mercaptoethanol, the lifetime measurement of this very viscous solution was also approximately the same, 4.46 ns.

Using acrylamide concentrations ranging from 0.1 to 0.5 M, we studied the quenching of the fluorescence of a 6-carboxyfluorescein-modified S-1 in comparison with the quenching of the fluorescence of a 6-carboxyfluorescein-modified S-1–F-actin complex. Under our conditions (50 mM Tris buffer, pH 7.5), a difference of only 10% in quenching was found.

ATPase Activities of 6-Carboxyfluorescein-Modified S-1. During the modification and after concentration in the Diaflow apparatus, we monitored the various ATP activities of the 6-carboxyfluorescein fluorescent S-1, using as a standard a native S-1 which had been exposed to the same EDC concentration and the same purification procedure. We conclude that this modification does not affect the K^+EDTA, Ca^{2+}, or Mg^{2+} ATPase activities.

Tryptic Cleavage of 6-Carboxyfluorescein-Modified S-1. We first studied the limited cleavage of labeled S-1 by trypsin (Figure 1A). The fluorescence which was originally only in the 95-kDa heavy chain of the S-1 (no fluorescence was detectable in the alkali light chain) progressively appears in a 75-kDa fragment and finally mainly in the "50K" domain. Fluorescence at the regular position of the "20K" domain, however, suggests that the dye is also slightly incorporated into the "20K" domain. After Coomassie blue staining (Figure 1B), the time course of the trypsinolysis of labeled S-1 shows progressive conversion of the 95-kDa heavy chain into three main domains, in the same two-step process observed with native S-1 (Mornet et al., 1984). The positions of the regular "50K", "27K", and "20K" domains are given in the gel.

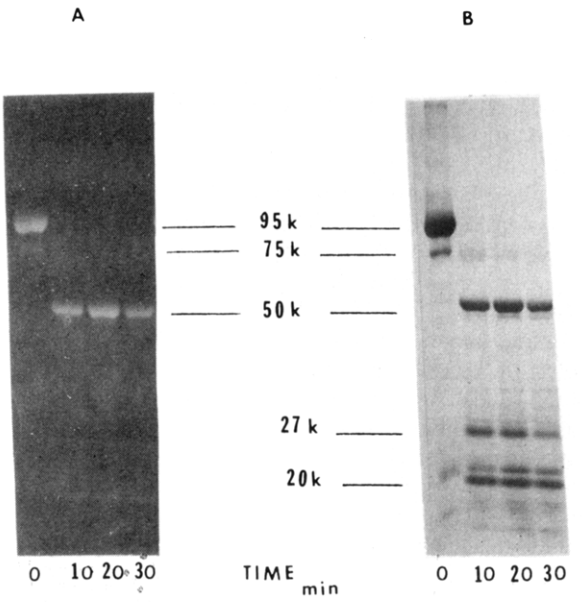

FIGURE 1: Correlation between trypsin cleavage of fluorescein-labeled S-1 and identification of the fragments: (A) gel viewed under ultraviolet light; (B) gel stained with Coomassie blue.

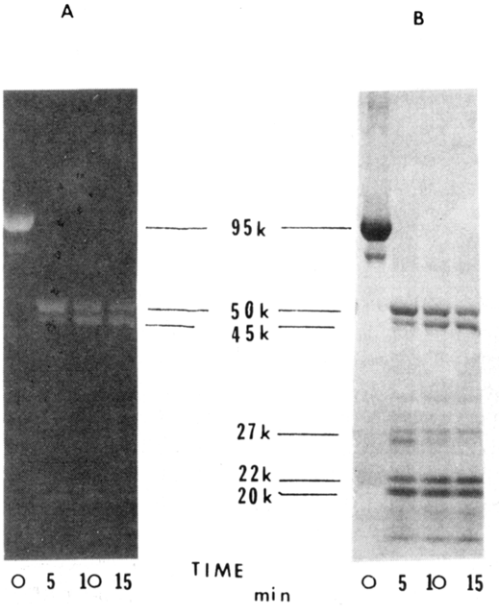

FIGURE 2: Time course of Mg^{2+}ADP-modulated tryptic cleavage of 6-carboxyfluorescein-labeled S-1: (A) gel viewed under UV light; (B) gel stained with Coomassie blue.

