Is SH1-SH2-Cross-Linked Myosin Subfragment 1 a Structural Analog of the Weakly-Bound State of Myosin?

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ABSTRACT Myosin subfragment 1 (S1) with SH1 (Cys^{707}) and SH2 (Cys^{697}) groups cross-linked by *p*-phenylenedimaleimide (pPDM-S1) is thought to be an analog of the weakly bound states of myosin bound to actin. The structural properties of pPDM-S1 were compared in this study to those of S1·ADP·BeF_x and S1·ADP·AlF_4^-, i.e., the established structural analogs of the myosin weakly bound states. To distinguish between the conformational effects of SH1-SH2 cross-linking and those due to their monofunctional modification, we used S1 with the SH1 and SH2 groups labeled with *N*-phenylmaleimide (NPM-S1) as a control in our experiments. The state of the nucleotide pocket was probed using a hydrophobic fluorescent dye, 3-[4-(3-phenyl-2-pyrazolin-1-yl)benzene-1-sulfonylamido]phenylboronic acid (PPBA). Differential scanning calorimetry (DSC) was used to study the thermal stability of S1. By both methods the conformational state of pPDM-S1 was different from that of unmodified S1 in the S1·ADP·BeF_x and S1·ADP·AlF_4^- complexes and closer to that of nucleotide-free S1. Moreover, BeF_x and AlF_4^- binding failed to induce conformational changes in pPDM-S1 similar to those observed in unmodified S1. Surprisingly, when pPDM cross-linking was performed on S1·ADP·BeF_x complex, ADP·BeF_x protected to some extent the nucleotide pocket of S1 from the effects of pPDM modification. NPM-S1 behaved similarly to pPDM-S1 in our experiments. Overall, this work presents new evidence that the conformational state of pPDM-S1 is different from that of the weakly bound state analogs, S1·ADP·BeF_x and S1·ADP·AlF_4^-. The similar structural effects of pPDM cross-linking of SH1 and SH2 groups and their monofunctional labeling with NPM are ascribed to the inhibitory effects of these modifications on the flexibility/mobility of the SH1-SH2 helix.

INTRODUCTION

Muscle contraction and actomyosin-based cell motility occur through the cyclic interactions of myosin and actin, coupled to myosin-catalyzed ATP hydrolysis. The actomyosin ATPase cycle can be described in simplified terms by Scheme 1 (Ma and Taylor, 1994), where AM is actomyosin and M is myosin.

$$\begin{array}{c} AM + ATP \leftrightarrow AM \cdot ATP \leftrightarrow AM \cdot ADP \cdot P_i \leftrightarrow AM \cdot ADP + P_i \leftrightarrow AM + ADP \\ \qquad\qquad\quad \updownarrow \qquad\qquad\quad \updownarrow \\ \qquad\quad M \cdot ATP \leftrightarrow M \cdot ADP \cdot P_i \end{array} \tag{1}$$

During the cycle, the actomyosin complex undergoes a transition between the weakly bound (AM·ATP, AM·ADP·P_i) and the strongly bound (AM·ADP, AM) states. This transition is accompanied by conformational changes in the myosin head, which are believed to play a key role in the actomyosin force generation process. The solution of the atomic structure of myosin subfragment 1 (S1) (Rayment et al., 1993) brought an opportunity to map such conformational changes at an atomic-level resolution. However, the weakly bound states of myosin are short-lived intermediates of the ATPase cycle. Thus, stable structural analogs of these states are required for crystallographic studies. Several such analogs have been reported to date. The most studied class of the stable analogs of weakly bound states of myosin is its complexes with ADP and phosphate analogs, such as vanadate (V_i), aluminum fluoride (AlF_4^-), and beryllium fluoride (BeF_x). It has been shown that S1·ADP·V_i binds weakly to actin and functionally and structurally resembles the S1·ADP·P_i intermediate complex (Wells and Bagshaw, 1984; Goodno and Taylor, 1982; Smith and Eisenberg, 1990; Bobkov and Levitsky, 1995). Similar conclusions were reached for S1·ADP·AlF_4^- and S1·ADP·BeF_x complexes (Werber et al., 1992; Maruta et al., 1993; Phan et al., 1993; Bobkov and Levitsky, 1995). However, despite the overall similarity between the S1·ADP·phosphate analog complexes, certain structural differences were observed between S1·ADP·BeF_x on the one hand and S1·ADP·V_i or S1·ADP·AlF_4^- on the other (Fisher et al., 1995; Smith and Rayment, 1996; Ponomarev et al., 1995; Maruta, 1994). Based on these observations and the kinetic study of chemical reactivities of SH1 (Cys^{707}) and SH2 (Cys^{697}) groups on myosin it was suggested that the S1·ADP·BeF_x complex is closer to the prehydrolyzed, S1·ATP state, while S1·ADP·V_i and S1·ADP·AlF_4^- resemble the posthydrolyzed, S1·ADP·P_i state (Fisher et al., 1995; Phan et al., 1997).

