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Biochemistry

Nucleotide trapping at the ATPase site of myosin subfragment 1 by a new interthiol crosslinking

(myosin head/interdomain interactions/reactive thiol)

PATRICK CHAUSSEPIED, DOMINIQUE MORNET, AND RIDHA KASSAB*

Centre de Recherches de Biochimie Macromoléculaire du Centre National de la Recherche Scientifique, Route de Mende, BP 5051, 34033 Montpellier Cedex, France

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ABSTRACT **When myosin subfragment 1 derivatives in which the reactive sulfhydryl SH1 has been blocked react with *N,N'-p*-phenylenedimaleimide or 5,5'-dithiobis(2-nitrobenzoic acid), the reactive sulfhydryl group SH2 of the 20-kDa domain is crosslinked with a thiol of the 50-kDa domain of the heavy chain. The crosslink induces the stable trapping of a significant amount of Mg^{2+}-nucleotide in the ATPase site.**

Energy transduction by actomyosin in muscle involves different conformations of the myosin head–nucleotide complexes formed by the hydrolysis of ATP and interacting with actin during the crossbridge cycle (1–4). A stable myosin-head form simulating the subfragment 1 (S-1)–ATP intermediate could be produced by covalent union of the reactive SH1 and SH2 thiols residing in a flexible peptide region within the 20-kDa carboxyl-terminal domain of the head (5). The bridging of these two sulfhydryls by any of several crosslinkers promotes the trapping of Mg^{2+}-nucleotide at the ATPase site (6–10). The nature of the structural changes linked to tight nucleotide binding to S-1 is unknown. Using the bifunctional fluorescent agent dibromobimane, Mornet *et al.* (11) have recently obtained another intramolecular crosslinking in S-1, which involves the SH1 group and a thiol located in the 50-kDa heavy-chain fragment. However, the covalent union of these two sulfhydryls did not result in a significant stable trapping of Mg^{2+}-nucleotide. Here we report the discovery of a new interthiol crosslinking process that occurs between the SH2 group and a thiol, designated SH*x*, located in the central, 50-kDa head region; this process leads to the entrapment of 0.60–0.80 molecule of Mg^{2+}-ADP in the active site. It is achieved by treating SH1-blocked S-1 with the usual crosslinkers, *N,N'-p*-phenylenedimaleimide [$Ph(NMal)_2$] and 5,5'-dithiobis(2-nitrobenzoic acid) (Nbs_2) in the presence of Mg^{2+}-ADP (12). The SH2–SH*x* crosslinked protein may represent a new S-1–nucleotide state. Its formation reveals the prominent cooperative role of the SH2 site and the proximal domain of 50 kDa in modulating the conformation of the ATPase site. This derivative is valuable, as it can include SH1 either free or substituted by various spectral probes.

MATERIALS AND METHODS

Chemicals. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin and untreated α-chymotrypsin were from Worthington. $Ph(NMal)_2$, ATP, and ADP were from Sigma. [^{14}C]ATP and [^{14}C]ADP were purchased from New England Nuclear. 1,N^6-Ethenoadenosine 5'-diphosphate and 5'-triphosphate were obtained from Pharmacia (Uppsala). All other chemicals were of the highest analytical grade.

S-1 and Actin. Rabbit skeletal myosin was prepared as described by Offer *et al.* (13). S-1 was prepared by digestion of myosin filaments with α-chymotrypsin (14) and was purified as usually (15). The split S-1 obtained by trypsin cleavage (S-1/trypsin weight ratio 1:25, at 25°C for 30 min in 0.05 M Tris Cl, pH 8.0) was purified as described by Mornet *et al.* (15). The concentration of the S-1 preparations was estimated by using $A_{280}^{1\%} = 7.5$ as described by Wagner and Weeds (16).

Rabbit skeletal muscle actin was prepared as described by Eisenberg and Kielley (17), and its concentration was estimated by using $A_{280}^{1\%} = 11.0$.

