Production and Properties of Skeletal Myosin Subfragment 1 Selectively Labeled with Fluorescein at Lysine-553 Proximal to the Strong Actin-Binding Site†

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ABSTRACT: We describe, for the first time, the reaction of skeletal myosin subfragment 1 (S-1) with the succinimido ester of 6-[fluorescein-5(and 6)-carboxamido]hexanoic acid (FHS), which takes place at pH 7.0, 20 °C, within a 15 min period, in the presence of 1.5−1.8-fold molar excess of reagent over protein. As a result, 0.9−1.0 mol of fluorescyl group/mol of S-1 was covalently incorporated exclusively into the 95 kDa heavy chain as monitored by spectroscopic measurements. The central 50 kDa segment included the main site of fluorescence attachment as assessed by gel electrophoresis. The extent of S-1−FHS conjugation is strongly sensitive to F-actin binding but not to the interaction of nucleotides. The formation of the rigor F-actin−S-1 complex decreased the level of S-1 labeling to 20% without any competition between actin and S-1 for FHS binding. The derivatization of S-1 did not alter the K^+-ATPase activity, but it enhanced the Ca^{2+}-ATPase and Mg^{2+}-ATPase to 150% and 225%, respectively, whereas it lowered the actin-activated ATPase to only 75% of the original activity. A double-reciprocal plot of the ATPase rate against actin concentration indicated a 2-fold decrease of the V_{max} value for modified S-1, while the K_m for actin was unchanged. Cosedimentation experiments did not reveal disruption of the rigor acto−S-1 interaction by the bound fluorophore. The labeled S-1 heavy chain was isolated, and its total tryptic digest was fractionated by reverse-phase HPLC. Only two fluorescent peptides, designated P-1 and P-2, containing 15% and 85%, respectively, of the initial fluorescence were found, and after purification they were entirely sequenced. The major P-2 peptide spanned the heavy chain sequence Ala-545−Lys-561 with Lys-553 identified as the FHS-hyperreactive residue; the sequence of the minor P-1 peptide corresponded to Gly-638−Lys-641 with Lys-640 being linked to FHS. The location of Lys-553 in the S-1 primary structure is of particular interest as it is relevant to the primary stereospecific and hydrophobic actin-binding site thought to involve the helix(Gly-516−Phe-542)−loop(Pro-543−Thr-546)−helix(Asp-547−His-558) motif residing in the lower subdomain of the 50 kDa region. Lys-553 is positioned at the end of the latter helix, and the fluorescyl group bound to it may represent a valuable landmark to probe the functioning and orientational properties of this strategic S-1 area during the acto−S-1-ATP interactions.

The cyclic interaction of the globular head of myosin or S-1[1] with actin filaments and ATP is essential for energy transduction during muscle contraction. The determination of the tertiary structure of S-1 has indicated that this protein motor is consisting of a catalytic domain and a light chain interactive domain (Rayment *et al.*, 1993a). The catalytic domain binds and hydrolyzes ATP within an active site ATPase pocket and interacts with F-actin at an opposite cleft which harbors primary actin-binding sites (Rayment *et al.*, 1993b). This actin-binding cleft subdivides the central 50 kDa heavy chain segment into lower and upper subdomains which are thought to undergo ATP-dependent movements promoting the opening and closing of the cleft. These movements would be, therefore, critical for the association−dissociation mechanism of the acto−S-1 complex during the power stroke, and their experimental detection and characterization would represent an important step in assessing the molecular aspects of the F-actin−S-1 interactions. The use of extrinsic probes for the spectroscopic analysis of these rearrangements would represent a powerful experimental approach. However, it requires the selective introduction of the reporter group into a well-defined locus residing at or near the primary actin-binding sites without altering the essential biological activities of the protein. To date, most of the known S-1 labels, whose sites of attachment in the primary structure have been established, are covalently bound to the side chain of cysteine-697 (SH2 thiol), cysteine-707 (SH1 thiol), or cysteines of the alkali light chains. All the derivatized residues are not structurally related to the primary actin-binding sites within the proposed model of the acto−S-1 complex (Rayment *et al.*, 1993b).

As a first part of the investigation aimed at probing, by fluorescence spectroscopy, the dynamics of the lower subdomain of the 50 kDa region containing the strong, hydrophobic, and stereospecific actin-binding site, we describe, in the present work, the selective, fluorescent labeling of this

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[1] Abbreviations: S-1, myosin subfragment 1; acto−S-1, actomyosin subfragment 1; FHS, 6-[fluorescein-5(and 6)-carboxamido]hexanoic acid succinimidyl ester; MOPS, 3-(*N*-morpholino)propanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; ATPase, adenosine-5′-triphosphatase; FH-S-1, 6-[fluorescein-5(and 6)-carboxamido]hexanoyl S-1.

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subdomain with 6-[fluorescein-5(and 6)-carboxamido]hexanoic acid succinimidyl ester. The major part of the S-1-incorporated fluorophore was covalently attached to the side chain of lysine-553, an amino acid located at the carboxyl-terminal edge of the helix−loop−helix motif of residues 516−558 thought to contribute to the tight, hydrophobic interaction of the S-1 heavy chain with actin subdomain-1 (Rayment *et al.*, 1993b). The characterized structural and enzymatic properties of the novel modified S-1 preparation make it a potentially useful tool for analyzing by energy transfer studies the orientational states of this functionally pivotal region during the F-actin−S-1 interactions in solution and in muscle fibers.

