Stepwise Modulation of ATPase Activity, Nucleotide Trapping, and Sliding Motility of Myosin S1 by Modification of the Thiol Region with Residues of Increasing Size†

Markus Tiepold, Werner Kliche, Jens Pfannstiel, and Heinz Faulstich*

Max-Planck-Institut für medizinische Forschung, Heidelberg, Germany

Received May 25, 1999; Revised Manuscript Received November 22, 1999

ABSTRACT: Rabbit muscle myosin S1 was modified either at SH1 alone or at both SH1 and SH2, using a series of alkylthiolating reagents of increasing size, designed for correlating gradually changing structural disturbances in the thiol region with functional impairments in the myosin head. The reagents were of the type $H(CH_2)_n$-S−NTB, (NTB = 2-nitro-5-thiobenzoate) (n = 1, 2, 5, 8, 9, 10, 11, and 12). Modification of only SH1 led to the expected activation of the Ca^{2+}-ATPase, but only with small reagents, while reagents with $n \geq 10$ caused inhibition of the Ca^{2+}-ATPase. Modification of both SH1 and SH2 showed the expected inhibition of Ca^{2+}-ATPase but likewise allowed considerable residual Ca^{2+}-ATPase activity if the residues were small. Trapping of the nucleotide, known to occur with cross-linking reagents, was seen also with monovalent reagents, provided their length exceeded n = 9 or 10. All S1 derivatives prepared in this study possessed an affinity for actin comparable to native S1 but lacked sliding motility in in vitro motility assays. The biochemical data of this study can be related to existing models of myosin S1 and recent structural data [Houdusse, A., Kalabokis, V. N., Himmel, D., Szent-Györgyi, A. G., and Cohen, C. (1999) *Cell* 97, 459−470] by making the assumptions that modification at SH1 prevents the formation of the SH1 helix mandatory for the transmission of conformational energy and that mobility of the thiol region is a prerequisite for ATPase activity. Immobilization of the thiol region by residues of increasing size apparently leads to lower enzyme activity and, finally, to inhibition of nucleotide exchange.

Myosin subfragment 1 (S1)[1] contains important properties of the myosin motor protein and has therefore been widely employed as a model for studying ATPase activity, its binding to actin, and the force-generating process (*1*−*4*). As shown by crystal structures obtained from various sources such as chicken striated muscle, chicken smooth muscle, and *Dictyostelium discoideum* (*4*−*6*), the three properties mentioned are located at different sites in the myosin head, but must be interconnected. This can be concluded, for example, from the fact that binding of ATP dissociates actin from the myosin head. Located between the nucleotide binding site on one hand and the converter domain (*7*) and the lever arm (*8*−*11*) on the other is the so-called thiol region. It comprises two short α-helices containing SH1 and SH2, which are the only thiol groups in the heavy chain of S1 exposed to solvent (*12*), and a highly conserved glycine residue that appears to be important for the power stroke (*13*−*15*), presumably by acting as a hinge region.

The significance of the thiol region was recognized very early from the observation that chemical modification of the two thiols had large effects on ATPase activity. In 1956, Kielley et al. (*16*) showed that modification of SH1 with organomercurials enhanced the ATPase activity in myosin when measured in the presence of Ca^{2+} (Ca^{2+}-ATPase), but decreased ATPase activity when measured in the absence of Ca^{2+} ions [K^+(EDTA)-ATPase]. Additional modification of SH2 inhibited ATPase activity completely. Such correlations were largely independent of the chemical nature of the reagent and have been confirmed many times using reagents either of the maleimide type (*17*, *18*) or of the alkylating (*19*−*24*) or arylating (*25*−*27*) type. Although ATPase was completely inhibited in most cases, trapping of the nucleotide was not reported for such monovalent thiol reagents. Most of the thiol modifications used so far were chemically irreversible, and thus did not allow detection of denaturation processes possibly caused by the modification, for example, by measuring the amount of ATPase activity recovered after the modification was removed.

A behavior similar to that after modification with monovalent reagents was seen when the two thiols were reacted with cross-linking reagents (*28*, *29*). As expected, Ca^{2+}-ATPase was found to be activated after the first reaction step of bivalent reagents with SH1. At the same time, K^+(EDTA)-ATPase was inhibited. In the second reaction step, Ca^{2+}-

† This work was supported by the Deutsche Forschungsgemeinschaft (DFG).

* To whom correspondence should be addressed: c/o Max-Planck-Institute of Cell Biology, D-68526 Ladenburg/Rosenhof, Germany. E-mail: hfaulst@zellbio.mpg.de.

[1] Abbreviations: S1, subfragment 1 of myosin; A1 and A2, myosin alkali light chain 1 and 2; $H(CH_2)_n$-S-NTB, (C_n) (n = 1, 2, 5, 8, 9, 10, 11, and 12), *n*-alkyl-[5-dithio-(2-nitrobenzoic-acid)]; ArSSAr, (C_0, Ellman's reagent), 5,5′dithio-bis-(2-nitrobenzoic-acid); PK, pyruvate kinase; LDH, lactate dehydrogenase; k_A, pseudo-first-order rate constants for the reactions with Cys_{374} in G-actin; k_S, second-order rate constants for the reaction with SH1 in S1; NEM, *N*-ethyl-maleimide; NTB^-, 2-nitro-5-thiobenzoate; pPDM, *N*,*N*′-*p*-phenylenedimaleimide; SH1 and SH2, Cys_{707} and Cys_{697} of the S1 heavy chain; SH_{LC}, reactive Cys of the A1 light chain; S1A1, myosin subfragment 1 with A1 light-chain bound; S1A1-C_n, S1A1 with SH1 reacted with C_n.

10.1021/bi991199z CCC: $19.00
Published on Web 01/21/2000

ATPase was inhibited also, reflecting the involvement of SH2 in the cross-linking reaction. Such correlations held also in the case of zero-length cross-linking between SH1 and SH2, for example, with Ellman's reagent (*30*), and for a new series of cross-linking reagents of varying length recently synthesized in our laboratory (*31*). In all cases, cross-linking of the two thiols caused trapping of the nucleotide (*32*, *33*).

In atomic models (*4*−*6*) showing the myosin head in conformations assumed to be related to the different steps of the working cycle, part of the thiol region, the SH2-helix, and the active site were found to be in close proximity, suggesting a close functional interrelationship between these two domains. The other part of the thiol region, the SH1 helix, was found to be in contact with the so-called relay domain (*34*), which may be involved in energy transmission to the converter domain, the part of the protein that is thought to move the lever arm. All models show that the thiol region is located right in the core of the motor protein and thus able to be involved in the energy transmission process, as emphasized most recently by Cohen and co-workers, who showed that the SH1 helix can adopt a nonhelical conformation (*34*).

Despite great progress in understanding the structure of this motor protein, a considerable number of biochemical findings established in the past remain unexplained by the structural data. For example, why is the Ca^{2+}-ATPase activated after substitution of SH1, or why is the ATPase activity inhibited after modification of SH2, given that SH2, though located in close vicinity to it is not part of the active site, and how do modifications in the thiol region cause nucleotide trapping or inhibition of the sliding motility in in vitro motility assays? In the present study, we try to answer these questions by measuring how functional parameters such as ATPase activation/inhibition or nucleotide trapping depend on the size of the residues introduced at SH1 and SH2, and we discuss how functional defects can be explained in terms of structural changes expected to occur after chemical modification of the two thiol groups.

EXPERIMENTAL PROCEDURES

Preparation of Thiol-Specific Reagents. The reagents were prepared from the corresponding thiol compounds HS-$(CH_2)_n$-H by the following procedure.

To 0.55 mmol of 5,5′-dithio-bis-(2-nitrobenzoic-acid) (Ellman's reagent), dissolved in 10 mL of 2% $NaHCO_3$ (pH 8), were added 0.5 mmol of the thiol compound in 10 mL of tetrahydrofuran (THF) under vigorous stirring. After the reaction was finished (ca. 10 min), the organic solvent was removed in vacuo. For reagents with $n = 8-12$, the aqueous solution was stored at 4 °C overnight in order to allow crystallization of the product, which was isolated by filtration and washed twice with ice-cold water. The pure product was dried in a desiccator over P_20_5.

For reagents with $n = 1$, 2, and 5, the tetrahydrofuran was removed and the solution adjusted to pH 0−1 with hydrochloric acid (32%) and extracted three times with ether. After drying the organic phase with anhydrous $CaCl_2$, the ether was partly removed in vacuo, and the residue purified on a silica gel column (25 × 2.5 cm) developed with $CHCl_3$/MeOH/HAc (2 N) (65:25:4). The pure material was crystallized from 70% NaCl and dried in a desiccator over P_2O_5.

For the preparation of $H(CH_2)S-NTB$, gaseous methanethiol was dissolved in THF, and the concentration measured in an aliquot by adding excess Ellman's reagent in 5% $NaHCO_3$.