Another frequently used analog of the myosin weakly bound state was introduced by Reisler et al. (1974b). They have reported that *p*-phenylenedimaleimide (pPDM) cross-links SH1 and SH2 groups on S1. Later it was shown that nucleotides are trapped in the active site by pPDM cross-linking (Wells and Yount, 1979). The resulting S1 species (pPDM-S1) has an affinity to actin, and its dependence on the ionic strength, similar to that of S1 + ATP (Burke et al., 1976; Chalovich et al., 1983). Based on these observations

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it was concluded that pPDM-S1 is an analog of the weakly bound state of myosin (S1·ADP·P_i).

However, a number of studies have shown that structural properties of pPDM-S1 are different from those of S1·ADP·P_i. X-ray scattering experiments revealed that the shape of pPDM-S1 is different from that of S1·ADP·P_i (Wakabayashi et al., 1992). In addition, pPDM-S1 has a lower intrinsic fluorescence intensity than S1 in the presence of ATP (Kirshenbaum et al., 1993). Finally, differential scanning experiments demonstrated that the conformation of pPDM-S1 is different from that of S1 in the complex with ADP and V_i (Levitsky et al., 1992). These observations indicate that pPDM-S1 may be a functional but not a conformational analog of the weakly bound state of S1.

To shed more light on this issue we have compared structural properties of pPDM-S1 to those of unmodified S1 in the S1·ADP·BeF_x and S1·ADP·AlF_4^- complexes. We have also studied the effects of BeF_x and AlF_4^- binding to pPDM-S1 on its conformation. To distinguish between the conformational effects of SH1-SH2 cross-linking and those due to their monofunctional modification, we have used NPM-S1 (in which the SH1 and SH2 groups are labeled with *N*-phenylmaleimide) as a control in our experiments. Our results show that the conformational state of pPDM-cross-linked S1 is different from that of the analogs of the weakly bound state, S1·ADP·BeF_x and S1·ADP·AlF_4^-.

MATERIALS AND METHODS

Reagents

pPDM and *N*-phenylmaleimide (NPM) were from Aldrich Chemical Co. (Milwaukee, WI). *N*-Ethylmaleimide (NEM) was obtained from Sigma (St. Louis, MO). 3-[4-(3-Phenyl-2-pyrazolin-1-yl)benzene-1-sulfonylamido]-phenylboronic acid (PPBA) was purchased from Polysciences (Warrington, PA).

Proteins

Myosin from the back and leg muscles of rabbits was prepared according to the method of Godfrey and Harrington (1970). S1 from rabbit myosin was prepared by digestion of myosin filaments with α-chymotrypsin (Weeds and Pope, 1977). The concentration of S1 was determined spectrophotometrically by using an extinction coefficient of $E^{1\%}_{280} = 7.5$ cm^{-1}. The concentration of modified S1 was determined by using the Bradford protein assay (1976).

ATPase activities

The ATPase activities of S1 were measured at 37°C, under steady-state conditions, using the Fiske and Subbarow (1925) phosphate determination assay. The Ca^{2+}-ATPase and K^+-EDTA-ATPase assay solutions contained 30 mM Tris-HCl (pH 7.5), 0.5 M KCl, and either 5.0 mM $CaCl_2$ or 5.0 mM EDTA.

Modifications of S1

S1 modifications were carried out in solutions containing between 20 and 30 μM S1, 30 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM ADP, and 20 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid (PIPES) (pH 7.0). The SH1 group on S1 was selectively labeled with a twofold molar excess of NEM over S1. SH1 and SH2 groups were cross-linked using a twofold molar excess of pPDM over S1. A fourfold molar excess of NPM over S1 was used to label both SH1 and SH2 groups on S1. The modification reactions were carried out over a period of 20–60 min. The extent of S1 labeling was estimated by measuring its Ca^{2+}- and K^+-EDTA-ATPase activities (Xie et al., 1997). In all cases we used S1 that was modified between 90% and 100%. The small variations in the extent of S1 modification had no apparent effect on our results.

Preparation of S1 complexes with AlF_4^- and BeF_x

The complexes of modified and control S1 with phosphate analogs were formed by incubation of 10–20 μM S1 with 1.0 mM ADP and 5.0 mM NaF + 0.5 mM $BeCl_2$ or 10 mM NaF + 0.5 mM $AlCl_3$. In some experiments these complexes were formed after S1 was modified with pPDM, NPM, or NEM. In other cases the complexes were formed with unmodified S1, after which the modifications were carried out on the S1 complexed with either ADP·BeF_x or ADP·AlF_4^-.