MATERIALS AND METHODS

Chemicals. 6-[Fluorescein-5(and 6)carboxamido]hexanoic acid succinimidyl ester (FHS) was purchased from Molecular Probes (Eugene City, OR). TPCK-treated trypsin and endoproteinase Glu-C were obtained from Worthington and ICN Biochemicals, respecitvely. PD 10 columns were from Pharmacia (Uppsala).

Proteins. Rabbit skeletal myosin was prepared as described by Offer *et al.* (1973). Chymotryptic S-1 was obtained according to Weeds and Taylor (1975) and was further purified over Sephacryl S-200 (Chaussepied *et al.*, 1986). Rabbit skeletal F-actin was prepared by the procedure of Eisenberg and Kielley (1974).

Protein concentrations were determined spectrophotometrically at 280 nm using the following extinction coefficients: S-1, $E^{1\%} = 7.50$ cm^{-1}; actin, $E^{1\%} = 11.0$ cm^{-1}; myosin, $E^{1\%} = 5.5$. For the labeled FH-S-1, the protein concentration was obtained after deduction of the absorption contribution at 280 nm from the probe using its molar absorbance at this wavelength that we determined as 2.04×10^4 M^{-1} cm^{-1}.

Labeling of Myosin-S-1 and Acto−S-1 with FHS. S-1 (50−75 μM) in 50 mM MOPS (pH 7.0) was supplemented with 1.5-fold molar excess of FHS dissolved immediatly before use in dimethylformamide (15 mM). The reaction mixture was incubated for 15 min at 20 °C in the dark. A glycine ethyl ester solution was then added to a final concentration of 10 mM to quench the reaction. After further incubation for 15 min at 20 °C, the labeled protein was isolated by gel filtration over a PD 10 column equilibrated in 20 mM MOPS (pH 7.5).

S-1 was labeled with FHS in the presence of F-actin as follows: the protein (50 μM) in 50 mM MOPS, 5 mM KCl, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ (pH 7.0) was mixed with varying concentrations of F-actin (0−200 μM). The reaction was initiated by the addition of FHS at a 1.5-fold molar excess of reagent to S-1. After 15 min at 20 °C, glycine ethyl ester was added to a final concentration of 10 mM. Following an additional incubation for 20 min at 20 °C, the reaction mixtures were supplemented with F-actin to bring their final actin concentration to the same value of 200 μM. After centrifugation at 180000*g* for 60 min at 4 °C, to pellet the acto−S-1 complex, the amount of unreacted FHS present in each supernatant was estimated spectrophotometrically at 495 nm.

Fluorescence Measurements and Estimation of FHS Labeling. Fluorescence emission spectra were recorded at 20 °C using a Kontron SFM 25 spectrofluorimeter (excitation, 495 nm). For each protein preparation, the amount of FHS covalently bound was determined at pH 7.5−8.0 by measuring the absorption of the fluorescein moiety and determining the concentration assuming an extinction coefficient of 6.8×10^4 M^{-1} cm^{-1} at 495 nm, as recommended by the manufacturer's instructions. Control measurements in the absence and presence of 0.1% $NaDodSO_4$ did not show any effect of the protein on the spectroscopic characteristics of the attached fluorophore as compared with those observed when it was free in solution. On the other hand, the indicated pH range used for absorbtion analyses was found prerequisite for accurate determinations of the labeling stoichiometry; in particular, acidic pH values strongly decreased the absorption of FHS.

ATPase Assays. The Ca^{2+}-ATPase activities were measured at 25 °C in the presence of 2.5 mM ATP, 250 mM KCl, 5 mM $CaCl_2$, and 50 mM Tris-HCl (pH 7.7). The K^+-EDTA ATPase activities were determined at 25 °C in 2.5 mM ATP, 1 M KCl, 5 mM EDTA, and 50 mM Tris-HCl (pH 7.5). The Mg^{2+}-ATPase and actin-activated ATPase activities were assayed in 5 mM ATP, 2.5 mM $MgCl_2$, 10 mM KCl, and 50 mM Tris-HCl (pH 8.0) in the absence and presence of 1 mg of F-actin, respectively. P_i liberated was determined colorimetrically as previously reported (Mornet *et al.*, 1979).

Binding Measurements. Rigor binding of FHS-labeled S-1 to F-actin was analyzed by sedimentation at 20 °C in a Beckman airfuge. FH-S-1 (3.3 μM) in 50 mM Tris-HCl, 10 mM KCl, and 2.5 mM $MgCl_2$ (pH 7.5) (ionic strength, 25 mM) was mixed with varying concentrations of F-actin (0−13.2 μM). The samples were then centrifuged at 180000*g* for 30 min, and the fraction of free FH-S-1 remaining in the supernatant was determined by densitometric measurements of the 95 kDa heavy chain present on Coomassie blue-stained electrophoretic gels.

Electrophoresis. $NaDodSO_4$−polyacrylamide gradient gel electrophoresis (5−18%) was carried out as previously described (Bertrand *et al.*, 1992). Fluorescent bands were located in the gels by illumination with a long wave ultraviolet light before staining with Coomassie blue. Densitometric scanning of the gels was performed with a Shimadzu Model CS-930 high-resolution gel scanner equipped with a computerized integrator.

Limited Proteolytic Digestions of FHS-Labeled Myosin-S-1. Restricted hydrolysis of FH-S-1 (5 mg/mL) with trypsin (enzyme:S-1 wt ratio of 1:100) or endoprotease V8 (enzyme: S-1 wt ratio of 1:50) was performed in 20 mM MOPS (pH 7.5) at 25 °C for 45 and 60 min, respectively. The proteolytic reactions were quenched by mixing 30 μL aliquots with 170 μL of boiling Laemmli buffer (pH 8.0), and the protein samples were then analyzed by $NaDodSO_4$−polyacrylamide gel electrophoresis.