Crosslinking Reactions. The reaction of 1.3-fold excess $Ph(NMal)_2$ in 50 mM Hepes (pH 8.0) was done with 50 μM modified or native enzyme in the presence or absence of 2.5 mM Mg^{2+}-ATP or Mg^{2+}-ADP at 4°C. The crosslinking of S-1 with Nbs_2 was performed under similar conditions, using a 5-fold excess of reagent. The reactions were quenched and the protein derivatives were purified by filtration over Sephadex G-50 (20 × 1.5 cm) in 50 mM Hepes, pH 7.0/200 mM KCl/0.5 mM NaN_3 at 4°C.

$NaDodSO_4$/PAGE. Tryptic fragments of modified S-1 were separated by electrophoresis in 0.1% $NaDodSO_4$/polyacrylamide slab gels containing a 5–18% (wt/vol) gradient of acrylamide (18). The running buffer was 50 mM Tris/100 mM boric acid (pH 8.0) (19). The densitometric scanning of the gels was carried out with a computerized model CS930 Shimadzu (Kyoto, Japan) gel scanner. The following were used as molecular mass markers: S-1 heavy chain (95 kDa), S-1 light chain 1 (LC1, 25 kDa), S-1 light chain 3 (LC3, 17 kDa), the three fragments obtained by tryptic cleavage of S-1 (50 kDa, 27 kDa, and 20 kDa), and actin (42 kDa).

Specific Modifications of S-1 Thiols. The specific fluorescence labeling of SH1 with 5-[2-(iodoacetyl)aminoethyl]aminonaphthalene-1-sulfonic acid (1,5-IAEDANS) was done according to the procedure of Takashi *et al.* (20). Under our conditions, 1.1 mol of the dye was incorporated per mol of S-1. Fluorescence gel scanning allowed us to quantify the labeling ratio as 0.2 in the light chains and 0.9 in the heavy chain.

The specific chemical modifications of SH1 by 2,4-dinitrofluorobenzene (N_2ph-F) and iodoacetamide were carried out as described by Reisler *et al.* (21) and by Kunz *et al.* (22), respectively. The thiolysis of N_2ph-S-1 was accomplished by incubation of 35 μM protein in 50 mM Hepes buffer (pH 8.0) in the presence of 10 mM dithioerythritol for 30 min at 20°C.

Thiol and Disulfide Titration. The total thiol content of native and modified S-1 in 0.1 M Hepes buffer (pH 8.0) was

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Abbreviations: S-1, subfragment 1; 1,5-IAEDANS, 5-[2-(iodoacetyl)-aminoethyl]aminonaphthalene-1-sulfonic acid; LC, light chain; Nbs_2, 5,5'-dithiobis(2-nitrobenzoic acid); $Ph(NMal)_2$, *N,N'-p*-phenylenedimaleimide; N_2ph, dinitrophenyl.

*To whom reprint requests should be addressed.

measured by using Nbs_2 (23) in the presence of 5 M guanidinium chloride. The disulfide bonds were determined from the absorbance at 412 nm of the thionitrobenzoate (Nbs) released during reaction of 5-fold excess Nbs_2 with the nondenatured proteins and after 30-min treatment of the purified Nbs_2-modified S-1 with 5 mM dithioerythritol at pH 8.0 and 20°C.

S-1 ATPase Activities. The K^+/EDTA- and Ca^{2+}-dependent ATPase activities of S-1 were measured as described (24).

RESULTS

Nucleotide-Dependent Inactivation of SH1-Blocked S-1 by $Ph(NMal)_2$ and Nbs_2. In a first step, the reactive SH1 group of chymotryptic S-1 (containing LC1 and LC3) was protected by three reagents that react quantitatively and specifically with this thiol. These were the fluorescent dye 1,5-IAEDANS (20), iodoacetamide (22), and N_2ph-F (21); N_2ph-F allows a reversible blocking of the SH1 thiol (25). The isolated S-1 derivatives exhibited about 5% the K^+-ATPase activity of unmodified S-1, whereas their Ca^{2+}-ATPase was enhanced to 350–450% the control value. Thiol titrations with Nbs_2 indicated for each derivative the loss of at least 0.9 mol of SH per mol of S-1. Thus, only SH1 was blocked, while SH2 remained unlabeled.