Comparison of panels A and B of Figure 1 shows that even though there is a slight fluorescence in the "27K" and "20K" domains, the bulk of the dye is introduced into the "50K" domain.

In a second experiment, we submitted the labeled S-1 to trypsin cleavage, using a trypsin/S-1 molar ratio of 1/5 in the presence of 5 mM Mg^{2+}ATP. In this case, there is the possibility of making two additional cleavages on the heavy chain: the conversion of the "27K" domain into a 22-kDa fragment and the degradation of the "50K" domain into a 45-kDa fragment (Hozumi, 1983); the latter was our principal interest. 6-Carboxyfluorescein-labeled S-1 undergoes the same MgATP-induced conformational change as native S-1. This change opens up a small stretch of polypeptide chain to trypsin. The resulting 50-kDa–45-kDa degradation can now be followed by fluorescence (Figure 2A) as well as Coomassie blue staining (Figure 2B).

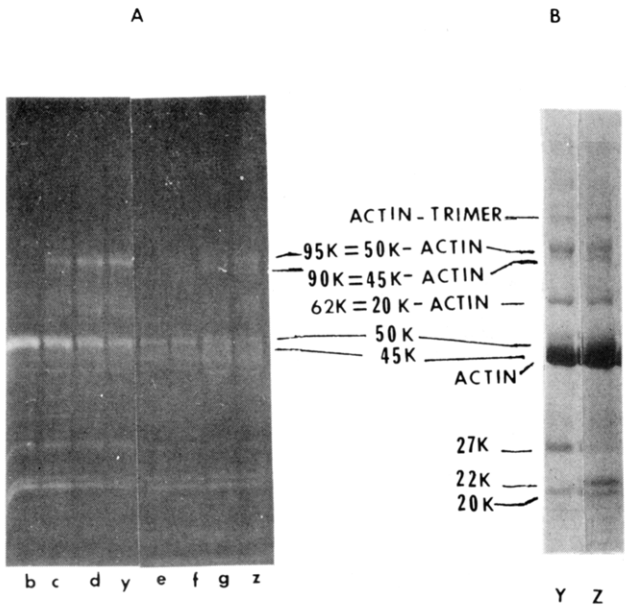

FIGURE 3: Time course of the cross-linked process between actin and different 6-carboxyfluorescein-split S-1's: (A) gel viewed under UV light; (B) gel stained with Coomassie blue. The different wells correspond to the following: (b) acto-split S-1 complex in which the "50K" domain is fluorescent (c, d, and y or Y) as a result of the EDC cross-linking process in the presence of F-actin after 5, 10, and 15 min, respectively; (e) acto-split S-1 complex in which the "50K" domain and its 45-kDa fragment are fluorescent; (f, g, and z or Z) result of the EDC cross-linking process in the presence of F-actin after 5, 10, and 15 min, respectively.

As a control for actin binding, we tested the protective effect of bound F-actin on the 6-carboxyfluorescein-modified S-1–F-actin complex during limited tryptic cleavage with a molar trypsin/S-1 ratio of 1/20. Good protection of the 50-kDa–20-kDa junction (not shown) was obtained. No "50K" fluorescent domain appears during the first 15 min of the cleavage, and we can easily follow the conversion of the 95-kDa fluorescent heavy chain into a stable 70-kDa fluorescent fragment as already known when native S-1 is used (Mornet et al., 1979).

Cross-Linking Experiments. As before (Kassab et al., 1981; Sutoh, 1982), we used the water-soluble carbodiimide EDC to obtain specific cross-links in the presence of F-actin. In the case of the 6-carboxyfluorescein-labeled S-1, a new fluorescent product of about 180 kDa appears (not shown) as the doublet shown previously with native S-1 (Mornet et al., 1981b). According to Sutoh (1983), this doublet can be explained as a covalent complex [of one actin (43 kDa) and one S-1 (95 kDa)] that migrates anomalously (this complex would have a molecular weight of 138 K on the basis of the molecular weight addition of the two constituents). The doublet was thought to arise from a cross-link of the actin with the "20K" domain (175-kDa product) or with the "50K" domain (185-kDa product) of S-1 (Sutoh, 1983).

