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Changes in the thermal unfolding of *p*-phenylenedimaleimide-modified myosin subfragment 1 induced by its 'weak' binding to F-actin

Olga V. Kaspieva[a], Olga P. Nikolaeva[a], Victor N. Orlov[a], Michael A. Ponomarev[b], Vladimir A. Drachev[a], Dmitrii I. Levitsky[a,b,*]

[a] *A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia*
[b] *A.N. Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow 117071, Russia*

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Abstract Differential scanning calorimetry (DSC) was used to analyze the thermal unfolding of myosin subfragment 1 (S1) with the SH1 (Cys-707) and SH2 (Cys-697) groups cross-linked by *N*,*N*′-*p*-phenylenedimaleimide (*p*PDM-S1). It has been shown that F-actin affects the thermal unfolding of *p*PDM-S1 only at very low ionic strength, when some part of *p*PDM-S1 binds weakly to F-actin, but not at higher ionic strength (200 mM KCl). The weak binding of *p*PDM-S1 to F-actin shifted the thermal transition of *p*PDM-S1 by about 5°C to a higher temperature. This actin-induced increase in thermal stability of *p*PDM-S1 was similar to that observed with 'strong' binding of unmodified S1 to F-actin. Our results show that actin-induced structural changes revealed by DSC in the myosin head occur not only upon strong binding but also on weak binding of the head to F-actin, thus suggesting that these changes may occur before the power-stroke and play an important role in the motor function of the head.

Key words: Actin–myosin interaction; Myosin subfragment 1; Thermal unfolding; Differential scanning calorimetry

1. Introduction

It is generally accepted that muscle contraction and actomyosin-based cell motility occur through the cyclic interactions of myosin heads with actin filaments, coupled to myosin-catalyzed ATP hydrolysis. The function of the myosin head as a 'molecular motor' is explained by significant conformational changes which occur in the head during ATPase reaction and alter the character of actin–myosin interaction [1,2]. These changes are related to the transition of actomyosin complex (AM) between two different states: from the so called weakly bound states (AM·ATP, AM·ADP·P_i) to the strongly bound states (AM·ADP, nucleotide-free AM). Direct electrostatic interaction between loop 2, a lysine-rich surface segment of the myosin head, and the negatively charged amino-terminal part of actin is mainly responsible for the weak binding of the myosin head to F-actin [3–5]. The following transition to the strongly bound states is accompanied by formation of many additional contacts between actin and myosin. The strongly bound states have been studied in detail as they are stable and long-lived, but comparatively little is known about the weakly bound states which are short-lived intermediates of the ATPase cycle. Thus, stable analogues of these states are required for structural studies. One of these analogues is the isolated myosin head, or myosin subfragment 1 (S1), that has reacted with the bifunctional thiol reagent, *N*,*N*′-*p*-phenylenedimaleimide (*p*PDM). Treatment of S1 with *p*PDM in the presence of ADP results in cross-linking the two reactive sulfhydryl groups, SH1 (Cys-707) and SH2 (Cys-697), of S1 [6], and trapping of ADP in the active site [7]. It was found that *p*PDM-modified S1 (*p*PDM-S1) binds to F-actin weakly, both in the presence and absence of ATP [8], with an affinity similar to that of unmodified S1 in the presence of ATP [9]. Therefore *p*PDM-S1 is often used for studies of the weak binding of the myosin head to actin.

Structural characterization of protein–protein interactions can be achieved by differential scanning calorimetry (DSC) as a direct method to measure the thermal unfolding of proteins interacting with each other [10]. This method was successfully used for probing the structural changes that occur in the myosin head due to its strong binding to F-actin in the presence of ADP. It was shown that the binding of skeletal S1 to F-actin significantly increased the thermal stability of S1 [11,12]. A very similar effect was observed by DSC with recombinant *Dictyostelium discoideum* myosin head fragment corresponding to the globular motor portion of the head [13]. It has also been shown that charge changes in the actin-binding surface loop 2 strongly affect the thermal unfolding of the myosin motor domain bound to F-actin [13].

In the present study we applied this DSC approach to examine the weak binding of *p*PDM-S1 to F-actin. Our results show that this binding increases the thermal stability of *p*PDM-S1, and this increase seems to be similar to that observed for strong binding of unmodified S1 to F-actin.