The concentration of reagent solutions was determined from the absorption at 412 nm ($\epsilon = 14\,150\ M^{-1}cm^{-1}$) after the addition of excess 2-mercaptoethanol. The starting material $H(CH_2)_{11}-SH$ was not commercially available and was prepared from $H(CH_2)_{11}-Br$ (Lancaster, Mühlheim) according to ref *35*.

Characterization of the Thiol Specific Reagents. The MS experiments were performed on a Fourier Transform-Ion-Cyclotron-Resonance mass spectrometer with high-resolution equipped with a standard electrospray (ESI) ion source. For all reagents, the negative full-scan mode gave a dominant $[M - H]^-$ with the expected mass-to-charge ratio.

The ^1H and ^{13}C NMR spectra were measured with a Bruker 500 MHz spectrometer at RT, using tetramethylsilane as internal standard.

Protein Purification. Myosin from rabbit skeletal muscle was prepared according to Margossian and Lowey (*36*) and stored in 50% glycerol at −20 °C. Myosin subfragment 1 (S1) was prepared by digestion of soluble myosin with α-chymotrypsin (Serva) according to Weeds and Taylor (*37*), frozen in liquid nitrogen, and stored at −70 °C. In this study, only the S1A1 isoform was used. A molecular weight of 115 000 (*38*) was assumed for S1A1. Protein concentrations of unmodified S1 were determined by measuring the absorbance at 280 nm [A_{280nm} 1% = 7.5 cm^{-1} (*39*)]. Protein concentrations of modified S1A1 were determined by the method of Bradford (*40*), using a calibration curve established with S1A1.

Actin was prepared from rabbit muscle as described by Spudich and Watt (*41*).

ATPase Measurements. Ca^{2+}-ATPase activity was measured at 25 °C according to Kodama et al. (*42*). For Ca^{2+}-ATPase measurements, a buffer containing 50 mM Tris-HCl, pH 7.5, 0.25 M KCl, 5 mM $CaCl_2$, and 0.2 mM ATP (Boehringer, Mannheim) was used. K^+(EDTA)-ATPase was measured in a buffer containing 50 mM Tris-HCl, pH 7.5, 1 M KCl, 5 mM EDTA, and 0.2 mM ATP. Standard calibration curves were established for each ATPase buffer using $KH_2PO_4 \times H_2O$. In the ATPase assays, the ATP concentration was reduced to 0.2 mM in order to avoid high background values. Ca^{2+}-ATPase activity with the lowered ATP concentration was checked for linearity by increasing the amount of S1A1. A typical ATPase assay contained 5 μL of 20 mM ATP and 495 μL of ATPase buffer containing $2-7 \times 10^{-3}$ mg of S1A1. After 1, 2, 3, 4, and 5 min incubation time, 500 μL of ice-cold 0.6 M perchloric acid was added, and the procedure continued as described by Kodama et al. (*42*). Twin values were fitted with the program Grafit II (Ericathus software). For ATPase measurements during protein modification, aliquots of 17 μL were taken at the appropriate time and diluted immediately in 83 μL ATPase buffer. From this solution, 10 μL aliquots were directly added to 485 μL of ATPase buffer together with 5 μL of 20 mM ATP. ATPase was measured in twin samples for 3 min.

Mg^{2+}-ATPase was measured using the PK/LDH-linked ATPase assay described by Trentham et al. (*43*). The assay was performed at 20 °C in a buffer containing 3 mM imidazole, 3 mM $MgCl_2$, 1 mM KCl, 0.25 mM NADH, 0.8

mM PEP, 8 μg/mL LDH, 20 μg/mL PK, 0.1 μM S1A1, and 3 mM ATP. The system was activated by adding 1−80 μM actin, and the extent of activation determined.

Protein Modifications. For protein modifications, the following buffers were used: 50 mTris/HCl (pH 8); 0.1 M KCl; 0.1 mM ADP and $MgCl_2$ (W/Y-buffer). Reactions in the absence of ADP were carried out in the same buffer without nucleotide. All protein modifications were performed at pH 8.

To S1A1 (10 μM) incubated for 30 min on ice in the appropriate buffer was adde 1 equiv of the thiol reagent. After the reaction was finished as indicated from the release of the calculated amount of 2-nitro-5-thiobenzoate (NTB^-, ϵ_{412} = 14 150 M^{-1} cm^{-1}), another equivalent of the reagent was added. Concentrations of the reagent stock solutions were likewise determined by measuring the absorption at 412 nm after adding an excess of 2-mercaptoethanol.

During the modification, aliquots were taken to measure the time course of change of the Ca^{2+}-ATPase activity.

BrCN Cleavage. In the $(C_{12})_3$- and $(C_1)_3$-derivatives of S1, the residual thiol groups were blocked with a 500-fold excess of NEM (ca. 6 h) before all disulfide bonds in the derivatives were cleaved by dialysis in W/Y-buffer containing 3 mM DTT (overnight). After this reaction, the solutions were dialyzed against 2 L of W/Y-buffer three times and for 2 h each and incubated with a 10-fold excess of pyrenyl maleimide for ca. 1 h (0 °C). Two controls were prepared: one without the DTT cleavage step, and the other one with native S1A1 instead of the derivatives. After centrifugation (10 min, 13 000 rpm) and the addition of 1 mM DTT (1 h), the protein was denaturated with 8 M urea (overnight) and purified on PD 10 columns (Pharmacia, Uppsala). The pyrenyl-modified myosin S1 was cleaved with BrCN in 70% formic acid at RT for 20 h using a 500-fold excess of BrCN over the calculated methionine content (*44*). The lyophilized peptides (ca. 3 mg) were dissolved in distilled water and submitted to electophoresis on a Laemmli gel (15%). They were transferred onto a poly-vinylidene difluoride membrane and the fluorescing band was analyzed by Edman.

Recovery Experiments. To show that the modification reactions of S1A1 with the thiol specific reagents had not caused denaturation, S1A1-C_ns were reduced by 24 h dialysis against 50 mM imidazole (pH 7) and 10 mM DTT at 4 °C, and the Ca^{2+}-ATPase and K^+(EDTA)-ATPase activities determined.

Trapping Experiments. All experiments (*45*) were carried out in the buffer used for the protein modification but with additional 10 μL of [^{3}H]ADP (NEM; 25 Ci/mmol). The purity of the [^{3}H]ADP was checked by HPLC analysis using a reversed-phase C18 column (Knauer, Nucleosil, 100 C18, 4 mm ID × 25 cm, 5 μm packing material) on a Waters HPLC system (Milford, Waters 996 photodiode array detector, Waters 600 pump, Waters 600 controller, software: Millennium). Solvent A was 0.1 M KH_2PO_4 with 8 M tetrabutylammoniumbromide (TBAB) and solvent B 70% CH_3CN with 30% A. The flow was 1.2 mL/min (20 °C). Gradient: 0 to 2.5 min, 100% A; 2.5 to 5.0 min, 0 to 20% B linear gradient; 5.0 to 10.0 min, 20 to 40% B linear gradient; 10.0 to 13.0 min, 40 to 100% B linear gradient; 13.0 to 18.0 min, 100% B. The nucleotides were detected by their absorption at 260 nm. We found a purity of 95%.

After incubation of S1A1 (10 μM) in ADP buffer for 30 min, a 3-fold excess of the thiol reagent was added and reacted overnight. The absorption at 412 nm was measured, and 2.5 vol of saturated $(NH_4)_2SO_4$-solution added. After centrifugation for 20 min at 40 krpm in order to remove the large exess of [^{3}H]ADP, the pellet was resuspended in 400 μL of nucleotide-free buffer and the untrapped ADP separated on a PD 10-column (Pharmacia, Uppsala). From 20 μL aliquots of each fraction, radioactivity was measured in 5 mL of Emulsifier-safe (Packard) using a TRI-CARB 4430 Liquid Scintillation system.

Stopped-Flow Experiments. Stopped-flow experiments for transient kinetics were performed at 20 °C with a High-tech Scientific SF 61 stopped-flow spectrometer. The observation cells of both systems had a 10 mm excitation path with fluorescence emission via 10 × 1.5 mm windows (*46*). Pyrenyl actin was prepared as described in ref *47* and the fluorescence excited a 365 nm and detected after passage through a KV 389-nm cutoff filter. All data were stored and analyzed using software provided by Hi-tech. Transients shown are the average of 3−5 consecutive shots of the stopped-flow machine. All concentrations refer to the concentration of the reactants after mixing in the stopped-flow observation cell.

In Vitro Motility Assays. In vitro motility assays were performed as described previously (*3*, *48*) with the modification that all buffers were without DTT. A Zeiss Axiovert 135 microscope with 1.3 NA 40× lens was used. Rhodaminyl phalloidin fluorescence (515−560 nm) was excited with a 75 W xenon arc-lamp (Wotan XBO 75/2), and the emission observed at 590 nm through a Zeiss Filterset 487714. Filaments were imaged by a Sony SSC-M370CE camera and recorded on a VHS recorder using an image processor (Hamamatsu Photonics) and time−date generator. All experiments were performed at 20 °C.