More specifically, we have studied the cross-linking process occurring between F-actin and the two kinds of split S-1: a "27K"–"50K"–"20K" split S-1 with its "50K" domain made fluorescent and a "22K"–"50K", "45K"–"20K" split S-1 with its "50K" domain and also its 45-kDa fragmented piece made fluorescent. As shown in Figure 3A (first four wells), when the fluorescent 50-kDa product is present there mainly appears a new fluorescent 95-kDa product, and (last four wells) when the fluorescent mixture of 50 and 45 kDa is present, there appear *two* new fluorescent 95- and 90-kDa bands. The slightly fluorescent "20K" domain cross-linked to actin also appears as a fluorescent 62-kDa product in both samples. In Figure 3B, only the resulting cross-linked products are stained with Coomassie blue, so we can easily follow the parallel formation of the condensates: 95 and 62 kDa, or the 95/90-kDa doublet and 62 kDa. In a different set of experiments, we used exactly the same conditions, but the two kinds of split S-1 were unmodified, and the fluorescence originated in the F-actin [modification of Cys-374 by 1,5-IAEDANS as described in Takashi (1979)]. Then, as shown in Figure 4, we could identify the same new products in the Coomassie blue pattern, enabling the following conclusions: (i) The 95-kDa fluorescent product contained fluorescent actin and therefore was a covalently linked "50K" domain and one actin monomer. (ii) The 90-kDa fluorescent product also contained actin and therefore was a covalently linked 45-kDa fragment and one actin monomer. (iii) The fluorescent 62-kDa product was as already described by Kassab et al. (1981) and by Sutoh (1982), a covalently linked "20K" domain and one actin monomer.

Although the fluorescence was clearly incorporated into the 50- and 45-kDa peptides, it was difficult to show the expected (on the basis of Coomassie blue staining) transfer for fluorescence into the cross-linked complexes. The fluorescence intensity of these new complexes was very low after cross-linking. We found that when EDC is added to a mixture of actin and 6-carboxyfluorescein-modified S-1, the fluorescence emission spectrum is reduced by 50%. Also, when EDC is added progressively to a solution of 6-carboxyfluorescein, in the absence of protein, there is a progressive loss of emission and a precipitation. Possibly EDC directly attacks the fluorescein rings, thus quenching emission and promoting polymerization; however, there may be other equally plausible explanations of this phenomenon.

Relationship between Actin Cross-Linking and Mg^{2+}-ATPase Activity. In these experiments, we have correlated the Mg^{2+}ATPase activity of covalent acto–S-1 complexes with the cross-linking process. The ability of S-1 covalently complexed to actin to hydrolyze ATP at a rate as high as that which would occur in the presence of an infinite actin concentration has already been described (Mornet et al., 1981b; Stein et al., 1983). Also, severing the 50-kDa–20-kDa junction was shown to produce a loss of actin activation at constant actin concentration (Mornet et al., 1979) because of a loss of actin affinity for split S-1 (Botts et al., 1982). Figure 5 shows that "split S-1" as well as S-1 attains the same stimulated ATPase activity.

Discussion

This work introduces the idea of "cross-linking" carboxyfluorescein to S-1 in much the same way as actin has been cross-linked to S-1, viz., by using EDC (Mornet et al., 1981a). Because activated EDC is targeted to amino groups, we had hoped that the labeling sites would be either of the following: First, there was the possibility of obtaining a label on the S-1 molecule at the actin-binding site, and probably inhibiting the recognition of actin by S-1. However, from the continuing actin protection against the trypsin cut at the 50-kDa–20-kDa junction, from the continuing possibility of covalently cross-linking S-1 or split S-1 with actin, and from the unchanged lifetime measurements, we conclude that there is no label in or near the actin-binding site. Second, there was the possibility of labeling various other lysine targets, viz., the 27-kDa–50-kDa and the 50-kDa–20-kDa junctions, or lysine-83 (Tong & Elzinga, 1983) which is on the "27K" and whose modification affects ATPase. All these targets are known to be easily accessible. However, from the unchanged pattern and kinetics of the trypsinolyis of modified S-1, no label seems to be in-