Chromatographic Separation and Sequencing of the FHS-Labeled Tryptic Peptides. The isolation of pure fluorescent S-1 heavy chain was carried out by subjecting FH-S-1 (1.2 mg) to reverse-phase HPLC on a microbore C-4 column (2 × 100 mm). Linear gradient elution was performed with 0−95% acetonitrile (solvent B) containing 0.1% aqueous trifluoroacetic acid (solvent A). The flow rate was adjusted to 0.2 mL/min, and 0.2 mL fractions were collected. The pH of each fraction was brought to 8.0 by the addition of 5 μL of 0.5 M NaOH, and the fluorescence was monitored at 520 nm with excitation at 495 nm. The fluorescent F-2 fractions were pooled, taken to dryness with a Speed Vac

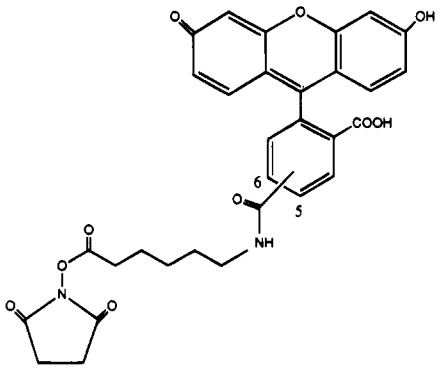


FIGURE 1: Structure of 6-[fluorescein-5(and 6)-carboxamido]-hexanoic acid succinimidyl ester (FHS) used for conjugation with skeletal myosin subfragment 1.

centrifuge, resuspended in 150 μL of 250 mM Tris-HCl and 2 M urea (pH 8.0), and digested at 37 °C with trypsin (enzyme:substrate wt ratio of 1:20). After 2 h reaction, a second equivalent amount of trypsin was added and the digestion was continued for 2 h. The resulting digest was fractionated by reverse-phase HPLC on an Aquapore C-8 Brownlee column (2 × 100 mm) eluted with 0−95% acetonitrile gradient (solvent B) containing 0.1% trifluoroacetic acid (solvent A), at a flow rate of 0.2 mL/min and collecting 0.2 mL fractions. Fluorescence measurements on the neutralized fractions were carried out as indicated above to identify the labeled peptides P-1 and P-2. These were further purified by reverse-phase HPLC under conditions specified in the legend of Figure 8. Amino acid sequencing of the pure fluorescent peptides was conducted using an Applied Biosystems model 470-A sequenator connected to an on-line model 120A phenylthiohydantoin amino acid analyzer (Hewick *et al.*, 1981).

RESULTS

F-Actin-Sensitive Labeling of Myosin-S-1 with 6-[Fluorescein-5(and 6)-carboxamido]hexanoic Acid Succinimidyl Ester. Following preliminary experiments, our attention was focused on FHS not only because of its spectroscopic advantages but primarily also because of the contribution of the hexanoyl chain in between the fluorescyl and succinimidyl ester groups (Figure 1) to the observed selectivity of the S-1 labeling by this reagent. The latter important feature was not encountered when reacting S-1 with the homologous compound 5-carboxyfluorescein succinimidyl ester, which lacked such a spacer arm.

When S-1 (A1 + A2) was incubated at pH 7.0 and room temperature with a concentration of FHS corresponding to only 1.5-fold molar excess of reagent to protein, it underwent a rapid acylation reaction the time course of which could be quantitatively monitored by measuring the absorbance at 495 nm of the isolated protein (Figure 2A). Under the employed optimum conditions, the reaction plateaued after 15 min with the progressive incorporation of an average of 0.9 fluorescyl group/mol of S-1. Comparative measurements of the modification extent using labeled protein samples isolated in the absence or presence of 0.1% $NaDodSO_4$ did not reveal noticeable adsorption of fluorescein to S-1 during the 15 min period of the process. This observation indicates that all the bound fluorophore was covalently conjugated to the myosin head. The separated S-1 (A1 and A2) isozymes behaved similarly, and increasing the ionic strength up to