The ATPases of premodified S-1 derivatives were then inactivated by $Ph(NMal)_2$ in the absence or presence of Mg^{2+}-nucleotide (ATP or ADP) under conditions that would be employed for SH1–SH2 crosslinking (12). Native S-1 was the control (Fig. 1*A*). In both AEDANS-S-1 and N_2ph-S-1 the presence of Mg^{2+}-ATP considerably accelerated the inactivation by $Ph(NMal)_2$ ($t_{1/2}$ < 2 min). The accelerations were even slightly higher than for unmodified S-1. In the absence of nucleotide, the N_2ph-S-1 was more sensitive to $Ph(NMal)_2$ than was AEDANS-S-1. S-1 substituted with iodoacetamide behaved much like AEDANS-S-1.

The ATPases of the SH1-blocked S-1 derivatives were also inactivated with Nbs_2 (Fig. 1*B*). No alteration of the elevated Ca^{2+}-ATPase of AEDANS-S-1 and N_2ph-S-1 occurred in the absence of Mg^{2+}-nucleotide, whereas a progressive increase of the original Ca^{2+}-ATPase of the native S-1 control was observed, as expected, owing to the rapid primary reaction of Nbs_2 with the free SH1 (9). In contrast, the addition of Mg^{2+}-nucleotide resulted in a rapid inactivation of the premodified S-1 derivatives, but at a rate lower ($t_{1/2}$ = 10–15 min) than with $Ph(NMal)_2$; during the first 70 min of reaction, $Ph(NMal)_2$ seems effective only in the presence of Mg^{2+}-nucleotide. The nucleotide-mediated inactivation of the Ca^{2+}-ATPase of the SH1-blocked S-1 molecules by $Ph(NMal)_2$ and Nbs_2 is consistent with the idea that these reagents are reacting with the critical SH2 thiol (25).

Nucleotide Trapping in $Ph(NMal)_2$- or Nbs_2-Treated SH1-Blocked S-1. Fig. 2 shows that the inhibition of AEDANS-S-1 by $Ph(NMal)_2$ or Nbs_2 is correlated with the incorporation of 0.80 and 0.70 mol of ^{14}C-labeled nucleotide per premodified S-1, respectively. As observed previously for native S-1, the nucleotide trapping was less than stoichiometric. Although the occurrence of some nonspecific modification cannot be directly ruled out, it is more likely that the substoichiometry results from the presence of S-1 molecules with altered active sites, as has been reported for the trapping via the SH1–SH2 crosslink (12, 26). Also, when the nucleotide employed was [^{32}P]ATP, only the ADP moiety was trapped (8, 12). When the binding of 1,N^6-ethenoadenosine 5′-triphosphate and 5′-diphosphate to $Ph(NMal)_2$-(AEDANS)-S-1 and to $Ph(NMal)_2$-S-1 was tested, using the acrylamide fluorescence quenching technique (27), no significant binding of the analogs to either of the two proteins was observed, strongly suggesting that the previously trapped nucleotide was bound at the active site of the SH1-blocked S-1 and not at a secondary site. Table 1 shows that the maximal values of [^{14}C]ATP and [^{14}C]ADP trapping within AEDANS-S-1 and N_2ph-S-1 by $Ph(NMal)_2$ and Nbs_2 compare well with those obtained for the native S-1 control (0.60–0.80 nucleotide/S-1). More trapping occurred at pH 8.0 than at pH 6.5. The $t_{1/2}$ of nucleotide release at 4°C, pH 7.0, was 3–4 days for