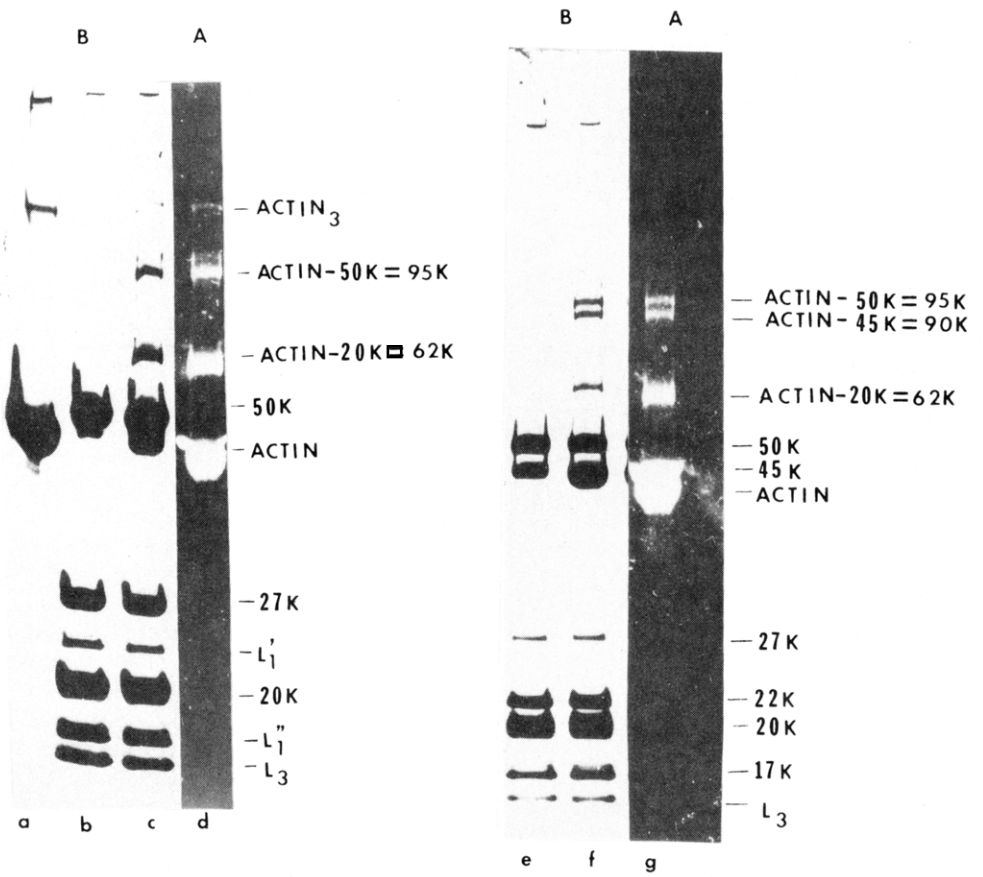

FIGURE 4: Time course of the cross-linked process between 1,5-IAEDANS-labeled F-actin and different native split S-1's: (A) gel viewed under UV light; (B) gel stained with Coomassie blue. The different wells correspond to the following: (a) actin after 15 min of EDC cross-linking; (b) native split S-1 ("27K"–"50K"–"20K") mixed with F-actin. (c or d) Split S-1 ("27K"–"50K"–"20K")–actin complex after 15 min of EDC cross-link. In (d), the fluorescent bands are actin, 20K–actin, and 50K–actin. (e) Split S-1 ("22K"–"50K", "45K"–"20K") mixed with F-actin. (f and g) Split S-1 ("22K"–"50K", "45K"–"20K")–actin complex after 15 min of EDC cross-link. In (g), the fluorescent bands are actin, 20K–actin, 50K–actin, and 45K–actin. The fluorescent gel has been obtained by applying 60 μg of the protein instead of 30 μg as in the Coomassie blue gels.