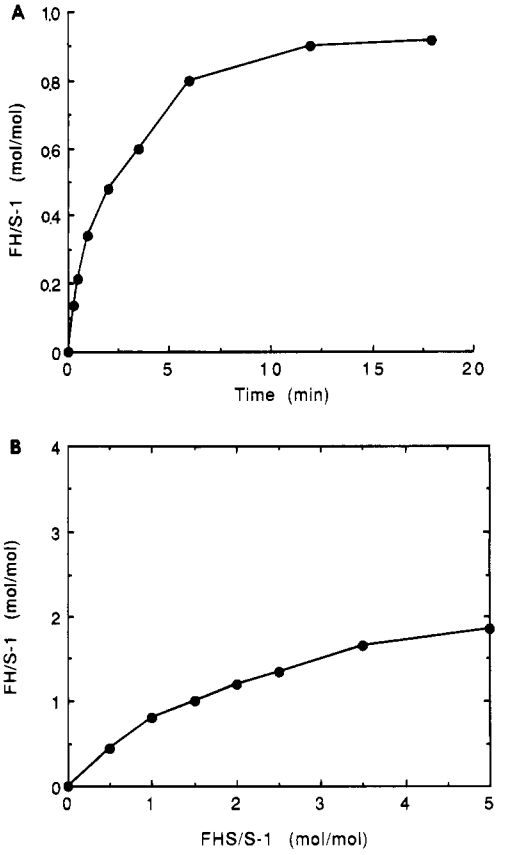


FIGURE 2: (A) Time course of the covalent reaction of myosin-S-1 with FHS. S-1 (75 μM) was incubated with 1.5-fold molar excess of FHS in 50 mM MOPS, pH 7.0, at 20 °C. At the indicated time intervals, aliquots were withdrawn, mixed with glycine ethyl ester to stop the reaction, and loaded on a PD 10 column as specified under Material and Methods. The stoichiometry of S-1 labeling was determined from the absorbance at 495 nm of the FH group bound to the isolated protein samples. (B) Extent of incorporation of the 6-[fluorescein-5(and 6)carboxamido]hexanoyl group into S-1 as a function of the FHS concentration. S-1 (70 μM) was reacted with up to 5-fold molar excess of FHS in 50 mM MOPS, pH 7.0. After 15 min at 20 °C, the protein samples were purified as in panel A and subjected to spectrophotometric measurements at 495 nm.

100 mM only slightly decreased the degree of labeling. Strikingly, increasing the initial concentration of FHS to 5-fold molar excess over S-1 did not result in an extensive substitution of the protein since, as shown in Figure 2B, the reaction tended to level off with the binding of maximally 2 fluorescyl groups/mol of S-1. Raising the pH of the reaction medium to 8 was to be avoided because it proved to alter the selectivity of the sublocalization of the fluorophore in the S-1 structure, as will be detailed below. Therefore, in routine work, S-1 labeling was conducted only at neutral pH and in the presence of 1.5−1.8-fold molar excess of reagent. Using these standard experimental conditions, we further examined the influence of nucleotides and F-actin on the production of the S-1−FHS conjugate.

The addition of millimolar concentrations of Mg^{2+}-ATP, Mg^{2+}-ADP, Mg^{2+}-AMPPNP, or Mg^{2+}-pyrophosphate did not significantly alter the kinetics of the reaction or the amount of FHS attached to S-1 (data not shown). In contrast, rigor binding of F-actin to S-1 dramatically changed the labeling process. As illustrated in Figure 3, F-actin strongly inhibited the fluorescence incorporation into S-1. At a molar ratio of actin:S-1 = 1, the level of S-1 substitution fell down below

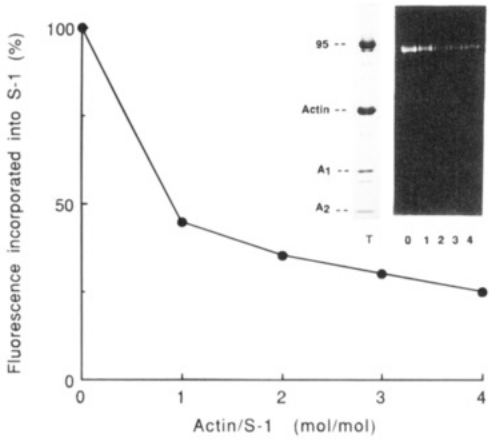


FIGURE 3: Protection of S-1 by F-actin against modification with FHS. S-1 (50 μM) was treated with 1.5-fold molar excess of FHS in 50 mM MOPS, 5 mM KCl, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$, pH 7.0, at 20 °C, for 15 min, in the presence of varying concentrations of F-actin (0−200 μM). After quenching of the reactions, the mixtures were brought to the same final actin concentration (200 μM) and then centrifuged, and the amount of unreacted FHS in the resulting supernatants was estimated spectrophotometrically at 495 nm, as described under Materials and Methods. The data are presented as percent of the control-labeled S-1 containing 0.90 mol of fluorescyl group/mol of protein. Inset, F-actin-modulated incorporation of the fluorophore into the S-1 heavy chain. Aliquots of the FHS-treated acto−S-1 complexes were analyzed by $NaDodSO_4$ gel electrophoresis. The fluorescent band pattern of S-1 labeled in the absence (lane 0) and presence of 1−4-fold-molar excess of F-actin (lanes 1−4) was established by illumination of the gels under UV light. The Coomassie blue-stained gel of the acto−S-1 complex labeled at an actin:S-1 molar ratio = 2 is also shown (lane T).

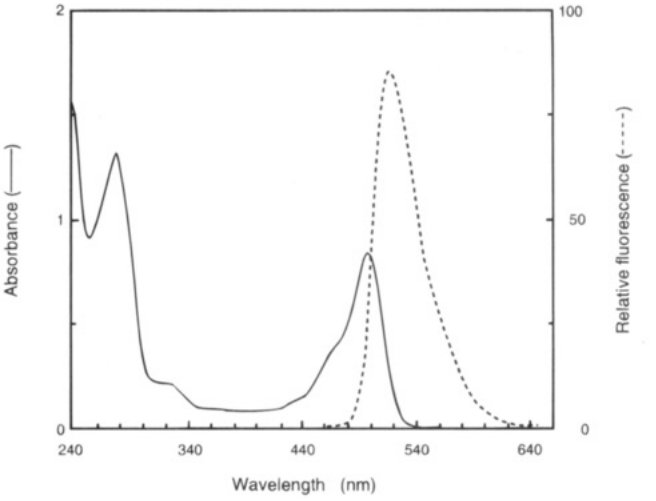


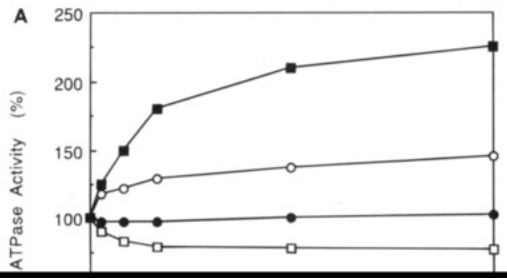
FIGURE 4: Absorption (solid line) and fluorescence emission (broken line) spectra of FHS-labeled, purified S-1. Spectra were obtained from samples containing modified S-1 (1.5 mg/mL) in 20 mM MOPS and 50 mM NaCl, pH 7.5, at 20 °C. The excitation wavelength was 495 nm.