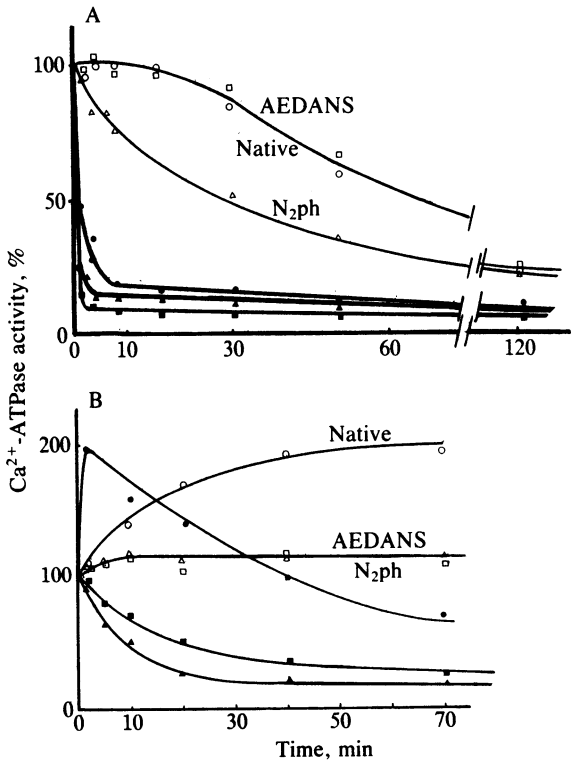

FIG. 1. Time-course of the inactivation of the Ca^{2+}-ATPase of various S-1 preparations (50 μM enzyme) in the absence (open symbols) and in the presence (filled symbols) of 2.5 mM Mg^{2+}-ATP, in 50 mM Hepes buffer at pH 8.0 and 4°C. (*A*) Inactivation with a 1.3-fold molar excess of $Ph(NMal)_2$. (*B*) Inactivation with a 5-fold molar excess of Nbs_2. Squares, AEDANS-S-1; triangles, N_2ph-S-1; circles, native S-1. Initial (100%) Ca^{2+}-ATPase activities (μmol of P_i per min per mg of protein) were as follows: AEDANS-S-1, 2.8; N_2ph-S-1, 2.6; native S-1, 0.7.

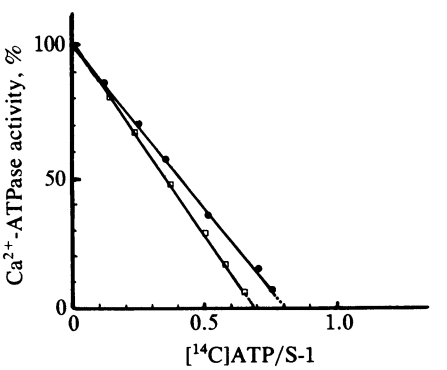

FIG. 2. Percentage of Ca^{2+}-ATPase activity remaining versus [^{14}C]ATP trapped per SH1-blocked S-1 during reaction with $Ph(NMal)_2$ (●) or Nbs_2 (□). AEDANS-S-1 was incubated for 80 min with $Ph(NMal)_2$ (0- to 1.3-fold molar excess) or 16 hr with Nbs_2 (0- to 10-fold excess), in the presence of 2.5 mM Mg^{2+}-[^{14}C]ATP (2500 cpm/nmol) under the conditions described for Fig. 1. S-1 samples were isolated by gel filtration and were assayed for ATPase activity and radioactivity.