PPBA spectra

Fluorescence spectra of PPBA bound to S1 in the presence of different nucleotides and phosphate analogs were obtained as previously described (Bobkov et al., 1997). Briefly, PPBA was added (from a 100 μM stock in *N,N*-dimethyl formamide) to a final concentration of 1.0 μM to S1 (between 10 and 20 μM S1) in solutions containing 20 mM PIPES (pH 7.0), 30 mM KCl, and 3.0 mM $MgCl_2$. The samples also contained one of the following compounds: 1.0 mM ATP, 1.0 mM ADP, 1.0 mM ADP + 5.0 mM NaF + 0.5 mM $BeCl_2$, or 1.0 mM ADP + 10 mM NaF + 0.5 mM $AlCl_3$. The excitation wavelength was set at 360 nm.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) experiments were performed on a 6100 N-DSC II differential scanning calorimeter (Calorimetry Sciences Corp., Provo, UT) with a cell volume of ~0.25 ml. All experiments were performed at a scanning rate of 1 K/min under 3.0 atm of pressure. Before measurements, all S1 samples were dialyzed against 30 mM HEPES (pH 7.3) and 1.0 mM $MgCl_2$. The dialysis buffer was used as a reference solution. The reversibility of the thermal transitions was checked by a second heating of the sample immediately after cooling, after the first scan. All thermal transitions were irreversible under the conditions used in this study. Because thermal denaturation of the protein samples studied by DSC was irreversible, only simple thermodynamic parameters and terms were used for the interpretation of results. The thermal stability of the proteins was described by the temperature of the maximum of thermal transition (T_m). The calorimetric enthalpy (ΔH_{cal}) was calculated as the area under the excess heat capacity function. Because these parameters can be obtained directly from experimental calorimetric traces after subtraction of the chemical baseline and concentration normalization, they can be used for the description of the irreversible thermal denaturation of S1.

RESULTS

PPBA probing of the nucleotide pocket in modified S1

It was shown before that the hydrophobic fluorescent probe PPBA, which binds noncovalently and stoichiometrically to S1, is a competitive inhibitor of the S1 ATPase activity, and

its fluorescent properties are sensitive to the state of the S1 nucleotide pocket (Hiratsuka, 1994). Thus PPBA appeared to be an attractive tool for monitoring the effects of pPDM and NPM modifications on the conformation of the nucleotide pocket of S1 in the S1·nucleotide complexes.

Fig. 1 demonstrates, as shown before (Hiratsuka, 1994; Bobkov et al., 1997), that ADP binding to S1 causes an increase in the intensity and a blue shift of the PPBA emission spectrum (*curve 2*). The formation of S1 complexes with ADP·BeF_x and ADP·AlF_4^- (*curves 3* and *4*) and, to a greater extent, ATP binding to S1 (*curve 5*) cause further increases in the intensity of the PPBA spectrum. Thus PPBA allows for an easy distinction between the states of S1 that bind weakly (S1·ADP·P_i; S1·ADP·BeF_x and S1·ADP·AlF_4^-) and strongly (S1·ADP, S1 alone) to actin.

Surprisingly, the spectrum of PPBA bound to S1 with SH1 and SH2 groups cross-linked by pPDM (pPDM-S1) (Fig. 2, *curve 2*) was quite different from the spectra observed for the weakly bound states of S1 (Fig. 1, *curves 3–5*) and even from the spectrum for S1·ADP (Fig. 1, *curve 2*). Because S1 modification by pPDM was always carried out in the presence of ADP, the pPDM-S1 used here contained ADP trapped at the active site. The spectrum of PPBA bound to pPDM-S1 resembled most closely the spectrum of PPBA bound to the nucleotide-free S1 (Fig. 1, *curve 1*). The pPDM-S1 complex with PPBA used in this experiment was prepared by using two different orders of PPBA addition, before and after the cross-linking of S1·ADP with pPDM. Because the resulting emission spectra do not depend on the order of PPBA addition, only one spectrum is shown for the pPDM-S1·PPBA complex in Fig. 2 (*curve 2*).This result excludes possible artifacts of PPBA binding to pPDM-S1. Thus, despite the fact that pPDM-S1 binds weakly to actin, the conformation of the nucleotide pocket on pPDM-S1 is different from that of the weakly bound states of unmodified S1.