2. Materials and methods

S1 from rabbit skeletal myosin was prepared by digestion of myosin filaments with α-chymotrypsin [14]. The concentration of S1 was determined spectrophotometrically, using $A^{1\%}$ at 280 nm equal to 7.5. S1

*Corresponding author. Fax: (7)-095-954 2732.
E-mail: inbio@glas.apc.org

Abbreviations: S1, myosin subfragment 1; *p*PDM, *N*,*N*′-*p*-phenylenedimaleimide; DSC, differential scanning calorimetry

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was modified with *p*PDM at 0°C in the presence of ADP [15]. The *p*PDM-modified S1 was further purified by sedimenting with F-actin in the presence of 0.1 M KCl [9]. The purified *p*PDM-S1 had NH_4^+-EDTA-ATPase activity less than 0.2% and Ca^{2+}-ATPase and actin-activated Mg^{2+}-ATPase activities less than 3% of unmodified S1.

Actin was prepared from rabbit skeletal muscle acetone powder [16]. Monomeric G-actin was stored in low-strength buffer composed of 2 mM Tris–HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol, and 0.01% NaN_3 (G buffer). Actin concentration was determined spectrophotometrically using $A^{1\%}$ at 290 nm equal to 6.3. G-actin was polymerized to F-actin in G buffer by the addition of 4 mM $MgCl_2$. F-actin was stabilized by the addition of a 2-fold molar excess of phalloidin (Sigma) to obtain a better separation of the thermal transitions of actin-bound S1 and F-actin on DSC thermograms. Specific binding of this cyclic heptapeptide to F-actin was shown to increase the temperature of the thermal denaturation of F-actin by 14°C [12,17,18]. Complexes of S1 or *p*PDM-S1 with F-actin were formed by mixing equal volumes of F-actin and S1 solutions. F-actin solutions contained G buffer, 3 mM $MgCl_2$, and 1 mM ADP. S1 solutions contained 30 mM HEPES, pH 7.3, and 1 mM $MgCl_2$. The final concentration of S1 or *p*PDM-S1 was 13 μM, and F-actin concentration varied from 30 to 130 μM.

The binding of *p*PDM-S1 to phalloidin-stabilized F-actin was determined by a cosedimentation assay [8,9]. The samples containing F-actin and *p*PDM-S1 or S1 were subjected to high-speed centrifugation, and protein composition of supernatants and pellets was analyzed by the sodium dodecyl sulfate (SDS)–gel electrophoresis [19]. The complexes of F-actin with *p*PDM-S1 were also examined by sedimentation velocity experiments in a Beckman model E analytical ultracentrifuge with a photoelectric scanning system at rotor speed from 30 000 to 60 000 rpm.

DSC experiments were performed on a DASM-4M differential scanning microcalorimeter (Institute for Biological Instrumentation, Pushchino, Russia) as described earlier [11–13,18,20]. All measurements were carried out in 15 mM HEPES, pH 7.3, containing 2 mM $MgCl_2$, 0.5 mM ADP, and twice-diluted G buffer, at a scanning rate of 1°C/min. The reversibility of the thermal transitions was verified by checking the reproducibility of the calorimetric trace in a second heating of the sample immediately after cooling from the first scan. The thermal denaturation of all protein samples studied was fully irreversible. The calorimetric traces were corrected for the instrumental background and for possible aggregation artifacts by subtracting the scans obtained from the second heating of the samples. The temperature dependence of the excess heat capacity was further analyzed and plotted using Origin software (MicroCal. Inc.). Transition temperatures (T_m) were determined from the maximum of thermal transition. Calorimetric enthalpies (ΔH_{cal}) were calculated from the area under the excess heat capacity curves. Because these parameters can be obtained directly from experimental calorimetric traces after simple treatments such as subtraction of instrumental background, concentration normalization, and chemical baseline correction, they can be used for the description of the irreversible thermal denaturation of S1. A molecular mass of 115 kDa was used for calculation of the excess molar heat capacity of S1 or *p*PDM-S1.

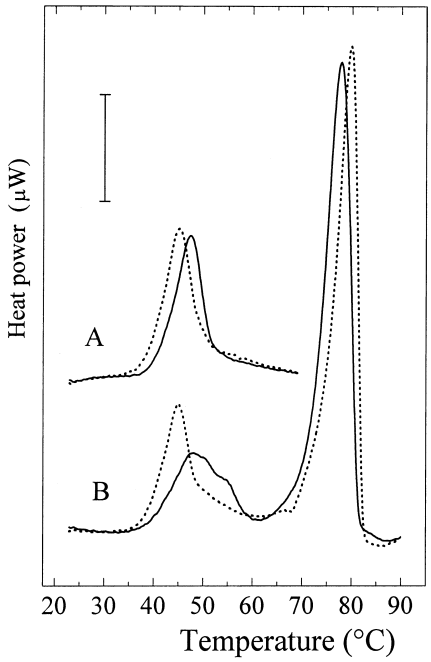

Fig. 1. The experimental DSC curves of *p*PDM-S1 (A) and its complex with F-actin stabilized by phalloidin (B). Conditions: 13 μM *p*PDM-S1, 40 μM F-actin, 80 μM phalloidin in 15 mM HEPES, pH 7.3, 2 mM $MgCl_2$, 0.5 mM ADP, and twice-diluted G buffer. Curves shown by dotted lines were obtained under the same conditions except that 200 mM KCl was added to the buffer. Heating rate 1 K/min. The vertical bar corresponds to 10 μW.