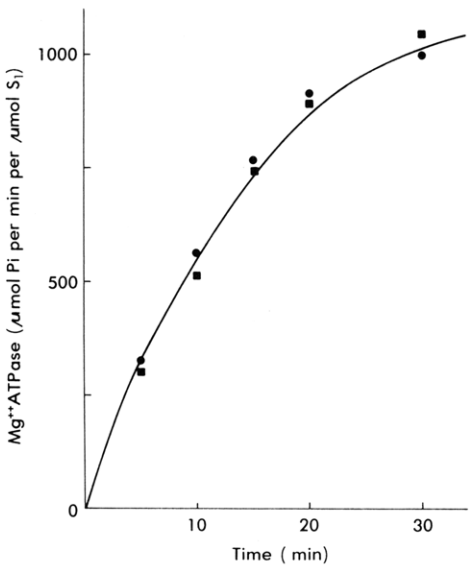

FIGURE 5: Comparative superactivation of the Mg^{2+}ATPase of native S-1 and split S-1 during covalent binding to the F-actin filament. (■) Split S-1 activities; (●) native S-1 activities in the presence of 1.5 mM Mg^{2+}ATP. Other conditions are described under Materials and Methods.

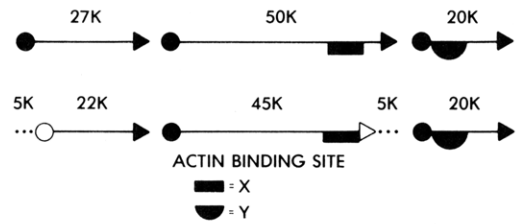

FIGURE 6: Scheme of the linear sequence of a three-domain heavy chain of the S-1 molecule: (●) and (▶) represent the N-terminal and C-terminal ends of each native segment. (○) and (▷) represent the N-terminal and C-terminal ends of new segments obtained by additional trypsin cleavage. (■) and (◡) represent the location of the two different actin-binding sites in the S-1 molecule. The letters X and Y do not imply an order of binding but only suggest two distinct actin-binding sites. There is a possibility that a tertiary loop could bring these two sites together to form a single binding site, as postulated by Sutoh (1983). If this "loop" conformation is in rapid equilibrium with a "straight" conformation, it is in principle possible either that a single actin could bind (to the loop) or that two actins could bind (to the straight conformation).

corporated in the "connector regions". Because ATPase activities did not change on labeling, and because "27K" was not made fluorescent, we concluded that Lys-83 also was unlabeled by activated carboxyfluorescein.

The same tests that excluded certain potential labeling sites also demonstrated that a "50K" fluorescent domain can be covalently cross-linked to the actin filament. The 45-kDa fragment, which also cross-links to actin, is formed after a loss of 5 kDa from the C-terminal end of the "50K" domain.

Several studies, including this one, show that S-1 touches actin via both its 20- and its 50-kDa domains. However, measurements of stoichiometry indicate that there is only one actin cross-linked per S-1. These observations suggest that the heavy chain may loop in such a way that parts of the 20-

and 50-kDa domains (distant at least 5 kDa from each other) come together around the same actin (Figure 6). Yet 20 and 50 kDa have somewhat independent characters. For example, as just shown, EDC reveals both contacts, but dimethyl suberimidate reveals only the latter (Yamamoto & Sekine, 1979; Labbé et al., 1982). More importantly, we have shown here with EDC that while both 20-kDa–actin and 50-kDa–actin unions are demonstrable, 20 kDa–actin–50 kDa is not. This would be expected if the normal reaction pathway called for making first one contact and then the other [cf. Chen et al. (1984)] and if cross-linking did not alter the steady-state concentrations of the two contacts (it must be remembered that EDC complexes have approximately the same ATPase as is obtained in the normal case using infinite actin concentration). Being able to place a relatively selective label on the "50K" domain is very important because it facilitates tracking the reactions of the "50K". Previously, it has only been surmised (Yamamoto & Sekine, 1979; Mornet et al., 1981ab; Labbé et al., 1982) that "50K" can bind to actin on the basis of finding a cross-linking product of 95 kDa that is fluorescent if labeled actin has been used; it has been assumed that this product is composed of 50 + 43 kDa. In experiments with various cross-linking agents, however, an actin dimer manifests itself as weighing from 85 to 103 kDa (Knight & Offer, 1978; Mockrin & Korn, 1981; Oharra et al., 1982; Fowler et al., 1984; Lehrer, 1981), thereby imperiling the foregoing interpretation of the 95-kDa fragment. the uncertainty of interpretation is totally removed in the present work, which shows that 95 kDa can be fluorescent when either fluorescent actin or fluorescent "50K" has been used.