50% of the control, and it was further reduced at higher actin:S-1 molar ratios. The actin effect was not due to a competition of the protein with S-1 for the uptake of the FHS reagent. When the treatment of the acto−S-1 complex with FHS was monitored by gel electrophoresis, the fluorescence was found associated only with the 95 kDa heavy chain both in the absence and presence of varying concentrations of F-actin which remained unsubstituted by the reagent even at the highest actin:S-1 molar ratio of 4 employed (Figure 3, inset). As expected also, the fluorescence intensity of the 95 kDa band decreased progressively in a manner dependent on the actin concentration. On the other hand, only trace amounts of fluoresence, if any, were detected in the two light chain bands. The overall findings clearly indicate that the interaction of F-actin with S-1 alters the accessibility and/or chemical reactivity of the S-1 amino acid(s) labeled by FHS, the location of which is confined to the heavy chain. Succinimidyl esters are known to react preferentially with amino groups, but under some circumstances, they may also modify hydroxyl amino acid residues (Miller *et al.*, 1994) and sulhydryls in proteins (Antolovic *et al.*, 1995). However, the ester or thioester bonds formed are alkali labile unlike the amide linkage involving the amino side chain. The observed stability of the S-1−FHS conjugate at high pH values suggests that the labeling of S-1 was most likely targetted at the ϵ-NH_2-lysine group.

Spectroscopic and Enzymatic Properties of the Purified S-1-FHS Conjugate. Figure 4 shows the absorption spectrum and fluorescence emission spectrum of S-1 containing about 0.9 fluorescyl group/mol of protein. Both spectra were quite similar to those of free FHS except for a 5 nm red shift of the maximum absorption wavelength found at 500 nm for the modified protein instead of 495 nm for the unattached fluorophore. Thus, the overall spectral characteristics of the (fluoresceincarboxamido)hexanoyl group were not much altered by its introduction into S-1.

We also determined the influence of the S-1 substitution with FHS upon all its enzymatic activities during the entire course of the reaction. The results, presented in Figure 5A, show that the K^+-ATPase remained unchanged whereas the Ca^{2+}-ATPase and Mg^{2+}-ATPase activities were both enhanced up to nearly 150% and 225% of the original values, respectively. In the presence of actin, the ATPase activity did undergo a progressive decrease but to only 75% of the control. Double-reciprocal plots of the actin-activated ATPases of native and isolated FHS-labeled S-1 indicated no change in the K_m for actin, whereas the V_{max} value for FH-S-1 was decreased 2-fold (2.7 versus 5.4 s^{-1}) (data not shown). This reduction of the acto−S-1 ATPase rate combined with the observed protection of S-1 by F-actin against FHS labeling led us to analyze quantitatively in airfuge pelleting experiments the rigor binding of the modified S-1 to increasing concentrations of F-actin. As seen in Figure 5B, the labeled S-1 bound to actin at stoichiometric 1:1 molar ratios suggested that the strong acto−S-1 interaction was not altered by the bound FHS. Essentially the same binding curve was previously obtained for the interaction of native S-1 with F-actin under identical experimental conditions (Bertrand *et al.*, 1989).

Localization of the FHS Fluorescence on the S-1 Heavy Chain Fragments. The fluorescyl-S-1 derivative was submitted to limited digestions using either trypsin or endoprotease V8. The data shown in Figure 6 revealed that the fluorescence initially associated with the 95 kDa heavy chain (Figure 6, lanes a and d) was transferred to the central 50 kDa fragment (Figure 6, lanes b and e) or 48 kDa peptide (Figure 6, lanes c and f), respectively, produced by these proteases. In contrast, very low levels of fluorescence were at the positions of the NH_2-terminal 27−28 kDa fragments and COOH-terminal 20−22 kDa peptides. Structurally, the trypsin-split S-1 differs from the endoprotease V8-generated S-1 by the loss of the two lysine-rich connector segments, joining the 50 kDa region to the 27 and 20 kDa fragments,