3. Results

Fig. 1 shows the calorimetric traces for the thermally induced unfolding of *p*PDM-S1 in the absence of actin (Fig. 1A) and in the presence of a 3-fold molar excess of F-actin stabilized by phalloidin (Fig. 1B). The solid line curves represent DSC scans obtained at low ionic strength, whereas the dotted line curves were obtained in the presence of 200 mM KCl. While the interaction of *p*PDM-S1 with F-actin had no appreciable influence on the thermal unfolding of phalloidin-stabilized F-actin which denatured with maximum at 78–80°C, this interaction significantly affected the thermal unfolding of *p*PDM-S1. This effect was observed only at very low ionic strength, i.e. under conditions favoring the weak binding

Table 1
Calorimetric parameters obtained from the DSC data for *p*PDM-S1, in comparison with those for unmodified S1

Preparation and conditions	T_m (°C)	ΔH_{cal} (kJ/mol)
In the absence of added ligands		
S1	47.8	1560
*p*PDM-S1	47.3	1310
In the presence of 0.5 mM ADP		
S1	50.1	1690
*p*PDM-S1	47.3	1430
In the presence of 0.5 mM ADP and 0.5 mM V_i		
S1	57.8	1950
*p*PDM-S1	47.5	1340
In the presence of 0.5 mM ADP and 0.5 mM BeF_x (5 mM NaF and 0.5 mM $BeCl_2$)		
S1	56.3	1800
*p*PDM-S1	48.7	1360
In the presence of 40 μM F-actin stabilized by phalloidin		
S1	54.2	2010
*p*PDM-S1	48.3 (47.3; 52–53)	1600

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

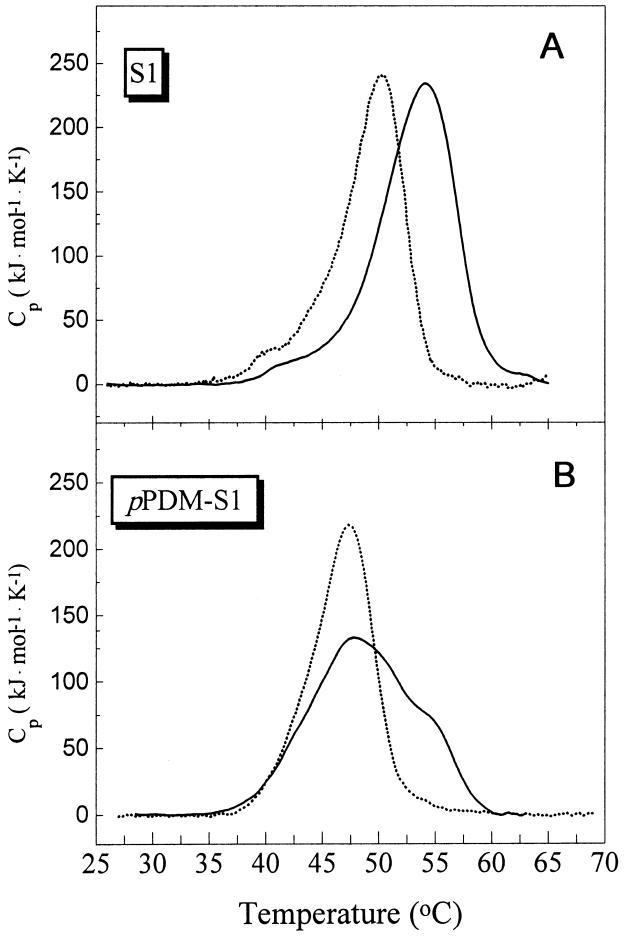

Fig. 2. Temperature dependences of excess heat capacity (C_p) for unmodified S1 (A) and *p*PDM-S1 (B) in the absence (dotted line curves) and in the presence (solid line curves) of F-actin. The temperature region above 65°C, corresponding to the region of thermally induced denaturation of phalloidin-stabilized F-actin, is not shown. Conditions were the same as in Fig. 1.

of *p*PDM-S1 to F-actin. The effect was not observed at higher ionic strength (200 mM KCl) which was shown to completely prevent the interaction of *p*PDM-S1 with F-actin [9,21] but not to prevent the strong binding to F-actin of unmodified S1 [21]. These results mean that the changes in the thermal unfolding of *p*PDM-S1 observed in the presence of F-actin at low ionic strength (Fig. 1) are caused by weak binding of *p*PDM-S1 to F-actin. Formation of the complex of F-actin with *p*PDM-S1 at low ionic strength was confirmed by sedimentation velocity experiments. The sedimentation coefficient of this complex measured at 20°C was equal to 92.0 ± 2.5 S, which is appreciably higher than the coefficient of free F-actin (65.5 ± 2.5 S) measured under the same conditions.