RESULTS

Reagents. All reagents (Table 1) were prepared from the corresponding thiol compounds and excess Ellman's reagent. When isolated in the protonated state, the reagents were white to yellowish solids, which were storable at 4 °C for long times. The reagents were identified by UV−vis absorption spectra (not shown), which were identical with the spectrum reported for the C_8 reagent by Faulstich et al. (*49*). Further characterization was obtained by mass and ^{1}H NMR spectrometry, the data being compiled in Table 1. When submitted to ^{13}C NMR, all reagents showed similar patterns of resonances; $H(CH_2)_{10}S-NTB$ (free acid; CD_3OD), for example, yielded the following data: δ 173.4 (C-1″), δ 146.4 (C-4′), δ 139.4 (C-1′), δ 145.4 (C-3′), δ 125.9 (C-2′), δ 125.8 (C-5′), δ 125.4 (C-6′), δ 39.8 [$S-CH_{2(alkyl)}$] δ 32.9−23.6 (8 C_{alkyl}), δ 14.4 [$-CH_{3(alkyl)}$].

Purity of the reagents was >95% in all cases, as concluded from their mass spectra. Like Ellman's reagent, the reagents with short hydrocarbon chains were easily soluble in buffers of pH 7 or 8, i.e., under the conditions used for the disulfide exchange reaction with protein thiols. Long reagents ($\geq C_{10}$) were handled in methanolic stock solution. Buffers of pH >8 were avoided because hydrolysis of the activated disulfides can occur.

Through the reaction with exposed protein thiols, a hydrocarbon chain is introduced into the protein together with the sulfur atom via a cystine-type disulfide bond. This

Table 1: Structure, Mass $[M - H]^-$, and ^1H NMR Data of the Thiol Reagents Used in This Study[a]

				δ (ppm)				
reagent	structure	mass $[M - H]^-$ calcd	mass $[M - H]^-$ found	$3H_{arom}$ (m)	$S-CH_2$ (t)	$S-CH_2-CH_2$ (m)	(CH_2) (m)	$-CH_3$ (t)
C_1	$H(CH_2)S-NTB$	243.974373	243.973710	7.9−7.7				2.4
C_2	$H(CH_2)_2S-NTB$	257.990023	257.989657	7.9−7.7	2.7			1.2
C_5	$H(CH_2)_5S-NTB$	300.036973	300.037331	7.95−7.5	2.7	1.75−1.6	1.45−1.2	0.85
C_8	$H(CH_2)_8S-NTB$	342.083923	342.084698	7.9−7.5	2.8	1.75−1.6	1.35−1.2	0.85
C_9	$H(CH_2)_9S-NTB$	356.099573	356.099333	8.0−7.5	2.8	1.75−1.6	1.4−1.2	0.8
C_{10}	$H(CH_2)_{10}S-NTB$	370.115223	370.116955	7.95−7.5	2.75	1.75−1.6	1.35−1.2	0.8
C_{11}	$H(CH_2)_{11}S-NTB$	384.130873	384.130196	7.9−7.5	2.75	1.75−1.6	1.4−1.2	0.8
C_{12}	$H(CH_2)_{12}S-NTB$	398.146523	398.146092	7.95−7.5	2.75	1.75−1.6	1.4−1.2	0.85

[a] NTB = 2-nitro-5-thiobenzoate.

Table 2: Reaction Rates of Reagents C1−C12 with Cys^{374} of Actin (k_A), (pseudo-first-order, 10 equiv of reagent/mol of actin), and with SH1 of S1A1 (k_S), (second-order, 1 equiv of reagent/mol myosin S1), at 22 °C and at pH 8

reagent	k_A (min^{-1})	k_S ($\mu M^{-1}min^{-1}$)
C_1	0.523	0.023
C_2	0.470	0.030
C_5	0.511	0.038
C_8	9.027	0.200
C_9	8.936	0.100
C_{10}	5.493	0.103
C_{11}	3.603	0.082
C_{12}	2.334	0.060

alkylthiolation reaction can be monitored spectrophotometrically at 412 nm by determining the amount of NTB^- released. The possibility of monitoring the reaction kinetics avoided overreaction and, in some cases, allowed specific thiol groups to be distinguished from their reaction rates. All residues introduced in this way into proteins could be removed under physiological conditions, for example, at pH 7.4, using either DTT or excess 2-mercaptoethanol. In the present study, DTT restored the activity of S1 to at least 90% in all cases (data not shown).

Using the series of the reagents described above, model reactions with the single exposed thiol group of actin, cys374, were performed. These reactions showed that short reagents of this type (C_1-C_5) reacted at rates similar to that found for Ellman's reagent, the rate constants (k_A, Table 2) being in the range of 0.2 min^{-1} (*31*). With reagents possessing longer hydrocarbon chains, like C_8, the reaction was accelerated by factors of up to 20, allowing the reaction to go to completion already after 2 min. The high reaction rate with the exposed thiol group in actin confirmed the conclusion of a previous study, in which the advantage of C_8 over Ellman's reagent, particularly for the titration of protein thiols, was demonstrated (*49*). Accelerated reaction rates as with C_8 were found for C_9 also, while for longer reagents such as C_{10}, C_{11}, and C_{12}, the reaction rates decreased again. The acceleration can be understood as an interaction of the hydrocarbon chains of the reagents with hydrophobic domains of the proteins. An acceleration similar to that in the reaction with the exposed thiol of actin was observed for the thiols of myosin S1 (k_s, Table 2), where again C_8 was the fastest reagent of all.

ATPase Activities after Modification of SH1, SH2, and SH_{Lc}. In the myosin fragment S1, the two SH groups located in the so-called thiol region, SH1 and SH2, are the most reactive thiol groups in the heavy chain. A third reactive

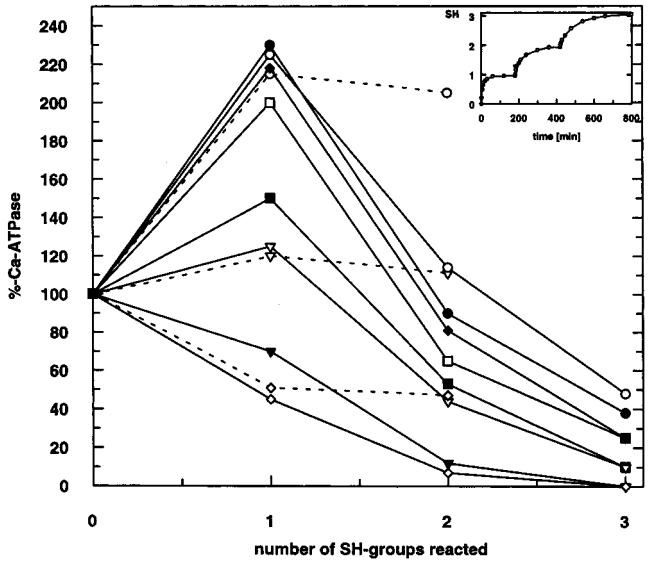

FIGURE 1: Ca^{2+}-ATPase activities of S1 (native enzyme = 100%) after full reaction of 1, 2, and 3 equiv of various reagents added at the times indicated (inset) in the presence of MgADP at pH 8. [C_1 (○); C_2 (●); C_5 (◆); C_8 (□); C_9 (■); C_{10} (▽); C_{11} (▼) and C_{12} (◇)]. The reaction of the 1 equiv represents complete reaction of SH1; reaction of 3 equiv represents full reaction of SH1, SH2, and SH_{LC}. The dashed lines show the titration of S1 with 1 and 2 equiv of [C_1 (○); C_{10} (▽); C_{12} (◇)] under the same conditions but in the absence of MgADP. Again the reaction of the 1 equiv represents complete reaction of SH1 while reaction of 2 equiv represents full reaction of SH1 and SH_{LC}. SH2 is not involved in the reaction, as seen from the unchanged Ca^{2+}-ATPase activity.

thiol is the single thiol group exposed by the light chain, SH_{LC}. To achieve as far as possible a selective modification of these three thiols, 3 equiv of reagent was added in three portions as shown in Figure 1 (inset).