Table 1. Efficiency of Mg^{2+}-nucleotide trapping in native S-1 and SH1-blocked S-1 by $Ph(NMal)_2$ and Nbs_2

		No. of cystine disulfides	No. of titratable SH groups	Mg^{2+}-[^{14}C]ATP, mol/mol of S-1	Mg^{2+}-[^{14}C]ADP, mol/mol of S-1
Native S-1	$Ph(NMal)_2$		7.5 (9.7)*	0.63	0.80
	Nbs_2	1.0 ± 0.2		0.65	0.80
AEDANS-S-1	$Ph(NMal)_2$		6.3 (8.6)*	0.60	0.70
	Nbs_2	1.1 ± 0.2		0.67	0.75
N_2ph-S-1	$Ph(NMal)_2$			0.62	0.76, 0.73†
	Nbs_2	0.9 ± 0.2		0.59	0.76

Trapping experiments were carried out as described for Fig. 2, except that $Ph(NMal)_2$ was incubated with native S-1 for 45 min and with SH1-blocked S-1 for 80 min. Data are averages of 2 or 3 experiments. SD for trapping measurements is 0.05.

*Values in parentheses represent total thiol group before reaction with $Ph(NMal)_2$.

†[^{14}C]ADP trapped in $Ph(NMal)_2$-(N_2ph)-S-1 before and after thiolysis, respectively.

$Ph(NMal)_2$-(AEDANS)-S-1 and 7–8 days for the $Ph(NMal)_2$-S-1 control. This suggests that the enzyme conformation around the bound nucleotide is not identical in the two kinds of complexes. Also, a total release of trapped Mg^{2+}-[^{14}C] nucleotide from $Ph(NMal)_2$-(AEDANS)-S-1 was obtained after mixing with a 5-fold molar excess of F-actin for 20 min at pH 7.6 and 22°C (μ = 25 mM), whereas a 20-molar excess of actin was needed under these conditions to expel the nucleotide from the $Ph(NMal)_2$-S-1 control. This suggests that the two S-1–nucleotide complexes have different affinities for actin, as will be shown elsewhere.

Relationship Between Nucleotide Trapping and the Crosslinking Between the 50-kDa and 20-kDa Fragments. The results in Table 1 indicate that in spite of the blocking of the SH1 thiol in AEDANS-S-1 and N_2ph-S-1, the treatment of these derivatives with Nbs_2 leads to the formation of a protein disulfide bond, as previously found for the native S-1 (9). As reported elsewhere (28), no such cystine bond was detected on reaction of Nbs_2 with the $Ph(NMal)_2$-S-1 control in which both SH1 and SH2 are masked. Also, the reaction of $Ph(NMal)_2$ with AEDANS-S-1 led to the disappearance of 2 thiols per molecule of S-1 derivative. These data suggest that the nucleotide trapping that we observed with the SH1-blocked S-1 derivatives coincides with formation of an intramolecular interthiol crosslink caused by Nbs_2 or $Ph(NMal)_2$ and that this link involves SH2 and another vicinal thiol in S-1. We tested this idea by $NaDodSO_4$/PAGE of the tryptic digests of $Ph(NMal)_2$-(AEDANS)-S-1 (containing 0.60 ATP/S-1) and of the $Ph(NMal)_2$-S-1 control (containing 0.75 ATP/S-1). Fig. 3 shows that the patterns obtained were different. The $Ph(NMal)_2$-S-1 had the same profile as native S-1, but $Ph(NMal)_2$-(AEDANS)-S-1 gave rise to a band at 78 kDa incorporating the fluorescence of the 20-kDa fragment. This product has a mobility similar to that of the 80-kDa species formed on crosslinking native S-1 with dibromobimane; that species is the union of the 20-kDa and 50-kDa domains of the heavy chain (11). When the trapping of nucleotide by $Ph(NMal)_2$ was carried out on the preformed, trypsin-split (27 kDa–50 kDa–20 kDa)S-1 and on AEDANS-(27 kDa–50 kDa–20 kDa)S-1 (Fig. 4*A*), the 78-kDa band was generated from the latter derivative only, and it grew at the expense of both the fluorescent 20-kDa and nonfluorescent 50-kDa components, as judged by densitometry (Fig. 4*B*). The production of the 78-kDa material was delayed relative to the rapid inactivation of S-1 reported in Fig. 1*A*, reaching its maximal extent after 60–80 min of reaction. This indicates that in the absence of free SH1, the crosslinker undergoes a rapid inhibitory anchorage on the fast-reacting SH2, which is then bridged to a less reactive thiol, henceforth called SH*x*, present in the 50-kDa region of S-1.