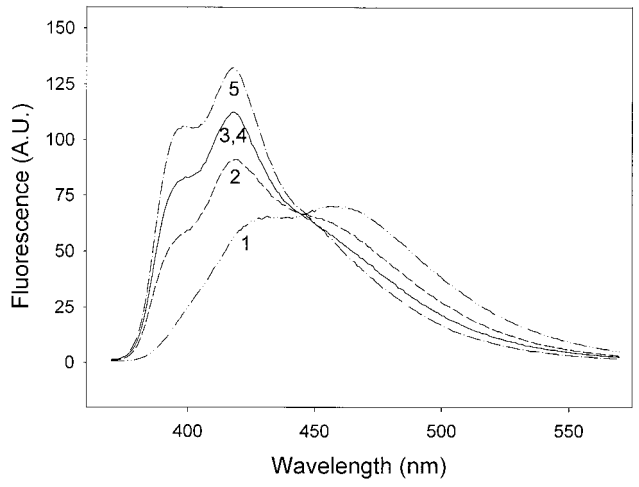

FIGURE 1 Fluorescence emission spectra of PPBA bound to nucleotide-free S1 (*1*), S1·ADP (*2*), S1·ADP·BeF_x (*3*), S1·ADP·AlF_4^- (*4*), and S1 + ATP (*5*). The assay solutions contained 10 μM S1, 1.0 μM PPBA, 20 mM PIPES (pH 7.0), 30 mM KCl, and 3.0 mM $MgCl_2$. The samples also contained one of the following compounds: 1.0 mM ATP, 1.0 mM ADP, 1.0 mM ADP + 5.0 mM NaF + 0.5 mM $BeCl_2$, or 1.0 mM ADP + 10 mM NaF + 0.5 mM $AlCl_3$. The excitation wavelength was set at 360 nm.

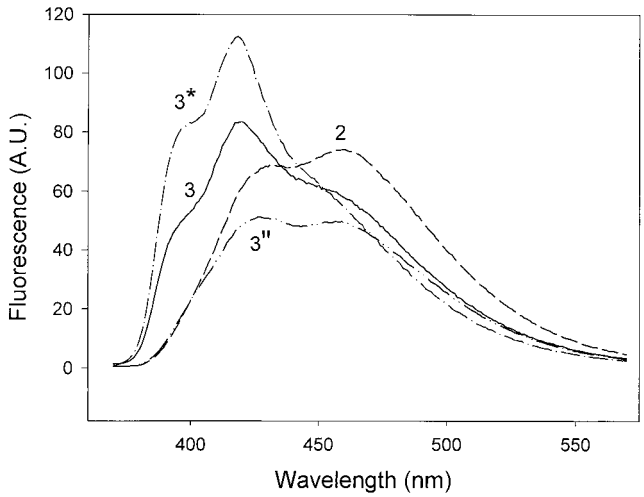

FIGURE 2 Fluorescence emission spectra of PPBA bound to SH1-SH2 cross-linked S1 (pPDM-S1). (*2*) S1·ADP complex cross-linked with pPDM. (*3″*) The same complex as in *2* after the addition of BeF_x. (*3*) S1·ADP·BeF_x complex cross-linked with pPDM. (*3**) Curve 3 from Fig. 1 (i.e., fluorescence emission spectrum of PPBA bound to S1·ADP·BeF_x), reproduced here for comparison. Experimental conditions are the same as in Fig. 1.

We have also tested the ability of pPDM-S1 to form complexes with ADP and BeF_x or AlF_4^-. Binding of BeF_x to pPDM-S1 caused a decrease in the intensity and a slight blue shift of the PPBA spectrum (Fig. 2, *curve 3″*). Binding of AlF_4^- had a similar effect on the probe spectrum (data not shown). Thus both phosphate analogs failed to induce structural changes in the nucleotide pocket of pPDM-S1 similar to those observed for the unmodified S1 (Fig. 1, *curves 3* and *4*; Fig. 2, *curve 3**). Again, the order in which PPBA was added to S1 did not change the results. Similar PPBA spectra were obtained when we first formed the S1·ADP·PPBA complex, then cross-linked it with pPDM, and finally added BeF_x/AlF_4^-, or, alternatively, first formed the S1·ADP complex and cross-linked it, then added PPBA, and finally added BeF_x/AlF_4^-.

However, the results were quite different when we reversed the order of cross-linking and S1·phosphate analog complex formation, i.e., when we first formed the S1·ADP·BeF_x complex in the presence of PPBA and then added pPDM (Fig. 2, *curve 3*). The resulting PPBA spectrum resembled in shape, although not in intensity, the spectra of unmodified S1 complexes with nucleotides (Fig. 1, *curves 2–5*; Fig. 2, *curve 3**). These results indicate that the formation of the S1·ADP·BeF_x complex protects to a certain extent the nucleotide pocket of S1 from the effects of

pPDM modification. It appears also that it is possible to obtain SH1-SH2 cross-linked S1 with somewhat different conformations of the nucleotide pocket, depending on the nature of the nucleotide bound to S1. We were unable to carry out similar experiments with S1·ADP·AlF, mainly because of the difficulty of achieving >50% cross-linking of such a complex. This difficulty is related to the much lower reactivity of the SH1 group in S1·ADP·AlF_4^- than in the S1·ADP and S1·ADP·BeF_x complexes (Hiratsuka et al., 1998).