Alkylthiolation at SH1. Reaction with the first equivalent of reagent was complete within at most 1 h for all reagents. For the reagent C_1, the reaction was associated with a pronounced increase in Ca^{2+}-ATPase activity (Figure 1), a finding that is well documented in the literature (*16*, *33*). Activation of Ca^{2+}-ATPase as associated with modification of SH1 became less as the length of the hydrocarbon chain increased, and turned into an inhibition for the longest reagents C_{11} and C_{12} (Figure 1). Nonetheless, it appeared that all reagents reacted with SH1 only. Evidence for selective modification at SH1 was obtained by monitoring the activity of the K^+(EDTA)-ATPase, which is known to be completely inhibited by modification of SH1 (*16*, *33*). We found that after release of 1 equiv of NTB^- the activity of the K^+(EDTA)-ATPase was indeed almost zero, inde-

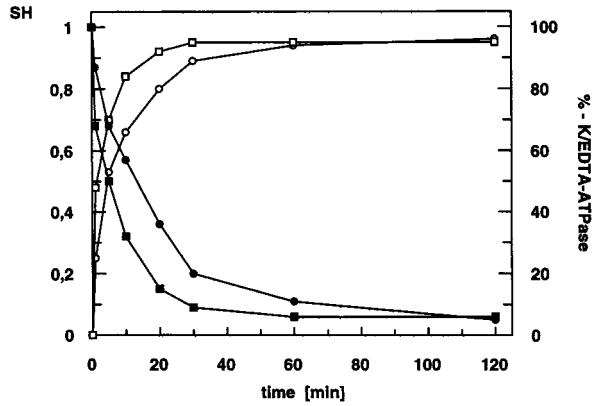

FIGURE 2: Comparison of the time courses of SH1 modification (○, □) and K^+/EDTA-ATPase inhibition (●, ■) in the reaction of myosin S1 with 1 equiv of C_1 (○, ●) or 1 equiv of C_{12} (□, ■). Native enzyme = 100%.

pendent of whether short (C_1) or long (C_{12}) reagents were used (Figure 2). Further evidence for the selective modification of SH1 came from comparison of the amounts of Ca^{2+}-ATPase activity measured after reaction of 1 equiv of reagent in the presence of MgADP (Figure 1), and in its absence (Figure 1, dotted lines), where SH2 is not reactive (*50*). For three selected derivatives, these activities were 230 and 205%, respectively, (C_1); 125 and 120%, respectively, (C_{10}); and 40 and 50%, respectively, (C_{12}). From the similarity of these values, we concluded that also with long reagents modification occurred exclusively at SH1 and that SH_{LC} and SH2 were not involved in the reaction. The results show that the enhancement of Ca^{2+}-ATPase described in previous reports as occurring after SH1 modification is seen only with small reagents at SH1, and that the activation effect may turn into inhibition if the residues introduced are too long. The results are in line with the only example reported in the literature where modification of SH1 alone caused deactivation of Ca^{2+}-ATPase, the introduction of the bulky bromobimane (*51*).

Alkylthiolation at SH2 and SH_{LC}. There is general agreement that modification of SH2 as well as SH1 leads to complete inhibition of all forms of ATPase activity (*33*). In accordance with this, we found that the Ca^{2+}-ATPase activity decreased for all reagents on adding the second equivalent of reagent (Figure 1). This decrease indicated that SH2 indeed participated in the reaction but left unanswered whether, and to what extent, the thiol group of the light chain was involved also. Because of this uncertainty, these mixtures were not further studied; instead, we investigated the S1 derivatives after reaction of 3 equiv of reagent (Figure 1, inset), where modification of SH2 (and SH_{LC}) can be assumed to be complete. Modification of thiol groups other than SH1 and SH2 (and SH_{LC}) could be excluded from the fluorescence patterns in SDS−PAGE of the BrCN fragments (Figure 3); the pattern obtained from native S1 treated with pyrenyl maleimide (lane a) resembled the pattern from S1 treated with pyrenyl maleimide after modification with 3 equiv of C_1 or C_{12}, NEM blocking of the remaining thiol groups, and DTT treatment (lanes c, and d, respectively). In all three lanes (a, c, and d), sequence analysis showed two peptides in the fluorescent gel band. The major component (aa 8−80) contained no cysteine while the second component corresponded to the 10 K fragment containing the two fast-

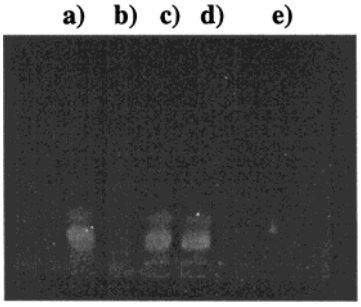

FIGURE 3: SDS−gel electrophoresis of BrCN-peptides of native myosin S1 labeled with pyrenyl maleimide (a) (control) or of S1 after modification with 3 equiv of C_1, treatment with excess NEM, reduction with DTT, and labeling with pyrenyl maleimide (c); (d) same as c, but modified with C_{12}; (b) same as c but without DTT treatment. (e) Cytochrome *c* as a 12.5 kDa fluorescent standard. Lanes c and d show no additional fluorescence bands with respect to lane a, indicating that in all samples only SH1 and SH2 were involved in the modification.

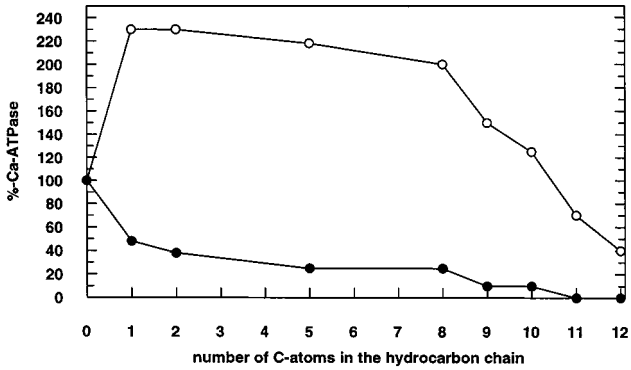

FIGURE 4: Ca^{2+}-ATPase activity of myosin S1 as dependent on the length of the hydrocarbon chain introduced, after modification of SH1 only (○), or SH1, SH2, and SH_{LC}, together (●). Native enzyme activity = 100%.

reacting thiols Cys_{697} and Cys_{707} (*52*, *53*). Since no other fluorescent fragments were found, the results prove that only these two cysteines were involved in the reaction.

When the myosin S1 derivatives modified at both SH1 and SH2 (as well as SH_{LC}) were submitted to Ca^{2+}-ATPase activity assays, we found that, in contrast to previous reports, modification at SH2 was not in all cases correlated with full inhibition of the Ca^{2+}-ATPase activity (Figure 1). While inhibition of the enzyme activity was complete with the largest reagents, C_{11} and C_{12}, reagents with $n < 11$ showed reduced, but not completely blocked, activities. For the smallest reagents, C_1 and C_2, residual activities were as high as 50 and 40%, respectively. In the absence of MgADP, the addition of a second equiv of reagent did not change Ca^{2+}-ATPase (Figure 1, dotted lines), suggesting that SH2 was excluded from the reaction, and that the second equivalent of NTB^- released reflected the reaction of the light-chain thiol group.

For comparison, the Ca^{2+}-ATPase activities for all S1 derivatives, either substituted at SH1 only or at both SH1 and SH2/SH_{LC}, were plotted vs the length of the reagents in Figure 4. Clearly, there exists an activation effect for all SH1 derivatives with the reagents between C_1 and C_{10}, which declines with reagents greater than C_8, reaches the value (100%) of native S1 around C_{10}, and falls further for C_{11} and C_{12}. In contrast, substitution at both SH1 and SH2 produced a strong inhibitory effect with even the smallest substituent, C_1, while further deactivation with chain elonga-

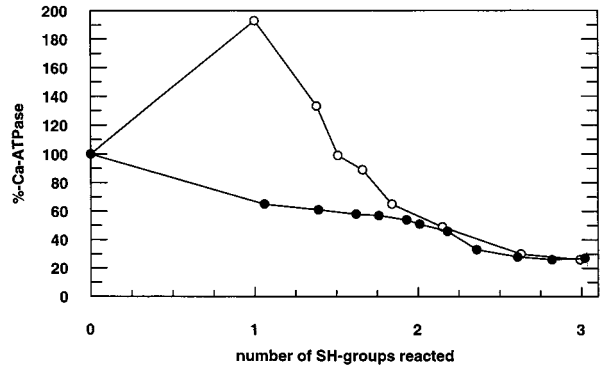

FIGURE 5: Kinetics of the decrease of Ca^{2+}-ATPase activities of myosin S1 premodified at SH1 with C_1, after the addition of 2 equiv of C_{12} (○) and of myosin S1 premodified at SH1 with 1 equiv of C_{12}, after the addition of 2 equiv of C_1 (●), both in the presence of MgADP at pH 8. In the last phase the two reactions show a similar course of Ca^{2+}-ATPase and end up with similar residual activities. Native enzyme activity = 100%.