A
250
200
150
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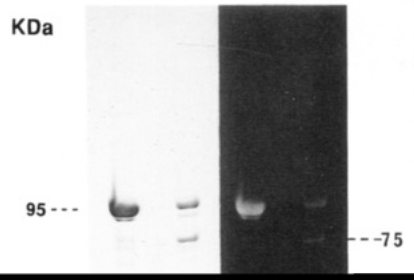
ATPase Activity (%)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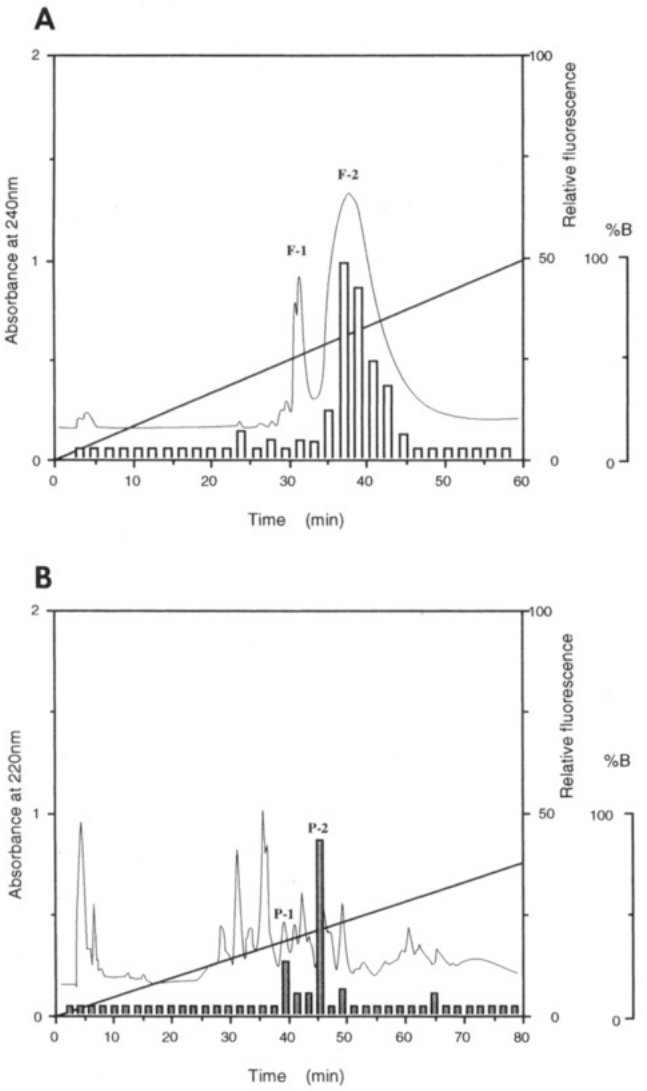

FIGURE 7: (A) Isolation by reverse-phase HPLC of the fluorescently labeled 95 kDa S-1 heavy chain. S-1 conjugated to FHS was prepared (75 μM), supplemented with trifluoroacetic acid to a final concentration of 0.5%, and loaded on a Microbore C-4 Brownlee column (2 × 100 mm) which was eluted for 60 min with a gradient (0−100%) of solvent B consisting of 95% acetonitrile in 0.1% trifluoroacetic acid. The fluorescence intensity (open bars) of the effluent adjusted to pH 8.0 was monitored (excitation at 495 nm and emission at 525 nm), and the fluorescent fractions, F-2, eluting between 36 and 44 min were collected for further analysis. (B) Reverse-phase HPLC separation of the fluorescent tryptic peptides from the labeled S-1 heavy chain. The total tryptic digest of the isolated modified S-1 heavy chain was prepared, as indicated under Materials and Methods, and then applied to a reverse-phase Aquapore C-8 Brownlee column (2 × 100 mm) eluted for 80 min with a gradient (0−80%) of solvent B consisting of 95% acetonitrile in 0.1% trifluoroacetic acid. The fluorescence elution profile (hatched bars) was monitored as in panel A. The fluorescent peaks, P-1 and P-2, were subjected to further purification.

The pure, fluorescent peptides P-1 and P-2 were submitted to microsequencing, and the structural information obtained for the major P-2 fragment is presented in Table 1. A single sequence was obtained for P-2 after 17 cycles of Edman degradation reactions. It was consistent with that for S-1 heavy chain residues 545−561, A-T-D-T-S-F-TMK-N-K-L-Y-E-Q-H-L-G-K (Tong & Elzinga, 1990). There was only one gap in the sequence at cycle 9, corresponding to Lys-553 on the heavy chain sequence, with no unusual PTH amino acid observed at this cycle. However, a normal value for leu-554 and all subsequent cycles was noticed. Since lysine was identified at the last cycle (17) and no further PTH derivatives were found, P-2 should represent a 17-residue tryptic peptide resulting from peptide bond cleavage at Lys-544-Ala-545 and Lys-561-Ser-562. The lack of proteolytic hydrolysis at the internal Lys-553 obviously indicates that the ϵ-amino group of this particular residue was blocked in the fragment. This feature and the absence of Lys-553 from cycle 9 strongly suggest that this was the amino acid derivatized with FHS. Furthermore, the apparent absence of the PTH derivative of the fluorescently substituted lysine at cycle 9 is consistent with the findings of control experiments which showed that the PTH derivative of synthetic ϵ-NH_2-(FHS)lysine was adsorbed to the filter of the sequencer and escaped detection with the PTH amino acid analyzer. The primary structure of P-2 was confirmed by additional sequence studies carried out on the same peptide isolated from the tryptic digest of the purified fluorescent 50 kDa fragment.

The sequence analysis of the minor peptide, P-1, revealed only three residues over four cycles. Glycine was identified at both the first and second cycles, whereas no PTH amino acid was observed at cycle 3. But a normal level of lysine was found at the last cycle (4) (data not shown). These data suggest that the peptide consists of a four-residue long sequence which could correspond only to residues 638−641 of the S-1 heavy chain, G-G-K-K. Most likely, it originates from tryptic cleavage of the peptide bonds at Lys-637-Gly-638 and Lys-641-Lys-642, located just within the 50 kDa−20 kDa junction. The substitution of the side chain of Lys-640 with the fluorophore explains the gap at cycle 3 as found for Lys-553 in the P-2 peptide. Our conclusions are consistent with the observed decrease of the bound fluorescence in the tryptic S-1 which, upon isolation, loses its connector peptides together with the fraction of label attached to it.