It is known that monofunctional modification of both the SH1 and SH2 groups drastically affects the functional properties of myosin (Reisler et al., 1974a; Xie and Schoenberg, 1998; Xie et al., 1999). To distinguish between the effects of cross-linking and monofunctional modification of SH1 and SH2 groups, we have examined the properties of S1 with the SH1 and SH2 groups modified by NPM (NPM-S1). The PPBA spectra of NPM-S1·ADP and NPM-S1·ADP·BeF_x complexes (Fig. 3) were very similar to those of the corresponding pPDM-S1 complexes (Fig. 2). As in the case of pPDM-S1 (Fig. 2, *curve 3*), when S1·ADP·BeF_x complex was formed first and then modified with NPM, the PPBA spectrum of such S1 revealed that ADP·BeF_x protected the nucleotide pocket in S1 from the effects of NPM modification (Fig. 3, *curve 3*). Overall, the effects of NPM modification monitored via PPBA spectra closely resembled the effects of pPDM modification on S1. Thus a double modification of the SH1-SH2 helix, rather than the cross-linking itself, may account for the effects of pPDM modification on the PPBA spectra of S1·nucleotide complexes.

Both NPM-S1 and pPDM-S1 have ATPase activities close to zero. Judging from the PPBA spectra, the binding of BeF_x and AlF_4^- to such S1s failed to induce the local conformational changes reported by this probe. The obvious question was whether such a desensitization of S1 also occurs after the labeling of SH1 alone, i.e., in the SH1-modified S1 (which retains some ATPase activity and the ability to form stable complexes with phosphate analogs). Fig. 4 shows the PPBA spectra of S1 labeled with NEM at the SH1 group (NEM-S1). The spectrum of PPBA bound to NEM-S1·ADP (Fig. 4, *curve 2*) closely resembled those of pPDM-S1·ADP (Fig. 2, *curve 2*) and NPM-S1·ADP (Fig. 3, *curve 2*). It was also similar to the spectrum of PPBA bound to nucleotide free S1 (Fig. 1, *curve 1*). This indicates that in analogy to NPM and pPDM modifications, the NEM modification of SH1 inhibits the ADP-induced conformational changes in the nucleotide pocket of S1. However, BeF_x (Fig. 4, *curve 3*) and, to a greater extent, AlF_4^- (Fig. 4, *curve 4*) induced increases in the intensity and blue shifts of the spectrum of PPBA bound to NEM-S1·ADP. The effects of AlF_4^- and BeF_x binding to NEM-S1 were similar but weaker than their effects on the unmodified S1 (Fig. 1, *curves 3* and *4*). Thus the ability of phosphate analogs to induce conformational changes in the nucleotide pocket of NEM-S1 was somewhat altered, but not abolished as in the pPDM-S1 and NPM-S1.

DSC on modified S1

PPBA spectra shown in Figs. 1 and 2 revealed that the conformation of the nucleotide pocket in pPDM-cross-linked S1 is different from that in the S1·ATP, S1·ADP·BeF_x, and S1·ADP·AlF_4^- complexes of unmodified S1. Moreover, BeF_x and AlF_4^- binding failed to induce conformational changes in pPDM-S1 and NPM-S1 similar to those observed in unmodified S1. To confirm these observations, we have employed DSC, which is a highly effective method for detecting nucleotide-induced conformational changes in myosin (Shriver and Kamath, 1990;

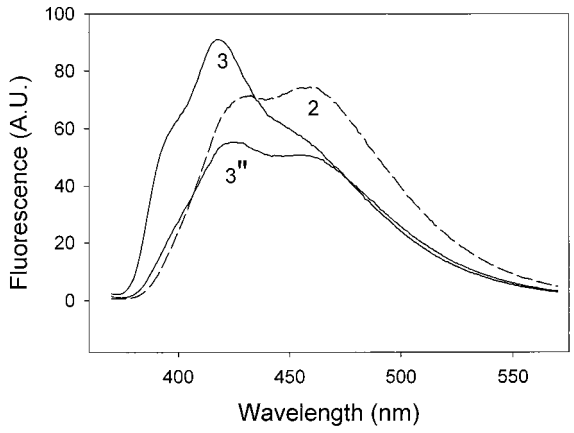

FIGURE 3 Fluorescence emission spectra of PPBA bound to S1 with SH1 and SH2 groups labeled with NPM (NPM-S1). (*2*) S1·ADP complex labeled with NPM. (*3″*) The same complex as in *2* after the addition of BeF_x. (*3*) S1·ADP·BeF_x complex labeled with NPM.