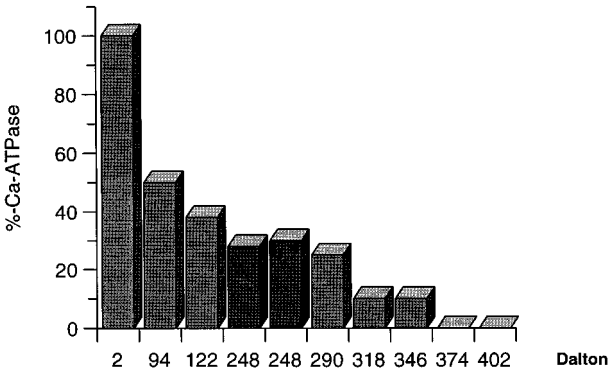

FIGURE 6: Ca^{2+}-ATPase activities (in percent, native enzyme = 100%) of myosin S1 as dependent on the sum of masses (Da) attached to SH1 and SH2, either in form of two identical substituents (light gray) or mixed substituents (dark gray). 2 Da = H + H; 94 Da = C_1 + C_1; 122 Da = C_2 + C_2; 248 Da = C_1 + C_{12} or C_{12} + C_1; 290 Da = C_8 + C_8; 318 Da = C_9 + C_9; 346 Da = C_{10} + C_{10}; 374 Da = C_{11} + C_{11}; 402 Da = C_{12} + C_{12}. (Note that each of the substituents contains one sulfur atom.)

tion proceeded slowly. Inhibition tended to completion when the reagents grew to greater than or equal to C_9.

Mixed Substituents at SH1 and SH2. The experiments described showed that large residues attached to SH1 inhibited rather than enhanced the Ca^{2+}-ATPase activity. In addition, we found that small substituents attached to SH2 did not abolish Ca^{2+}-ATPase activity completely but allowed considerable residual activities. Since our technique allowed selective modification of SH1 in a first step with subsequent modification at SH2 and SH_{LC} in a second, we used this possibility for the preparation of S1 derivatives with mixed substituents, for example, C_1 at SH1, and C_{12} at SH2/SH_{LC}, and vice versa. The graph in Figure 5 shows that a residual activity of ca. 30% results, regardless of wether the ATPase was initially increased (C_1) or decreased (C_{12}) by substitution at SH1. This suggests that Ca^{2+}-ATPase activity depends on the sum (probably of the masses) of the substituents at the two thiol atoms rather than on their position. This idea was substantiated by the graph in Figure 6 showing that the Ca^{2+}-ATPase activities, when plotted against the sum of the molar masses at SH1 and SH2, decreased continuously and independently of whether the substituents at SH1 and SH2 were the same or different.

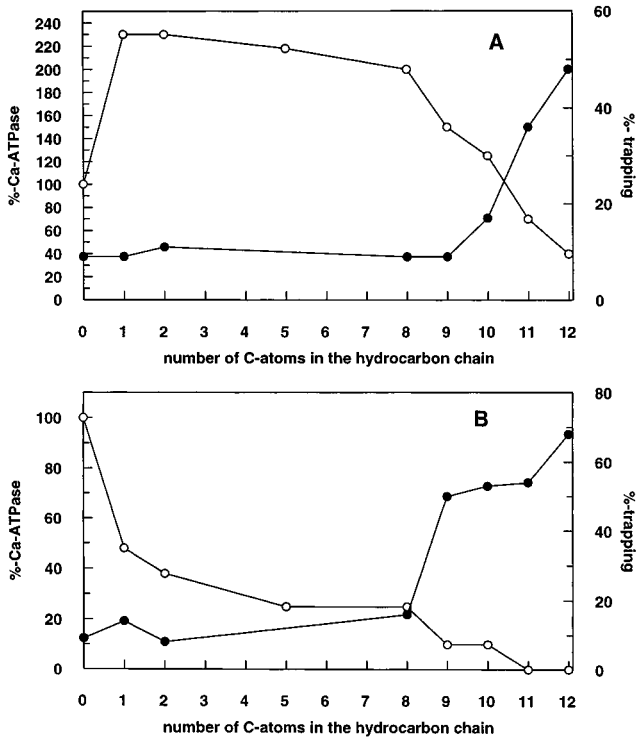

FIGURE 7: Ca^{2+}-ATPase activities [(○) native enzyme = 100%] and trapping of Mg[^{3}H]ADP (●), as dependent on the length of the hydrocarbon chain attached to SH1 alone (A), or to SH1, SH2, and SH_{LC} together (B).

Trapping of the Nucleotide. Trapping of the nucleotide is a phenomenon reported to occur when the two exposed thiol groups in myosin S1 are bridged by cross-linking reagents (*45*, *54*−*56*). We assayed here whether monovalent alkylthiolating reagents of increasing length, either at SH1 alone or at SH1 and SH2/SH_{LC} together, would cause trapping as well. As shown in Figure 7, native myosin S1 itself traps 8−10% of the [^{3}H]ADP under the assay conditions, an effect that was similarly reported by others (*57*). Trapping increased steeply, however, and rose to ca. 50% when the residues introduced became larger, as with C_{10}, C_{11}, and C_{12}, (Figure 7A). Interestingly, enhanced trapping started at a point where the Ca^{2+}-ATPase was no longer enhanced but lay close to, or below, the value of the native enzyme (100%). This point was reached with the reagent C_{10} (Figure 7A).

In experiments where both thiols, SH1 and SH2, were modified (Figure 7B), the inhibition of ATPase caused by small reagents such as C_1, C_2, C_5, or C_8 (down to ca. 25%), was not associated with trapping. Only with $n \geq 9$ was a steep increase in trapping observed, raising the amount of trapped nucleotide from ca. 15% to ca. 50%. Strongest trapping was seen when the large C_{12} residue was attached to both thiols (ca. 70% of buried nucleotide).

The conclusion to be drawn from these experiments is that inhibition of Ca^{2+}-ATPase grows with increasing size of the substituents, either at SH1 alone or at SH1 and SH2 together, but with small reagents is not accompanied by nucleotide trapping. Only with large residues, $n > 8$, was nucleotide trapping observed, which may be the cause of enzyme inhibition. Trapping was also studied for the mixed S1 derivatives (C_1/C_{12} and C_{12}/C_1). In a plot showing the variation of trapping with mass of the residue, the mixed

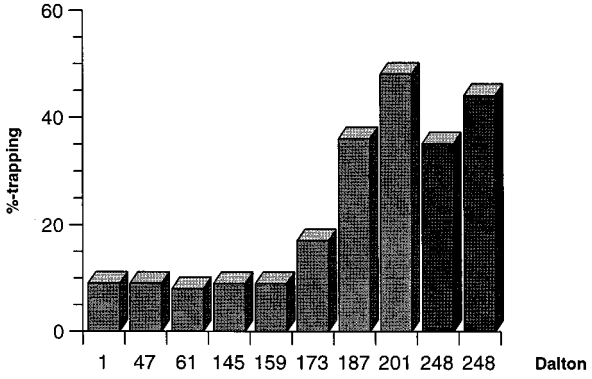

FIGURE 8: Percent trapping of nucleotide (native enzyme = 100%) of myosin S1 derivatives either monosubstituted at SH1 (light gray) or bisubstituted at SH1 and SH2 with C_1/C_{12} or C_{12}/C_1 (dark gray). 1 Da = unmodified; 47 Da = C_1; 61 Da = C_2; 145 Da = C_8; 159 Da = C_9; 173 Da = C_{10}; 187 Da = C_{11}; 201 Da = C_{12}; 248 Da = $C_1 + C_{12}$ or $C_{12} + C_1$. (Note that each of the substituents contains one sulfur atom.)

Table 3: Steady-State Kinetic Parameters of MgATPase of the Myosin S1 Derivatives Substituted Either at SH1 Alone or at SH1 and SH2 (and SH_{LC}) Together[a]

myosin derivative	basal (s^{-1})	K_m (μM)	V_{max} (s^{-1})
S1A1	0.069	26	18
S1A1(C_1)	0.140	10	7
S1A1(C_{12})	0.049	10	6
S1A1(C_1)$_3$	0.041	5	0.5
S1A1(C_{12})$_3$	0.006	2	0.03
S1A1(C_1)(C_{12})$_2$	0.025	3	0.4
S1A1(C_{12})(C_1)$_2$	0.013	7	0.4

[a] Values for k_m and V_{max} were calculated from fitting the data to the Michaelis−Menten equation. All measurements were performed at 25 °C. Abbreviations: unmodified S1, S1A1; C_1 at SH1, S1A1(C_1); C_{12} at SH1, S1A1(C_{12}); C_1 at SH1, SH2, and SH_{LC}, S1A1(C_1)$_3$; C_{12} at SH1, SH2, and SH_{LC}, S1A1(C_{12})$_3$; C_1 at SH1 and C_{12} at SH2, SH_{LC}, S1A1(C_1)(C_{12})$_2$; C_{12} at SH1 and C_1 at SH2, SH_{LC}, S1A1(C_{12})(C_1)$_2$.