To determine whether the nucleotide trapping is associated with the rapid phase of SH2 blocking by $Ph(NMal)_2$ or with the subsequent slow crosslinking process between SH*x* and SH2, we measured both the amount of [^{14}C]ADP trapped and the extent of conversion of the 50-kDa peptide into the 78-kDa component during the reaction of $Ph(NMal)_2$ with AEDANS-S-1. Fig. 4*C* shows a close correlation between the fraction of reacted 50-kDa fragment and the amount of trapped nucleotide. In particular, after 10 min of reaction and a total loss of the Ca^{2+}-ATPase (implying a complete modification of the SH2 group), we measured only 0.20 mol of bound ADP and 0.25 mol of reacted 50-kDa peptide. This indicates that the stable nucleotide trapping observed after 80 min of reaction (Fig. 2) did not result just from the initial blocking of SH2 and SH1 monofunctionally in $Ph(NMal)_2$-(AEDANS)-S-1. Further, the data in Table 1 show that the amount of trapped nucleotide in $Ph(NMal)_2$-(N_2ph)-S-1 was not significantly changed upon release of the N_2ph group (on SH1) by reduction with dithioerythritol, with the unmasking of at least 0.80 mol of SH1 as determined by thiol titration. Thus, the prior blocking of SH1 was not contributing to the observed trapping of nucleotide. The thiolysis of $Ph(NMal)_2$-(N_2ph)-S-1 provides a new protein derivative in which the SH1 thiol is free and the nucleotide is trapped mainly through interthiol crosslinking between the 50-kDa and 20-kDa heavy-chain segments.

DISCUSSION

The nucleotide trapping that we have described results mainly from the crosslinking of the 20-kDa and 50-kDa peptides of the S-1 heavy chain and not simply from the

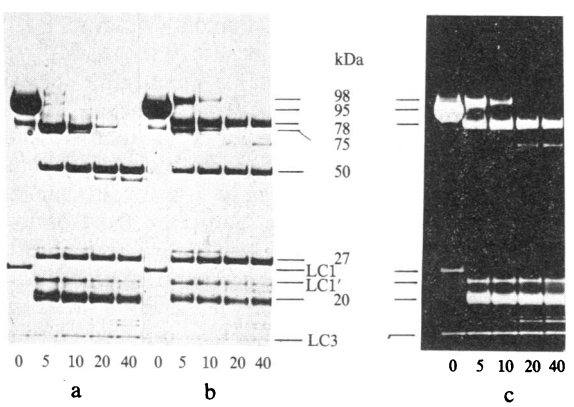

FIG. 3. Time-course of the digestion of $Ph(NMal)_2$-S-1 (*a*) and $Ph(NMal)_2$-(AEDANS)-S-1 (*b* and *c*) with trypsin (protease/S-1 ratio 1:100, wt/wt) in 50 mM Hepes (pH 8.0) at 20°C. Digests were analyzed by electrophoresis in 5–18% acrylamide slab gels; length of digestion, in min, is given below each lane. Gels were viewed using long-wave UV light (*c*) and stained with Coomassie blue (*a* and *b*). The heavy chain of $Ph(NMal)_2$-(AEDANS)-S-1 migrates at 98 kDa, whereas that of $Ph(NMal)_2$-S-1 migrates, like the native heavy chain, at 95 kDa.