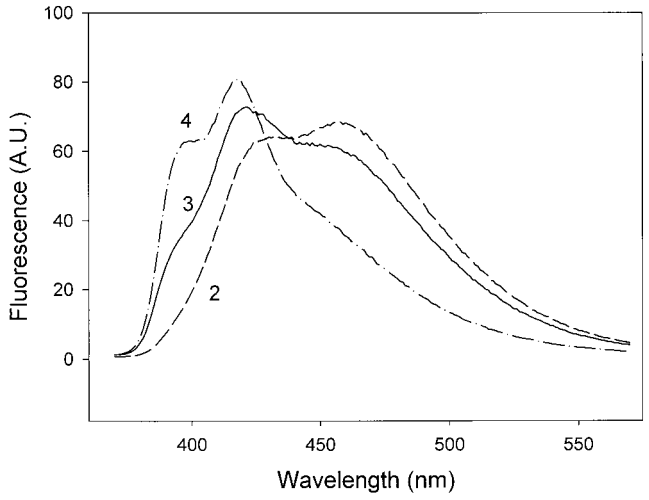

FIGURE 4 Fluorescence emission spectra of PPBA bound to SH1-labeled S1 (NEM-S1). (*2*) NEM-S1·ADP. (*3*) NEM-S1·ADP·BeF_x. (*4*) NEM-S1·ADP·AlF_4^-. Experimental conditions are the same as in Fig. 1.

Levitsky et al., 1992; Bobkov and Levitsky, 1995). It has been shown before that the changes revealed by DSC occur in the catalytic domain of the myosin head (Levitsky et al., 1998b) and are sensitive to the nature of the bound nucleotide (Bobkov and Levitsky, 1995).

As previously reported (Bobkov et al., 1993; Bobkov and Levitsky, 1995), the binding of BeF_x to the S1·ADP complex induced a significant increase in the thermal stability and a considerable increase in the calorimetric enthalpy of S1 (Fig. 5 *A*, *curve 2*, and Table 1). Binding of AlF_4^- to the S1·ADP complex caused a similar effect (Table 1), in agreement with previous observations (Levitsky et al., 1998a). In contrast to expectations, previous DSC experiments (Levitsky et al., 1992) revealed that the conformation of pPDM-S1 is quite different from that of S1 complexed with ADP and phosphate analogs. In agreement with that observation, in our hands pPDM-S1 had significantly lower thermal stability and calorimetric enthalpy than S1·ADP·AlF_4^- and S1·ADP·BeF_x (Fig. 5 *C*, *curve 1*, and Table 1). In analogy to our results with the PPBA probe, calorimetric features of pPDM-S1 were closer to those of nucleotide free S1 than to those of the S1·nucleotide complexes (Table 1).

TABLE 1 Thermodynamic parameters obtained from DSC scans for S1 · nucleotide complexes of unmodified S1, pPDM-S1, and NPM-S1

	T_m (°C)	ΔH_{cal} (kcal/mol)
S1	48.8	274
S1·ADP	50.9	290
S1·ADP·BeF_x	57.3	342
S1·ADP·$A1F_4^-$	58.5	320
NPM-S1·ADP	50.4	250
NPM-S1·ADP·BeF_x	53.3	304
NPM-S1·ADP·$A1F_4^-$	51.9	313
pPDM-S1·ADP	48	210
pPDM-S1·ADP·BeF_x	49.5	200
pPDM·S1·ADP·$A1F_4^-$	49.3	190

The absolute error in T_m values did not exceed ±0.2°C; the relative error in ΔH_{cal} values did not exceed ±10%.

The addition of BeF_x or AlF_4^- had a marginal effect on the thermal denaturation of pPDM-S1, with only a slight increase in the thermal stability and no effect on the calorimetric enthalpy of pPDM-S1 (Fig. 5 *C*, *curve 2*, and Table 1). Thus DSC confirmed our results from PPBA experiments that binding of phosphate analogs does not induce conformational changes in pPDM-S1 similar to those observed in unmodified S1. However, in contrast to the fluorescence data (Fig. 2), the order in which S1 was cross-linked and combined with BeF_x did not affect the DSC results. The DSC curves were similar irrespective of whether we first cross-linked S1·ADP with pPDM and then added BeF_x or first formed the S1·ADP·BeF_x and then cross-linked it with pPDM.