Table 4: Equilibrium Dissociation Constants of Rabbit Muscle F-Actin and Myosin S1 Derivatives Substituted at SH1 or at Both SH1 and SH2 (and SH_{LC}), with Small Substituents (C_1), or Large Substituents (C_{12}), as Determined by Kinetic Analysis (k_{on}, k_{off}) at 20 °C[a]

myosin derivative	k_{on} 10^6 (M^{-1} s^{-1})	k_{off} (s^{-1})	$k_d = k_{off}/k_{on}$ (nM)
S1A1	0.6386	0.0973	152
S1A1(C_1)	0.3100	0.0311	100
S1A1(C_1)$_3$	0.3926	0.0291	74.1
S1A1(C_{12})	0.3589	0.0241	67.1
S1A1(C_{12})$_3$	0.5000	0.0249	49.8

[a] Abbreviations as in Table 3.

derivatives did not fit into the series of S1 derivatives modified at both thiols (not shown); they fitted much better into the series of derivatives with only monomodification at SH1 (Figure 8).

Kinetic Analysis. Some of the S1 derivatives substituted at SH1, or at SH1 and SH2/SH_{LC}, were submitted to kinetic analysis of their Mg^{2+}-ATPase activities, to determine the K_m and V_{max} values (Table 3) as well as the affinities to F-actin (Table 4).

Mg^{2+}-ATPase Assay. All myosin S1 derivatives were able to hydrolyze ATP in the presence of Mg^{2+} ions. Like the Ca^{2+}-ATPase, the Mg^{2+}-ATPase activity (Table 3, basal activity) depended on length and position of the thiol

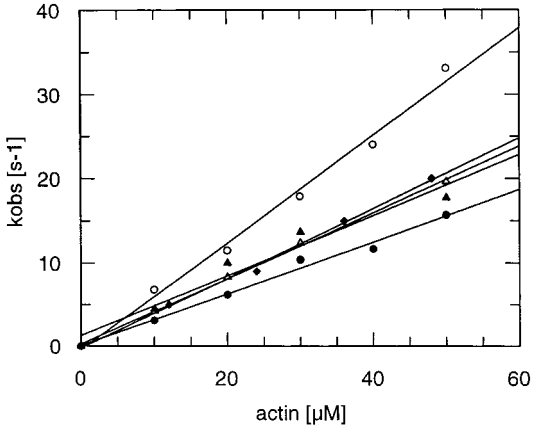

FIGURE 9: Dependence of actin binding of S1 (○) and four derivatives [S1−C_1 (●); S1-(C_1)$_3$ (△); S1−C_{12} (▲), and S1-(C_{12})$_3$ (◆)] on pyrene actin concentration measured with stopped-flow experiments. The data were fitted to a straight line, the slope of which gives a second-order binding constant (k_{on}, Table 4). All intercepts are close to zero since the rate of dissociation is slower than the rate of association determined by these plots.

reagents. Interestingly, in one of the derivatives, S1A1(C_1), substitution caused an activation of the basal ATPase, while all others showed decreased activities. While V_{max} was strongly reduced (≥10 times) by substitution at SH2, K_m was less sensitive to substitution at both SH1 and SH2 and decreased by factors of 2−3.

Actin Complexes of the Myosin Derivatives. The fluorescence of the pyrene reporter group is quenched by ca. 70% upon binding of myosin head fragments (*58*). We used this effect to monitor the rate of actin binding by observing the decrease of the pyrene fluorescence that follows mixing of the myosin derivatives (1.5 μM) with excess of pyrene-labeled actin. Fitting to two exponentials (*46*) gave a better fit than to one (*47*). Rates (k_{obs}) were plotted against actin concentration and found to vary linearly over the concentration range 10−50 μM actin (Figure 9). The second-order association rate constants (k_{on}) as obtained from the slopes (*46*) are listed in Table 4 for S1 and four selected derivatives. All derivatives show only slight differences relative to unmodified S1, indicating that actin binding of the derivatives was not significantly different from the native protein. The rate constant of actin dissociation (k_{off}) from the myosin derivatives and S1 under rigor conditions was determined by displacing pyrene actin from the pyrene actomyosin complex with an excess of unlabeled actin. The observed values could be fitted to a single exponential function and corresponded directly to k_{off} (Table 4). All k_{off} values of the derivatives were three to four times lower than that of the unmodified myosin S1. The equilibrium dissociation constants (K_d) for actin binding to our derivatives were calculated from the ratios of k_{on} and k_{off} (*47*, *59*), and found to be about three times lower than that of native S1. In general, the affinity to actin was the higher as more hydrophobic hydrocarbon material was introduced at the two thiol groups.

Sliding Motility of the Myosin Head Derivatives. In in vitro motility assays, we checked whether our derivatives were able to support movement of F-actin filaments, i.e., had the capacity to generate mechanical force. For the native enzyme, we measured sliding velocity values between 1 and 4 μm/s (Figure 10). This value was essentially not changed when DTT was added. In contrast, none of the derivatives,

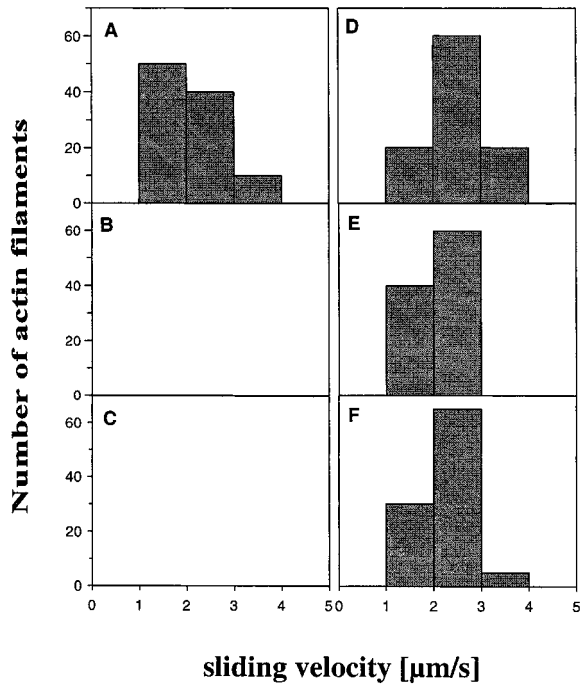

FIGURE 10: Sliding velocity of actin filaments over native skeletal myosin S1 (A), myosin S1 after methylthiolation of SH1 (B), and after methylthiolation of both SH1 and SH2 (C) using the reagent C_1. Corresponding experiments in the presence of DTT (D, E, F). The number of filaments observed was 100 in each case.

modified either at SH1 alone, or at SH1 and SH2 together, exhibited any sliding motility. This indicates that modification of only SH1, even with the smallest reagent available, suffices to abolish completely the sliding motility capacity. Figure 10 also shows that sliding motility was almost completely restored when the experiments were performed in the presence of DTT. This proves that all modifications made with our reagents were fully reversible and allowed recovery of the active myosin head after reductive cleavage of the substituents.

DISCUSSION

Reagents. Several members of the reagent family used in this study, like $H(CH_2)S-NTB$, $H(CH_2)_2S-NTB$, and $H(CH_2)_8S-NTB$, were investigated in previous studies (*60*, *61*). It was shown, for example, that the reagents can be used, like Ellman's reagent, to determine the number of exposed thiol groups in a protein. In such reactions, the octyl reagent, $H(CH_2)_8S-NTB$, yielded the same results as Ellman's reagent but at distinctly shorter reaction times, thus diminishing denaturation effects and possible exposure of thiol groups normally buried (*49*). As shown with papain, for example, the reagents were likewise useful for blocking essential thiol groups in the active site of an enzyme under spectrophotometric control. The biological activity of the inhibited enzyme could easily be restored when excess of low molecular weight thiol compounds, like cysteine (*49*), or DTT, was added. The most promising application of this family of reagents so far was, however, their use for introducing selected residues at defined positions in thiol-exposing proteins under spectrophotometric control, as shown for example for cys374 of actin and in studying the functional changes caused by the residues introduced (*60*, *61*).

In the present study, we extended the latter approach to myosin S1. For this, we first prepared some missing members of the reagent family C_n with, e.g., $n = 5$, 9, 10, 11, and 12, which completed the series of our reagents. In total, we had at our disposal reagents of 3.9−20.4 Å in length, corresponding to 47−201 Da in mass. This series was introduced at SH1 and SH2 of the myosin head in order to study ATPase activity, nucleotide trapping, and sliding motility in dependence of the growing size of the reagents. We took advantage of the fact that all reagents of the series differed in length or mass but were similar in chemical nature, a situation that would allow us to correlate functional changes to exclusively the parameter of size. Clearly, such a correlation is more easily established when the reagents differ in size only and not in chemical nature as well, as in many previous studies. Since the size of reagents changed in small steps only, we expected that functions of myosin S1 affected would be modulated in a more or less continuous manner also. From continuously changing functional parameters, in correlation with the continuously changing size of residues at defined positions, we wanted to gain insight into structural alterations attributable to ATPase inhibition, nucleotide trapping, or impairment of energy transmission. Crystallographic data now available for the myosin head (*4*−*7*, *34*) play a key role in this attempt.