Until now, the "27K" N-terminal domain was seen as a nucleotide-binding domain (Szilagy et al., 1979; Walker et al., 1982; Okamoto & Yount, 1983) having some interactions with the light chain (Labbé et al., 1981), and the "20K" C-terminal domain was seen as an actin-binding domain (Mornet et al., 1981a; Sutoh, 1981) also having some interactions with the light chain (Labbé, 1982; Burke et al., 1983). Recently it was reported that points on the "50K" domain are within 6–7 Å of the ATP-binding site (Mahmood et al., 1984). This suggests that the "50K" domain may be both a nucleotide-binding domain and an actin-binding domain. This domain also bears an essential carboxyl group (Korner et al., 1983). We anticipate also that our placement of a fluorescent dye on this domain will be helpful in the purification and renaturation of this "50K" domain (Muhlrad & Morales, 1984).

Another reaction has been followed with the help of this new label. Using subtilisin cleavage, we have been able to obtain among the degraded peptides of 50 kDa mainly one or two strongly fluorescent fragments of 8–10 kDa. This result indicates that our labeling is probably restricted to a rather small area of the "50K" domain. However, it is left to a future investigation to define the nature of the modified amino acid residue and to locate that residue in the primary sequence.

ACKNOWLEDGMENTS

We express our appreciation to Manuel F. Morales and Andras Muhlrad for invaluable discussions and suggestions during the performance of this work. We also acknowledge the assistance of Dr. Peter Torgerson, who measured for us the excited-state lifetime of the attached carboxyfluorescein.

Registry No. ATPase, 9000-83-3; 6-carboxyfluorescein, 3301-79-9.

REFERENCES

Amos, L. A., Huxley, H. E., Holmes, K. C., Goody, R. S., & Taylor, K. A. (1982) *Nature (London) 299*, 467–469.

Ando, T., Duke, J., Tonomura, Y., & Morales, M. F. (1982) *Biochem. Biophys. Res. Commun. 109*, 1–6.

Babcock, D. F., & Kramp, D. C. (1983) *J. Biol. Chem. 258*, 6389.

Botts, J., Muhlrad, A., Takashi, R., & Morales, M. F. (1982) *Biochemistry 21*, 6903–6905.

Burke, M., Sivaramakrishnam, M., & Kamalakamnan, V. (1983) *Biochemistry 22*, 3046–3053.

Chaussepied, P., Bertrand, R., Audemard, E., Pantel, P., Derancourt, P., & Kassab, R. (1983) *FEBS Lett. 161*, 84–88.

Chen, T., Applegate, D., & Reisler, E. (1984) *Biophys. J. 45*, 222a.

Duke, J., Takashi, R., Ue, K., & Morales, M. F. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 302–306.

Eisenberg, E., & Kielley, W. W. (1974) *J. Biol. Chem. 249*, 4742–4748.

Fowler, W. E., Buhle, E. L., & Aebi, V. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1669–1673.

Greene, L. E. (1984) *Biophys. J. 45*, 222a.

Hozumi, T. (1983) *Biochemistry 22*, 799–801.

Kassab, R., Mornet, D., Pantel, P., Bertrand, R., & Audemard, E. (1981) *Biochimie 63*, 273–289.

Knight, P., & Offer, G. (1978) *Biochem. J. 175*, 1023–1032.

Korner, M., Vanthiem, N., Cardinaud, R., & Lacombe, G. (1983) *Biochemistry 22*, 5843–5847.