Similar to pPDM, NPM modification strongly inhibited the conformational changes in S1 detected by DSC upon formation of S1 complexes with ADP and phosphate analogs (Fig. 5 *B*, *curve 2*, and Table 1). As for pPDM-S1, the DSC results were independent of the order of S1·ADP modification with NPM and the addition of BeF_x. However, while pPDM-S1 and NPM-S1 showed virtually identical PPBA fluorescence responses, DSC revealed some structural differences between these forms of modified S1. Judging from the DSC data (Fig. 5 and Table 1), NPM modification had a somewhat smaller effect on the conformation of S1·nucleotide complexes. The T_m and ΔH_{cal} values for NPM-S1·nucleotide complexes (Table 1) were higher (and closer to those of unmodified S1) than those of pPDM S1. However, it is important to note that both the cross-linking with pPDM and the dual modification of SH1 and SH2 groups with NPM inhibited much more strongly the thermal stabilization of S1 by ADP·AlF_4^- and ADP·BeF_x than the

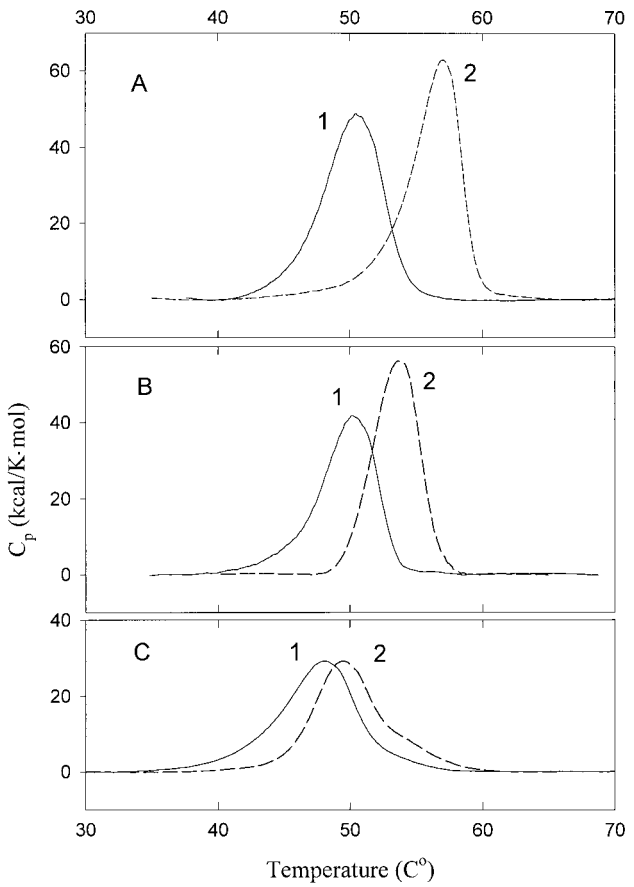

FIGURE 5 DSC scans obtained for unmodified S1 (*A*), S1 modified monofunctionally at SH1 and SH2 groups with NPM (*B*), and S1 with SH1 and SH2 groups cross-linked with pPDM (*C*). For each panel curves 1 and 2 were obtained in the presence of 1.0 mM ADP and 1.0 mM ADP + 5.0 mM NaF + 0.5 mM $BeCl_3$, respectively. The assay solutions contained 1.7 mg of S1, 30 mM HEPES (pH 7.3), and 2.0 mM $MgCl_2$. The heating rate was 1 K/min.

single-site SH1 or SH2 modifications (Golitsina et al., 1996; Ponomarev et al., unpublished data).

DISCUSSION

According to current views, force generation by myosin occurs upon the release of ATP hydrolysis products, when the light chain binding domain (LCBD) swings relative to the catalytic domain of S1, acting like a lever arm. An important goal in muscle biochemistry has been to document such a movement. The solution of several atomic structures of S1 (Rayment et al., 1993; Fisher et al., 1995; Smith and Rayment, 1996; Dominguez et al., 1998; Houdusse et al., 1999) led to the description of three conformational states of S1 with different positions of the lever arm (Houdusse et al., 1999). However, important questions remain open. The same conformational state was observed for the nucleotide-free and nucleotide-bound S1 structures (Fisher et al., 1995; Gulick et al., 1997). In addition, the same S1·ADP·BeF_x complex was crystallized in different conformational states for *Dictyostelium* (Fisher et al., 1995) and smooth muscle (Dominguez et al., 1998) myosins. Moreover, the unique S1 conformation state with the melted SH1-SH2 helix visualized in the atomic structure of scallop myosin S1 complexed with ADP is believed to correspond in fact to the prehydrolysis, S1·ATP state (Houdusse et al., 1999). Clearly, the assignment of different conformational states seen in myosin atomic structures to intermediate complexes in the myosin ATPase cycle is rather difficult at this stage.

pPDM-S1 was considered as a candidate for atomic structure determination in an attempt to define the conformational state of myosin in the S1·ATP complex (Houdusse et al., 1999). pPDM-S1 is known to bind weakly to actin and is believed to be an analog of the myosin weakly bound states (Burke et al., 1976; Chalovich et al., 1983). The fact that the SH1-SH2 helix on S1 is stabilized by pPDM cross-linking in a presumably melted/bent state makes pPDM-S1 especially attractive for structural studies. However, there is also evidence showing that the conformation of pPDM-S1 is different from that of S1·ADP·P_i (Chaussepied et al., 1986; Wakabayashi et al., 1992; Kirshenbaum et al., 1993) and S1·ADP·V_i (Levitsky et al., 1992).