DISCUSSION

In this investigation, we have defined experimental conditions for the selective covalent derivatization and fluorophore labeling of Lys-553 in the S-1 heavy chain with the succinimido ester of 6-[fluorescein-5(and 6)-carboxamido]-hexanoic acid. The main functional and structural properties of the resulting novel modified S-1 preparation were also characterized. A remarkable feature of this conjugation process was the hyperreactivity of Lys-553 toward the FHS molecule at neutral pH, which has made possible its extensive substitution without modification of other residues, except a small fraction of Lys-640. A specific protein microenvironment around Lys-553 might have enhanced its nucleophilic character thus favoring its chemical reaction with FHS. However, other tested succinimido esters of fluorescein which differed from FHS only by the absence of the hexanoyl chain did not react as selectively as the latter reagent. They were found to label evenly all three proteolytic fragments of the S-1 heavy chain. Therefore, the hydrophobic, six-carbon long hexanoyl moiety of FHS seems also to play a major role in directing the reagent to the side chain of Lys-553.

In contrast to other fluorescent labels previously introduced into the 50 kDa region of S-1 but without precise determination of their distribution and localization in the fragment

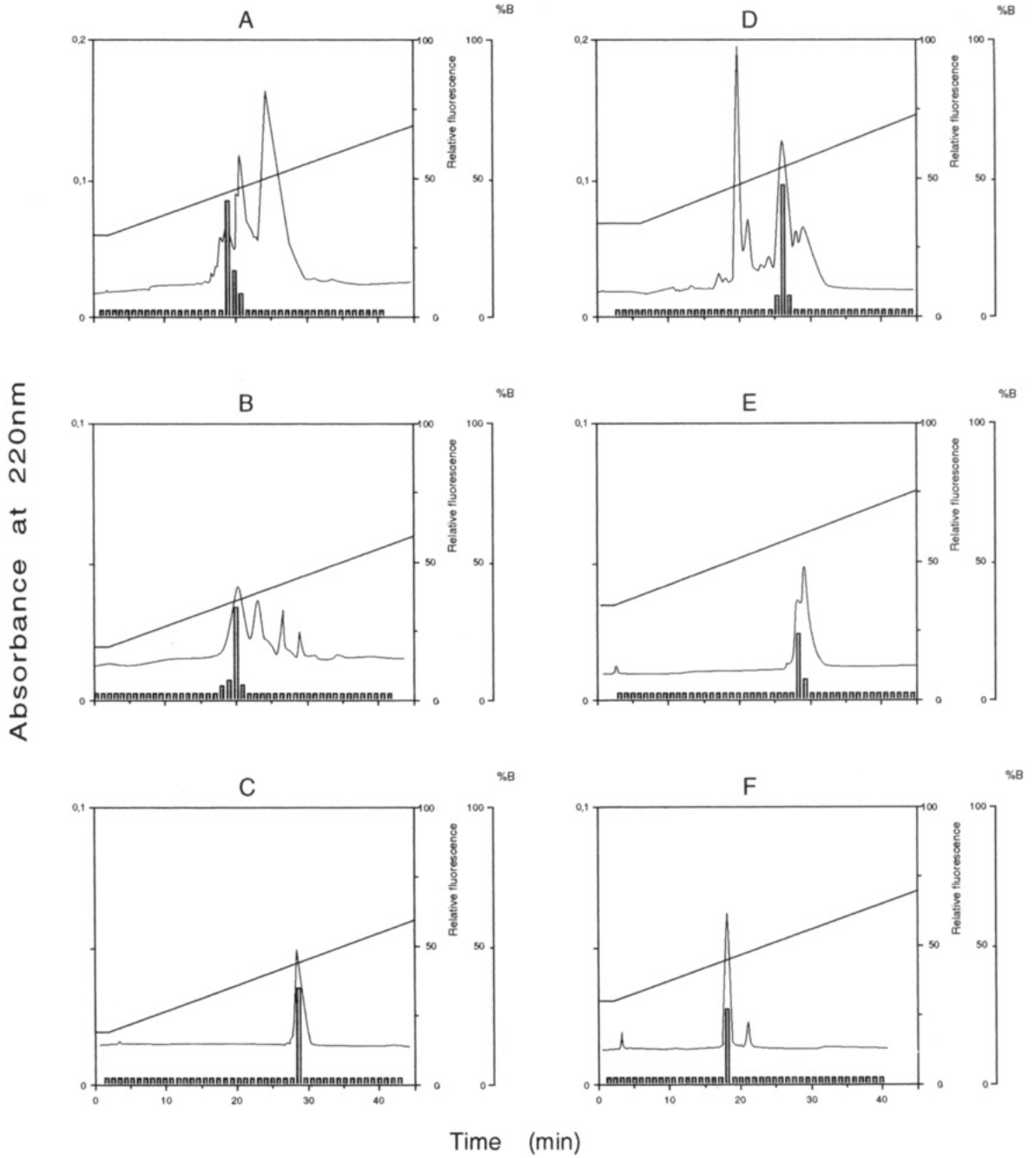

FIGURE 8: Reverse-phase HPLC purification of the fluorescent tryptic peptides P-1 and P-2. The two collected peptide fractions, P-1 and P-2, were concentrated, and each peptide mixture was submitted to three successive HPLC, the fluorescence elution profiles (hatched bars) of which are shown in panels A−F, respectively. For P-1, use was made of (A) a Brownlee C-18 column eluted with a 30−70% gradient of B (B = 60% acetonitrile, and 0.1% trifluoroacetic acid), (B) a Brownlee C-4 column eluted with 20−60% of B, and (C) a Brownlee C-18 column eluted with 20−60% of B containing 0.005% trimethylamine. Purification of P-2 was achieved using (D) a Brownlee C-18 column eluted with 35−75% of B, (E) a Brownlee C-18 column eluted with 35−75% of B containing 0.005% trimethylamine, and (F) a Brownlee C-4 column eluted with 30−70% of B. All columns (2 × 100 mm) were eluted for 40 min.