Selectivity of Substitutions at SH1 and SH2. In rabbit muscle myosin S1, the thiol group of cysteine 707 (SH1) reacts fastest. A preference of most of the reagents for this thiol group is well documented in the literature and seems to be independent of the chemical nature of the reagent (*17*−*27*, *33*). Even cross-linking reagents (*31*, *62*), including Ellman's reagent (*30*), were shown to react with this thiol group first. On the basis of this evidence, we believed that the reagents investigated in this study would also preferentially react with SH1. This was indeed the case, as concluded from the significantly enhanced Ca^{2+}-ATPase activity found after reaction of 1 equiv of C_1, for example, amounting to 230%, a value that is in good accordance with corresponding values reported for other small-sized thiol reagents (*33*, *63*). Further proof for this was given by fragmentation and identification of the thiol groups involved in the reaction.

Enhancement of Ca^{2+}-ATPase activities in this range was also seen for longer reagents such as C_2, C_5, and C_8, but not for C_9 or larger, which reduced the Ca^{2+}-ATPase activation effect. This reduction of the activation effect seemed to be caused by the increasing length or mass of the reagents, rather than by a partial modification of SH2. The latter possibility was ruled out by the observation that the Ca^{2+}-ATPase values obtained after reaction of 1 equiv of C_{10} or C_{12} in the presence of MgADP were roughly equal to those obtained with 2 equiv of these reagents in the absence of nucleotide, where, according to numerous previous studies (*50*, *62*, *64*), SH2 is excluded from reaction. Proof of exclusive and complete reaction of SH1, not only with short (C_1) but also with long (C_{12}) reagents, came finally from the finding that after the release of 1 equiv of NTB^-, both C_1 and C_{12} produced full inhibition of K^+(EDTA)-ATPase (Figure 2).

While selective modification of SH1 in the presence of SH2 and SH_{LC} is possible, specific modification of SH2 in the presence of SH_{LC} is not. We found that the major part of the second equivalent of reagent added may have reacted with SH2, but that SH_{LC} also reacted to some extent (data

not shown). Such undefined mixtures of SH2 and SH_{LC} derivatives were not further investigated, but were reacted with a third equivalent of reagent, thus completing the modification of SH2 and SH_{LC}. Since the literature does not report any other thiol group reactive under these conditions [reaction of cys522 (*51*, *52*, *65*), for example, required higher temperature and was observed under cross-linking conditions only, we assumed that the myosin S1 species obtained after full reaction of 3 equiv of reagents were well-defined, representing myosin S1 derivatives with modification at SH1 and SH2 [as well as at SH_{LC}, which does not affect ATPase activity (*66*)].

Functional Defects after Substitution at SH1. Activation of Ca^{2+}-ATPase appears to be a direct consequence of SH1 modification. Since its discovery by Kielley et al. (*16*), this effect has frequently been used to show that modifications in myosin S1 had occurred at the fast-reacting thiol group. In the present study, the smallest substituents used, C_1 (3.9 Å, 47 Da), C_2 (5.4 Å, 61 Da), caused the strongest activation of Ca^{2+}-ATPase. This activation decreased only slightly with reagents such as C_5 (9.9 Å, 61 Da) and C_8 (14.4 Å, 145 Da). Significant reduction of the effect was seen when the reagents grew to greater than or equal to C_9. With the reagent C_{10}, for example, Ca^{2+}-ATPase was reduced to the activity of the native enzyme (ca. 100%), but the state of the enzyme reached at that point is probably not the same as that of the unmodified myosin S1. Further elongation of the substituents, as with C_{11} and C_{12}, caused further deactivation of the enzyme to below the activity of the native enzyme. It is worth noting that it was only at this point that significant trapping of nucleotide was observed. This happened at a length of around 18 Å or a mass of ca. 190 Da (C_{11}). The coincidence between nucleotide trapping and the value of *n* at which Ca^{2+}-ATPase fell below the native value suggested that the smaller reagents had no significant effect on nucleotide binding or release of the reaction products. On the other hand, larger reagents slowed the release of products from the active site. Considering the length of the reagents and the distance between SH1 and the nucleotide-binding site, we believe that the interaction of the reagents is not a direct one but is mediated by domains located between SH1 and the active site as, for example, the SH2 helix, which may contact the reagents. Such interaction finally caused ADP trapping; this effect may be understood as steric hindrance of domains that must move in order to achieve release of the nucleotide.

In in vitro motility assays, sliding motility of the S1 fragment is significantly lower than that of HMM (*3*) but large enough to show whether modification of thiol groups in myosin S1 with our reagents impaired mechanical quality. In agreement with findings of Reisler's group (*67*, *68*) and Marriott and Heidecker (*69*), we found that selective modification of SH1, even with the smallest reagent of our series, caused a complete loss of mechanical competence. Steric perturbation as exerted by a residue composed of only one sulfur atom and a methyl group appears to lead to complete loss of sliding motility, a finding that points to the thiol region as a key player in energy transmission. As outlined above, loss of sliding mobility is accompanied by a 2−3-fold increase in ATPase activity. This coincidence suggests that modification of SH1 may cause uncoupling of the active site from energy-transmitting parts of the motor protein, which, similar to an idling engine, would cause an increase of ATPase turnover rates.

The importance of the thiol region for energy transmission was also suggested by recent atomic models of the myosin head (*4*−*7*, *34*), which may provide the basis for relating biochemical and structural data. According to one of these structures, the atomic structure of scallop myosin S1 (*34*), part of the thiol region, the so-called SH1-helix, can lose its helical structure as concluded from the finding that the region corresponding to the SH1 helix remained undefined in the X-ray analysis. This observation may be of importance for understanding myosin function, since transmission of conformational ernergy in a motor protein is more likely to be achieved by a rigid helix than by a flexible loop. Provided the SH1 helix really functions in this way and provided also that substitution of SH1 disturbs the formation of the SH1 helix, chemical modification at SH1 could explain the mechanical incompetence observed in S1 derivatives modified at this thiol group. This speculation seems reasonable since it was suggested that the SH1 helix is stabilized by the HP helix via a contact area located around the thiol group of SH1 (*34*). Clearly, it would be of great interest to crystallize such S1 derivatives, to see whether modification at SH1 indeed causes unwinding of the SH1 helix.

Ca^{2+}-ATPase and Trapping after Modification of Both SH1 and SH2. Modifications at SH2, which according to atomic models is much closer to the nucleotide binding site, have a greater effect on ATP turnover than modifications at the remote thiol group of SH1. Thus, activity fell to ca. 50% and ca. 40% for the C_1 derivative and the C_2 derivative, respectively. While in derivatives modified only at SH1 the inhibitory effect may be an indirect one, modifiers at SH2 may come close enough to directly interfere with the active site. Full inhibition of the enzyme after modification of SH1 and SH2 was seen when reagents exceeded a length of 14−16 Å ($\geq C_9$). In derivatives modified at SH1 and SH2, similar to those modified at SH1 alone, the critical point was reached with reagents with $n > C_9-C_{10}$, which caused nucleotide trapping in both cases. Again, the most reasonable explanation for this effect is a mechanical disturbance of the mobility of the thiol region: while small residues cause a partial immobilization of the thiol region, larger residues such as C_{10}, C_{11}, and C_{12} apparently lead to a complete, or almost complete, immobilization of this region.

Direct evidence for mobility of the thiol region as an essential parameter came from our experiments with mixed substituents at SH1 and SH2, (C_1/C_{12} and C_{12}/C_1), which were almost equal to each other with respect to Ca^{2+}-ATPase activity and nucleotide trapping (Figures 5 and 8). This indicates that the disturbance caused by modification of SH1 and SH2 is correlated with the sum (of the masses) of the two substituents rather than with the position they occupy, and suggests that, correspondingly, functional parameters attributable to the whole thiol region may be of importance in this respect. One such parameter could be the ability of the thiol region to adopt a conformation necessary for enzyme function, which, with increasing mass attached to the thiol region, may form less readily. Another parameter changed by the modification may be the mobility of the thiol region. While small residues at SH1 and SH2 could cause a restriction of mobility associated with a slowing of the ATPase, large residues could cause complete immobilization of dy-

namic structures of the enzyme leading to trapping of the nucleotide and total inhibition of the ATPase, as observed.

We cannot distinguish whether the disturbed mobility is caused by steric effects or mass effects, as outlined above. A third mode of interaction, the unspecific hydrophobic interaction between long reagents at SH1 and/or SH2 and neighboring hydrophobic domains, can also not be excluded. The latter explanation seems possible because the strongest effects were observed with reagents greater than or equal to C_8, a chain length at which a sudden increase in hydrophobicity occurs, as shown in Table 2. As for the S1 derivatives modified at SH1, the derivatives with two thiol groups modified would be interesting candidates for X-ray analysis, which would also reveal the mode of interaction of the residues at the two thiols with functional domains of the protein.