To evaluate the possible use of pPDM-S1 as a structural analog of the weakly bound (to actin) states of S1, we have compared the properties of pPDM-S1 and S1·ADP·AlF_4^- and S1·ADP·BeF_x complexes by two methods: the fluorescence of the nucleotide pocket probe (PPBA) and the DSC of S1. It is pertinent to note that PPBA reports on the local environment of the nucleotide pocket, whereas DSC monitors the thermal stability of S1, which reflects the conformational state of the entire S1 molecule. Both methods clearly distinguish between the states of S1 that bind strongly (nucleotide-free S1, S1·ADP) and weakly (S1·ADP·BeF_x, S1·ADP·AlF_4^-, and, in case of PPBA experiments, S1·ADP·P_i) to actin. However, PPBA probing failed to resolve between the S1·ADP·BeF_x and S1·ADP·AlF_4^- complexes. Thus, either under the conditions of our experiments the conformations of the nucleotide pockets in S1·ADP·BeF_x and S1·ADP·AlF_4^- are similar, or this method is not sensitive enough to distinguish between them.

The methods employed in this study not only provided new evidence that the conformation of pPDM-S1 is different from that of S1 in the weakly bound states, but also helped to localize the structural effects of pPDM cross-linking on S1. PPBA spectra demonstrated that the state of the nucleotide pocket of pPDM-S1 is different from that in S1·ADP·BeF_x, S1·ADP·AlF_4^-, and S1·ADP·P_i complexes and resembles that of nucleotide-free S1. Our observation that pPDM cross-linking desensitizes S1 to the effects of phosphate analogs and ADP·BeF_x partially protects the nucleotide pocket of S1 from the effects of cross-linking indicates that the SH1-SH2 helix in pPDM-S1 is in a state different from that in the S1·ADP·AlF_4^- and S1·ADP·BeF_x complexes. It may be deduced that the formation of these complexes requires flexibility of the helix, which is compromised by its cross-linking.

Interestingly, the effects of SH1 and SH2 modification with NPM and their cross-linking with pPDM on the S1 conformation were very similar. This can be rationalized in terms of the effects of these modifications on the flexibility of the SH1-SH2 helix. The SH1 group is located near Gly^{710} and SH2 is near Gly^{699} in the SH1-SH2 helix. These conserved glycines were shown to serve as pivot points that allow the SH1-SH2 helix to rotate (Dominguez et al., 1998). Modification of SH1 and SH2 groups with NPM could limit the helix flexibility and rotation around Gly^{710} and Gly^{699} and thus produce an effect similar to that of pPDM cross-linking. However, the DSC results indicate that the conformational effects of pPDM and NPM modifications on S1, although similar with respect to some properties of the nucleotide site, are less similar for S1 thermal stability.

Previously we proposed that the inhibitory effect of a monofunctional SH1 or SH2 modification on the motor function of myosin can be explained if it is assumed that these modifications affect the flexibility of the SH1-SH2 helix at Gly^{710} and Gly^{699} positions, respectively, thus uncoupling the lever arm from the catalytic domain of S1 (Bobkova et al., 1999). Such modifications, however, affect only marginally the conformational changes induced in S1 by the formation of S1·ADP·BeF_x and S1·ADP·AlF_4^- complexes (Golitsina et al., 1996; Ponomarev et al., unpublished data). Thus, while SH1 or SH2 modification is sufficient to completely abolish the motor function of myosin (Bobkova et al., 1999), dual modification or cross-linking of the SH1-SH2 helix is required to inhibit the conformational changes induced in S1 by AlF_4^- or BeF_x.

Overall, this work presents new evidence that the conformational state of pPDM-cross-linked S1 is different from that of the analogs of the weakly bound states, S1·ADP·BeF_x

and S1·ADP·AlF_4^-. Some of the structural properties of pPDM-S1 observed in our experiments are in fact similar to those of S1 in the strongly bound states. We may speculate that the cross-linking of SH1-SH2 helix alters the coupling between the lever arm, the nucleotide, and actin binding sites on S1. Thus the weakening of S1 affinity for actin by pPDM cross-linking may not be accompanied by conformational changes in S1 that are similar to those observed upon ATP binding.

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