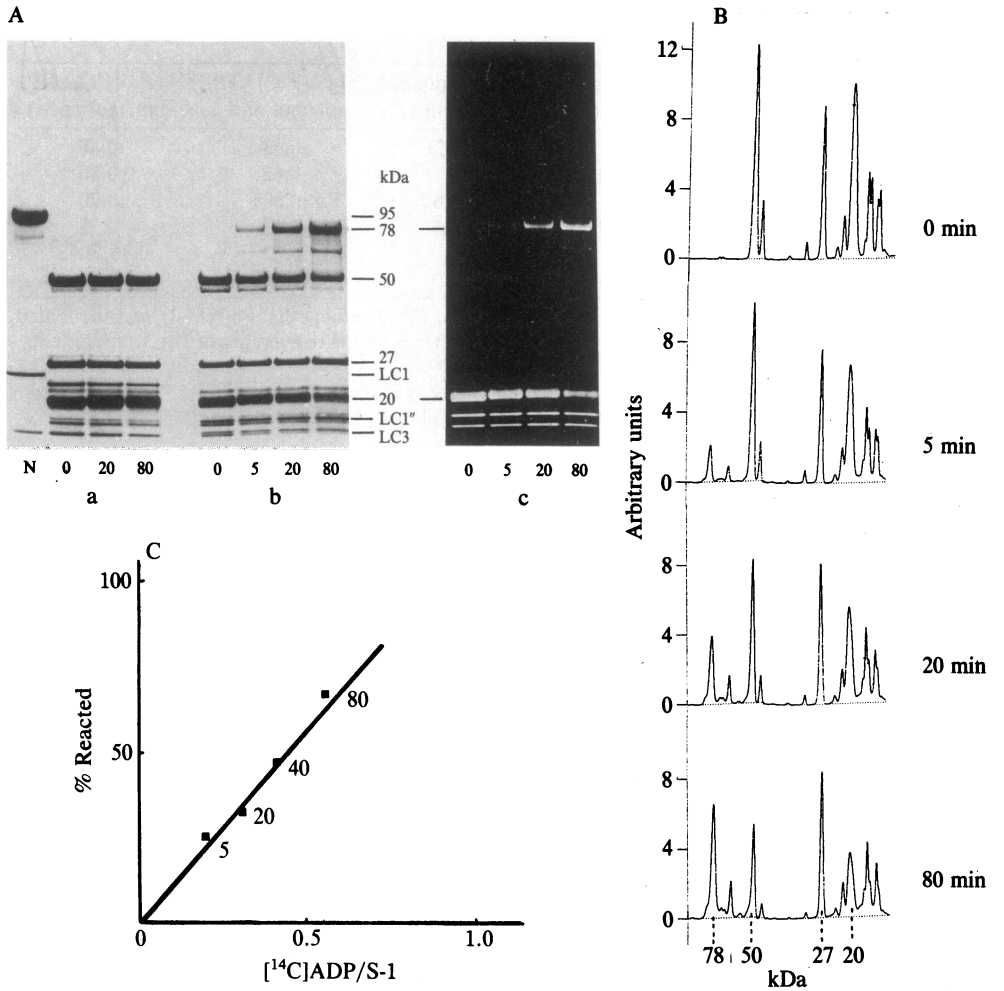

FIG. 4. (*A*) Production of the 78-kDa species formed during reaction of $Ph(NMal)_2$ with AEDANS-(27 kDa–50 kDa–20 kDa)S-1 (*b* and *c*). The modification of (27 kDa–50 kDa–20 kDa)S-1 with $Ph(NMal)_2$ was used as control (*a*). At the indicated times of the crosslinking reactions, the proteins were subjected to gel electrophoresis as in Fig. 3. Bands were located by fluorescence (*c*). The gels were then stained with Coomassie blue (*a* and *b*). Lane N: native S-1. (*B*) $Ph(NMal)_2$-promoted crosslinking between the 20-kDa and 50-kDa fragments of AEDANS-S-1 as assessed by the densitometric scanning of the gel (*b*) shown in *A*. (*C*) Relationship between nucleotide trapping and the crosslinking of the 50-kDa heavy-chain fragment. The amount of 50-kDa peptide that had reacted was deduced from the amount of residual peptide measured by densitometry (see *B*). The trapped [^{14}C]ADP was measured in protein samples isolated after reaction times of 5, 20, 40, and 80 min.