ACKNOWLEDGMENT

We thank Prof. K. C. Holmes for continuous support; G. Helmig for kindly providing actin acetone powder and myosin; S. Zobeley for technical assistance; J. Wray for critical reading of the manuscipt; M. Furch for his help with the stopped-flow experiments; S. Fujita-Becker for friendly support with the in vitro motility assays; and R. Bruns and T. Möhring (University of Oldenburg), and D. Schmid and Prof. G. Jung (University of Tübingen) for measuring the NMR, and MS data, respectively.

REFERENCES

1. Mueller, H., and Perry, SV. (1962) *Biochem. J. 85*, 431−436.
2. Mornet, D., Bonet, A., Audemard, E., and Bonicel, J. (1989) *J. Muscle Res. Cell Motil. 10*, 10−24.
3. Toyoshima, Y. Y., Kron, S. J., McNally, E. M., Niebling, K. R., Toyoshima, C., and Spudich, J. A. (1987) *Nature 328*, 536−539.
4. Rayment, I., Rypniewski, W. R., Schmidt-Base, K., Smith, R., Tomchik, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G., and Holden, H. M. (1993) *Science 261*, 50−58.
5. Smith, C. A., and Rayment, I. (1996) *Biochemistry 35*, 5404−5417.
6. Fisher, A. J., Smith, C. A., Thoden, J. B., Smith, R., Sutoh, K., Holden, H. M., and Rayment, I. (1995) *Biochemistry 34*, 8960−8972.
7. Houdusse, A., and Cohen, C. (1996) *Stucture 4*, 21−32.
8. Holmes, K. C. (1997) *Curr. Opin. Struct. Biol. 6*, 781−789.
9. Holmes, K. C. (1997) *Curr. Biol. 7*, R112−R118.
10. Dominguez, R., Freyzon, Y., Trybus, K. M., and Cohen, C. (1998) *Cell 94*, 559−571.
11. Whittaker, M., Wilson-Kubalek, E. M., Smith, J. E., Faust, L., Miligan, R. A., and Sweeney, H. L. (1995) *Nature 378*, 748−751.
12. Barany, M., and Barany, K. (1959) *Biochim. Biophys. Acta 35*, 293−309.
13. Patterson, B., and Spudich, J. A. (1996) *Genetics 143*, 801−810.
14. Kinose, F., Wang, S. X., Kidambi, U. S., Moncman, C. L., and Winkelmann, D. A. (1996) *J. Cell Biol. 134*, 895−909.
15. Batra, R., Geeves, M. A., and Manstein, D. J. (1999) *Biochemistry 38*, 6126−6134.
16. Kielley, W. W., and Bradley, L. B. (1956) *J. Biol. Chem. 208*, 653−659.
17. Miyanishi, T., and Borejdo, J. (1989) *J. Biochem. 28*, 1287−1294.
18. Root, D. D., Cheung, P., and Reisler, E. (1991) *Biochemistry 30*, 286−294.
19. Takashi, R., Duke, J., Ue, K., and Morales, M. F. (1976) *Arch. Biochem. Biophys. 175*, 279−283.
20. Kunz, P. A., Walser, J. T., Watterson, J. G., and Schaub, M. L. (1977) *FEBS Lett. 83*, 137−140.
21. Muhlhern, S. A., and Eisenberg, E. (1978) *Biochemistry 17*, 4419−4425.
22. Borejdo, J., Putman, S., and Morales, M. F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6346−6350.
23. Lu, R. C., Moo, L., and Wong, A. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6392−6396.
24. Ajtai, K., and Burghardt, T. P. (1989) *Biochemistry 28*, 2204−2210.
25. Bailin, G., and Bárány, M. (1972) *J. Biol. Chem. 247*, 7815−7821.
26. Muhlrad, A., Lamed, R., and Oplaka, A. (1975) *J. Biol. Chem. 250*, 175−181.
27. Sekine, T., Takahashi, S., Hikita, S., Sutoh, N., and Satake, K. (1984) *J. Biochem. 96*, 27−33.
28. Hiratsuka, T. (1993) *J. Biol. Chem. 268*, 24742−24750.
29. Nitao, L. K., and Reisler, E. (1998) *Biochemistry 37*, 16704−16710.
30. Reisler, E. (1980) *J. Mol. Biol. 138*, 93−107.
31. Kliche, W., Pfannstiel, J., Stoeva, S., Tiepold, M., and Faulstich, H. (1999) *Biochemistry 38*, 10307−10317.
32. Wells, J. A., and Yount, R. G. (1980) *Biochemistry 19*, 1711−1717.
33. Reisler, E. (1982) *Methods Enzymol. 85*, 84−93.
34. Houdusse, A., Kalabokis, V. N., Himmel, D., Szent-Györgyi, A. G., and Cohen, C. (1999) *Cell 97*, 459−470.
35. Organicum, Deutscher Verlag der Wissenschaften, Berlin (1990) 18. Auflage, pp 206−207.
36. Margossian, S. S., and Lowey, S. (1982) *Methods Enzymol. 85*, 55−71.
37. Weeds, A. G., and Taylor, R. S. (1975) *Nature 257*, 54−55.
38. Weeds, A. G., and Pope, B. (1977) *J. Mol. Biol. 111*, 129−145.
39. Wagner, P. D., and Weeds, A. G. (1977) *J. Mol. Biol. 109*, 455−473.
40. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
41. Spudich, J. A., and Watt, J. (1971) *J. Biol. Chem. 246*, 4866−4871.
42. Kodama, T., Fukui, K., and Kometani, K. (1986) *J. Biochem. 99*, 1465−1472.
43. Trentham, D. R., Bardsley, R. G., Eccleston, J. F., and Weeds, A. G. (1972) *Biochem. J. 126*, 635−644.
44. Elzinga, M., and Collins, J. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4281−4284.
45. Wells, J. A., and Yount, R. G. (1982) *Methods Enzymol. 85*, 93−123.
46. Furch, M., Geeves, M. A., and Manstein, D. J. (1998) *Biochemistry 37*, 6317−6326.
47. Criddle, A. H., Geeves, M. A., and Jeffries, T. (1985) *Biochem. J. 232*, 343−349.
48. Kron, S. J., Yoyoshima Y. Y., Uyeda T. Q. P., and Spudich J. A. (1991) *Methods Enzymol. 196*, 399−416.
49. Faulstich, H., Tews, P., and Heintz, D. (1993) *Anal. Biochem. 208*, 357−362.
50. Yamaguchi, M., and Sekine T. (1966) *J. Biochem. (Tokyo) 59*, 24−33.
51. Mornet, D., Ue, K., and Morales, M. F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1658−1662.
52. Chaussepied, P., Morales, M. F., and Kassab, R. (1988) *Biochemistry 27*, 1778−1785.
53. Ue, K. (1987) *Biochemistry 26*, 1889−1894.
54. Wells, J. A., Sheldon, M., and Yount, R. G. (1980) *J. Biol. Chem. 255*, 1598−1603.
55. Liang, W., and Spudich, J. A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 12844−12847.
56. Cremo, C. R., Neuron, J. M., and Yount, R. G. (1990) *Biochemistry 29*, 3309−3319.

57. Wells, J. A., and Yount R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966−4970.
58. Ritchie, M. D., Geeves, M. A., Woodward, S. K. A., and Manstein, D. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8619−8623.
59. Marston, S. B. (1982) *Biochem. J. 203*, 453−460.
60. Faulstich, H., and Heintz, D. (1995) *Methods Enzymol. 251*, 357−366.
61. Drewes, G., and Faulstich, H. (1993) *Eur. J. Biochem. 212*, 247−253.
62. Polosukhina, K., and Highsmith, S. (1997) *Biochemistry 36*, 11952−11958.
63. Sekine, T., and Kielley, W. W. (1964) *Biochim. Biophys. Acta 81*, 336−345.
64. Phan, B. C., Peyser, Y. M., Reisler, E., and Muhlrad, A. (1997) *Eur. J. Biochem. 243*, 636−642.
65. Agarwald, R., and Burke, M. (1991) *Arch. Biochem. Biophy. 290*, 1−6.
66. Marsh, D. J., and Lowey, S. (1980) *Biochemistry 19*, 774−784.
67. Root, D. D., and Reisler, E. (1992) *Biophys. J. 63*, 730−740.
68. Bobkov, A. A., Bobkova, E. A., Homsher, E., and Reisler E. (1997) *Biochemistry 36*, 7733−7738.
69. Marriott, G., and Heidecker, M. (1996) *Biochemistry 35*, 3170−3174.

BI991199Z