primary structure (Mornet & Ue, 1985; Hiratsuka, 1986), the FH group we have attached to the same region has been shown in this work to be exclusively bound to Lys-553. This finding allows one to further consider the positioning of this amino acid relative to the ATPase- and actin-binding sites within the crystal structure of S-1 (Rayment *et al.*, 1993a) and in the model of the rigor acto−S-1 complex (Rayment *et al.*, 1993b). Such a consideration helps to better understand the observed functional consequences of the modification as well as the effects displayed by the S-1 ligands on the chemical reaction. The location of the peptide segment including Lys-553 is of particular interest as it is relevant to that proposed for the primary, stereospecific, and hydrophobic actin-binding site of S-1. This important site would involve the helix(Gly-516−Phe-542)−loop(Pro-543−Thr-546)−helix(Asp-547−His-558) motif residing in the lower subdomain of the 50 kDa region. The hydrophobic patches of this motif are solvent exposed and thought to determine the strong attachment of the S-1 heavy chain to complementary hydrophobic side chains at the bottom surface of actin subdomain-1. Lys-553 is a highly conserved residue which is always located at the end of the second helix of the motif and, therefore, would be placed close to the strong actin-binding site of S-1. This proximity is consistent with

Table 1: Sequence Analysis of the Fluorescent Tryptic Peptide P-2 Isolated from FHS-Labeled Myosin-S-1[a]

cycle	amino acid	yield of phenylthiohydantoin derivative (pmol)[b]
1	Ala	35
2	Thr	ND
3	Asp	20
4	Thr	ND
5	Ser	ND
6	Phe	30
7	TM-Lys[c]	qi
8	Asn	16
9	Lys	
10	Leu	19
11	Tyr	14
12	Glu	11
13	Gln	11
14	His	ND
15	Leu	9
16	Gly	10
17	Lys	7

545 553 561

deduced sequence: **A-T-D-T-S-F-TMK-N-K-L-Y-E-Q-H-L-G-K**

FH

[a] The P-2 peptide was purified to homogeneity by reverse-phase HPLC as specified in Figures 7B and 8. [b] Sequencing was performed with an average yield/cycle of 94%. [c] TM-Lys is trimethyllysine which was qualitatively identified in the chromatographic system used to separate PTH amino acids. ND means not determined by sequence.

and makes it understandable the observed efficient protection of S-1 against FHS labeling by rigor binding to F-actin which must shield Lys-553 either directly or by an induced local conformational change affecting its chemical reactivity. On the other hand, the hydrophobic influence of the components of the actin-binding site near Lys-553 might have contributed to the observed preferential substitution of this residue with FHS.

Although fluorescein is a large and bulky group, its attachment to Lys-553 did not at all interfere with the rigor binding of FH-S-1 to F-actin. This property could be a consequence of the relatively long and flexible tether from the aromatic groups of fluorescein to the protein backbone, which allows a proper access of F-actin to its hydrophobic strong binding site on the modified S-1. However, the acto–S-1 ATPase reaction seems to be more sensitive to the bound fluorophore as indicated by the moderate decrease of the ATPase rate suggesting that a kinetic step subsequent to the binding of S-1-ATP to actin was somewhat altered by the attached fluorescein moiety. It would be interesting to examine the impact of the modification on the sliding of actin filaments over S-1 in the *in vitro* motility assay. The minor binding of the FH group to Lys-640 was another striking feature of the FHS–S-1 coupling as this amino acid is located in the flexible peptide loop of residues 626–647 also involved in the binding of S-1 to actin (Rayment *et al.*, 1993b). Because of its disorder, the precise structure of the loop is unknown, but it stays at the bottom of the actin-binding cleft not far from Lys-553. In native S-1, the flexible ϵ-NH_2 side chain of Lys-640 could sporadically come close to the hydrophobic region around Lys-553 and undergo a marginal reaction with the FHS reagent. In this regard, while tryptic cleavage of FH-S-1 resulted in a protein derivative labeled only at Lys-553, the reaction of FHS with preformed tryptic S-1 was observed to generate S-1 modified at the latter residue as well as at other lysines. Thus, the selectivity of the Lys-553 reaction with FHS seems to require the structural integrity of the vicinal 50 kDa–20 kDa junction.

Finally, the fact that Lys-553 is on the side of S-1 opposite to the active site pocket (Rayment *et al.*, 1993a) also rationalizes the lack of nucleotide effects on the coupling of FHS to S-1. Conversely, the absence of FHS-induced changes in the K^+-ATPase activity and the lack of inhibitory effect on the Mg^{2+}-ATPase or Ca^{2+}-ATPase activitiy indicate that the ATPase site of the modified S-1 was not significantly affected. The observed acceleration of the two latter enzymatic activities could be only a manifestation of the well-known communication in S-1 between the actin-binding site and the ATPase site (Botts *et al.*, 1989) which should be sensitive to any structural event occurring at or near the former site, such as the chemical modification of Lys-553. Recently, fluorescent ADP analogs covalently attached to the S-1 ATPase site without interfering with ATP binding and hydrolysis were described as potential specific probes of the S-1 active site cleft (Luo *et al.*, 1995). Our present study suggests that the (fluoresceincarboxamido)hexanoyl group linked to Lys-553, adjacent to the strong actin-binding subsite and not much perturbing the actin–S-1 rigor binding or the actin-activated ATPase activity, may also represent a valuable probe to monitor the dynamics and orientational properties of this functionally important region during the acto–S-1 interactions. Experiments along these lines of investigations, using fluorescence resonance energy transfer, are currently underway.

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