Labbe, J. P., Mornet, D., Vandest, P., & Kassab, R. (1981) *Biochem. Biophys. Res. Commun. 102*, 466–475.

Labbe, J. P., Mornet, D., Roseau, G., & Kassab, R. (1982) *Biochemistry 21*, 6897–6902.

Labbe, J. P., Bertrand, R., Audemard, E., & Kassab, R. (1984) *Eur. J. Biochem. 143*, 315–322.

Lehrer, S. S. (1981) *J. Cell Biol. 90*, 459–466.

Mahmood, R., Nakemaye, K., & Yount, R. G. (1984) *Biophys. J. 45*, 283a.

Mendelson, R. A., Putman, S., & Morales, M. F. (1975) *J. Supramol. Struct. 3*, 162–168.

Mercola, D. A., Morris, J. W. S., & Aeguila, E. R. (1972) *Biochemistry 11*, 3860–3865.

Mockrin, S. C., & Korn, E. D. (1981) *J. Biol. Chem. 256*, 8228–8233.

Mornet, D., Der Terrosian, E., Pradel, L. A., Kassab, R., & Barnan, T. E. (1977) *FEBS Lett. 84*, 362–366.

Mornet, D., Pantel, P., Audemard, E., & Kassab, R. (1979) *Biochem. Biophys. Res. Commun. 89*, 925–932.

Mornet, D., Pantel, P., Audemard, E., & Kassab, R. (1980) *FEBS Lett. 117*, 183–188.

Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981a) *Biochemistry 20*, 2110–2120.

Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981b) *Nature (London) 292*, 301–306.

Mornet, D., Ue, K., & Morales, M. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 736–739.

Muhlrad, A., & Morales, M. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1003–1007.

Offer, G., Moos, C., & Starr, R. (1973) *J. Mol. Biol. 71*, 653–676.

Oharra, O., Takahashi, S., Ooi, T., & Fujiyoshi, Y. (1982) *J. Biochem. (Tokyo) 91*, 1999–2012.

Okamoto, Y., & Yount, R. G. (1983) *Biophys. J. 41*, 298a.

Stein, L. A., Greene, L. E., Chock, P. B., & Eisenberg, E. (1983) *Biophys. J. 41*, 310a.

Sutoh, K. (1981) *Biochemistry 20*, 4800–4804.

Sutoh, K. (1982) *Biochemistry 21*, 3654–3661.

Sutoh, K. (1983) *Biochemistry 22*, 1579–1585.

Szilagyi, L., Balint, M., Sreter, F. A., Gergely, J. (1979) *Biochem. Biophys. Res. Commun. 87*, 936-945.
Takashi, R. (1979) *Biochemistry 18*, 5164-5169.
Takashi, R., Duke, J., Ue, K., & Morales, M. F. (1976) *Arch. Biochem. Biophys. 175*, 279-283.
Tong, S. W., & Elzinga, M. (1983) *J. Biol. Chem. 258*, 13100-13110.
Torgerson, P. (1984) *Biochemistry 23*, 3002-3007.
Wagner, P. D., & Weeds, A. G. (1977) *J. Mol. Biol. 109*, 455-473.
Walker, J. E., Saraste, M., Runswick, M., & Gay, N. J. (1982) *EMBO J. 1*, 945-951.
Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54-56.
West, J. J., Nagy, B., & Gergely, J. (1967) *J. Biol. Chem. 242*, 1140-1145.
Yamamoto, K., & Sekine, T. (1979) *J. Biochem. (Tokyo) 86*, 1863-1868.
Yamamoto, K., & Sekine, T. (1983) *J. Biochem. (Tokyo) 94*, 2075-2078.