blocking of both SH1 and SH2 with bulky groups. It was reported that the substitution of these two thiols by *N*-ethylmaleimide traps a fractional amount of an ATP analog (29). In our case, however, at least 0.70 mol of Mg^{2+}-ADP remained bound in our crosslinked S-1 even after unmasking of the SH1 thiol. This is a new phenomenon in S-1; it contrasts, in particular, with a different, dibromobimane-induced crosslinking between the same heavy chain peptides in native S-1 that does not cause significant nucleotide trapping (11). However, in the latter case the crosslinking occurred through a bridge between SH1 and a cysteine in the 50-kDa peptide, whereas in the present case crosslinking is through a union of SH2 and one of the three thiols of the 50-kDa fragment. Although $Ph(NMal)_2$ can also crosslink a sulfhydryl group to a lysine residue (30), the loss of two thiols that we observed upon complete reaction of $Ph(NMal)_2$ with AEDANS-S-1 and the data we obtained with Nbs_2 strongly indicate the participation of only SH groups. Although the initial reaction of SH2 with both $Ph(NMal)_2$ and Nbs_2 was obvious on the basis of the nucleotide-dependent inactivation of the Ca^{2+}-ATPase, we do not know whether both reagents bridge SH2 to the same thiol of the 50-kDa domain. It is noteworthy, however, that Nbs_2 and $Ph(NMal)_2$ crosslink the same pair of SH1–SH2 thiols in native S-1 (31).

Our study reveals another conformational orientation of the SH2 site. Being located in a highly flexible peptide segment (11), it apparently can move to close proximity not only with the SH1 site but also with a site on the 50-kDa region. The participation of SH2 in interthiol crosslinking seems to be essential for nucleotide trapping, whereas this is not apparently the case for SH1. This suggests that the SH2 region has a particular relationship with the S-1 ATPase site. It is indeed well known that nucleotides have different effects on the chemical reactivity of SH2 and SH1 and, conversely, the blocking of these thiols has different consequences on the ATPase activities (25). Because the 50-kDa peptide has been shown to interact with actin and nucleotides (19, 32), its involvement together with SH2 in modulating the conformation of the ATPase site is of particular interest. It has been proposed that the myosin ATPase site is likely to include parts of the three fragments constituting the S-1 heavy chain (15). Specific interactions between the three regions would be expected to affect the conformation of the ATPase site. The tight binding of Mg^{2+}-nucleotide consequent to the crosslinking between two specific sites within the 20-kDa and 50-kDa domains is in agreement with this idea. The site around the 50-kDa-domain thiol, together with SH2, may constitute a flexible protein conformation communicating with the ATPase site [assuming that the 50-kDa domain was cross-

linked by both $Ph(NMal)_2$ (12 Å) and Nbs_2 (2 Å)]. It is possible that the well-known influence of SH2–SH1 crosslinking on the ATPase site is actually mediated by the 50-kDa segment. At least, the present findings show that the nucleotide trapping is not a peculiarity of crosslinking SH1 and SH2 but, more likely, that it results from structural alterations in a region that includes SH1, SH2, and a thiol of the 50-kDa domain.

When compared to the SH1–SH2 crosslinked S-1, the SH2–SH*x* crosslinked derivative showed a 2-fold greater rate of release of the trapped nucleotide and a higher affinity for actin. Spectroscopic studies and crosslinking experiments between actin and both derivatives, to be described elsewhere, revealed differences that suggest that the two interthiol-crosslinked proteins exhibit different conformations.

The possible substitution of the SH1 site by a variety of spectral probes, previously employed with native S-1, makes the SH2–SH*x* crosslinked S-1 useful for studies on the S-1 and acto-S-1 conformations in the presence of trapped nucleotides. SH2–SH*x* crosslinked S-1 complements the conventional SH1–SH2 crosslinked S-1, on which such investigations are obviously not possible.

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