Effect of Tryptic Cleavage on the Stability of Myosin Subfragment 1. Isolation and Properties of the Severed Heavy-Chain Subunit†

Morris Burke* and Vedhachalam Kamalakannan‡

Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106
Received May 8, 1984

ABSTRACT: The procedure of thermal ion-exchange chromatography has been used to examine the effect of prior tryptic cleavage on the stability of myosin subfragment 1 (SF1). Although it is found that digestion does destabilize the subunit interactions at physiological temperatures, the heavy-chain subunit can be isolated either as an equimolar complex comprised of 50K, 27K, and 21K fragments or as one comprised of 50K, 27K, and 18K peptides. Thus, the interactions within the heavy chain are considerably more stable than those between the two subunits. Both forms of the free severed heavy chain exhibit ATPase properties similar to those of the parent tryptic SF1. The V_{max} for the actin-activated MgATPase of the free severed heavy chain is the same as that for both undigested and tryptic SF1(A2). Since its K_m for actin is similar to that of tryptic SF1(A2), it may be concluded that changes in the affinity of SF1 for actin induced by trypsin [Botts, J., Muhlrad, A., Takashi, R., & Morales, M. F. (1982) *Biochemistry 21*, 6903-6905] are not dependent on the presence of the associated alkali light chain. Furthermore, the communication between the SH1 site and the ATPase site is also shown to be independent of the associated alkali light chain, and it persists despite the cleavages present in the free heavy chain. Studies on the ability of these severed heavy chains to reassociate with free A1 and A2 chains indicate that the binding site is retained in the 21K-severed heavy chain but is lost in the 18K form.

The subfragment 1 (SF1)[1] cross-bridge region of the myosin molecule can be isolated as a stable complex comprised of a 95-kdalton polypeptide, representing the amino-terminal half of a myosin heavy chain, together with a single alkali light chain which exists in two chemically related forms called A1 and A2 (Yagi & Otani, 1974; Weeds & Taylor, 1975). Both the 95K chain and its associated light chain are folded into a very compact pear-shaped head which is approximately 190 Å long and about 40 Å at its widest point (Elliott & Offer, 1978). Within this head reside two distinct sites—an ATPase site and an actin binding site—whose coupling appears to be essential for contraction. The manner in which these sites communicate with one another remains obscure in the absence of detailed knowledge of the folded structure of the head, but in recent years, significant gains in characterizing the topology of SF1 have been made possible by the application of limited proteolysis together with photoaffinity labeling and with limited cross-linking approaches.

Limited tryptic digestion of SF1 is confined primarily within two small stretches of between 1 and 2 kilodaltons located at about 75K (T1) and 27K (T2) from the amino-terminal end of the heavy chain, and it gives rise to a stable tryptic SF1 complex comprised of the light chain and a severed heavy chain consisting of three protease-insensitive peptides of 27K, 50K, and 21K arranged in this order in the linear sequence (Balint et al., 1975). Although the ATPase site has not been unequivocally characterized, it appears from photoaffinity labeling that regions of the 27K (Szilagyi et al., 1979; Okamoto & Yount, 1983) and 50K (Mahmood et al., 1984) segments are involved here. The actin binding sites in the rigor acto-SF1 complex have been characterized by chemical cross-linking and are located at the 21K and 50K segments, each of which can bind to actin (Mornet et al., 1981a; Yamamoto & Sekine, 1979; Sutoh, 1982, 1983).

Functionally, cleavages at the T1 and T2 sites do not have a significant effect on the ATPase properties of SF1 (Mornet

†Supported by a grant from the National Institutes of Health (NS-15319). Preliminary accounts of some aspects of this work have been presented at the Biophysical Society Meeting, San Diego, CA, 1983.
‡Present address: Department of Biological Sciences, Rutgers, State University of New Jersey, Busch Campus, Pistcataway, NJ 08854.

[1] Abbreviations: DEAE, diethylaminoethyl; SF1, SF1(A1), and SF1(A2), myosin subfragment 1 and the A1- and A2-containing isoenzymes, respectively; A1 and A2, two alkali light chains; T1 and T2, two regions located at about 75 and 27 kilodaltons, respectively, from the amino terminus of the heavy chain and which are vulnerable to tryptic attack; HC, heavy chain of SF1; 18K-severed heavy chain and 21K-severed heavy chain, 27K, 50K, and 18K and 27K, 50K, and 21K forms of the fragmented heavy chain, respectively; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

0006-2960/85/0424-0846$01.50/0