Fig. 2 shows the excess heat capacity curves for S1 (Fig. 2A) and *p*PDM-S1 (Fig. 2B) obtained in the absence and in the presence of F-actin. In the absence of actin both S1 and *p*PDM-S1 demonstrate sharp thermal transitions. The T_m and ΔH_{cal} values for *p*PDM-S1 were lower than those of S1 (Table 1), in good agreement with earlier published results [20,22]. Strong binding of S1 to F-actin in the presence of ADP increases the thermal stability of S1 substantially by shifting whole the thermal transition by more than 4°C, from 50.1 to 54.2°C (Fig. 2A). Cosedimentation experiments (Fig. 3) indicated that S1 was completely bound to F-actin under the conditions used for the DSC experiments. Under the same conditions only about 40% of *p*PDM-S1 was bound to F-actin, and about 60% of *p*PDM-S1 remained in the supernatant (Fig. 3). Cosedimentation studies also showed that interaction of *p*PDM-S1 with F-actin is insensitive to ATP addition, that is a characteristic of the weak binding of *p*PDM-S1 to F-actin [8]. The binding of *p*PDM-S1 to F-actin results in the appearance of a high-temperature shoulder at 53–57°C on the calorimetric peak of *p*PDM-S1 (Fig. 2B). As this shoulder was only observed in the presence of F-actin and only under conditions favoring the binding of *p*PDM-S1 to F-actin (Figs. 1 and 2B), it corresponds to the thermal unfolding of actin-bound *p*PDM-S1, which is more thermostable than free *p*PDM-S1.

Thus, one can propose that in the presence of F-actin the thermal transition of *p*PDM-S1 comprises two overlapping peaks, the peak characteristic of free *p*PDM-S1, and the peak corresponding to *p*PDM-S1 bound to F-actin. In order to check this assumption we have applied a simple computer simulation to reveal the peak of actin-bound *p*PDM-S1 within experimental DSC curve of *p*PDM-S1 obtained in the presence of F-actin. Cosedimentation measurements (Fig. 3) were used to estimate the fraction of *p*PDM-S1 unbound to actin under the conditions of the DSC experiment. The corresponding fraction of the heat sorption curve for *p*PDM-S1 in the absence of actin was subtracted from the curve of *p*PDM-S1 obtained in the presence of actin (Fig. 4). This simple procedure was justified by our observation that the thermal unfolding of free *p*PDM-S1 was almost independent of protein concentration (data not shown). As a result of this subtraction we obtained the peak of actin-bound *p*PDM-S1 (Fig. 4, curves shown by dashed lines). At *p*PDM-S1/actin molar ratio equal

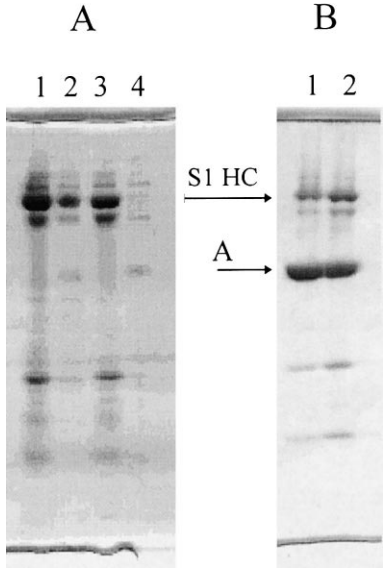

Fig. 3. Cosedimentation of S1 or *p*PDM-S1 with F-actin. 40 μM F-actin stabilized by phalloidin was mixed with 13 μM S1 or *p*PDM-S1 in 15 mM HEPES, pH 7.3, 2 mM $MgCl_2$, 0.5 mM ADP, and twice-diluted G buffer. The actin was then pelleted at 100 000 × *g* for 120 min. Equivalent samples of the pellet and supernatant were run on SDS–PAGE and stained with Coomassie blue. (A) Supernatants of *p*PDM-S1 (1, 2) and S1 (3, 4) after centrifugation in the presence (2, 4) or absence (1, 3) of F-actin. (B) The pellets of F-actin after centrifugation in the presence of *p*PDM-S1 (1) and S1 (2). A, actin; S1 HC, S1 heavy chain (95 kDa).

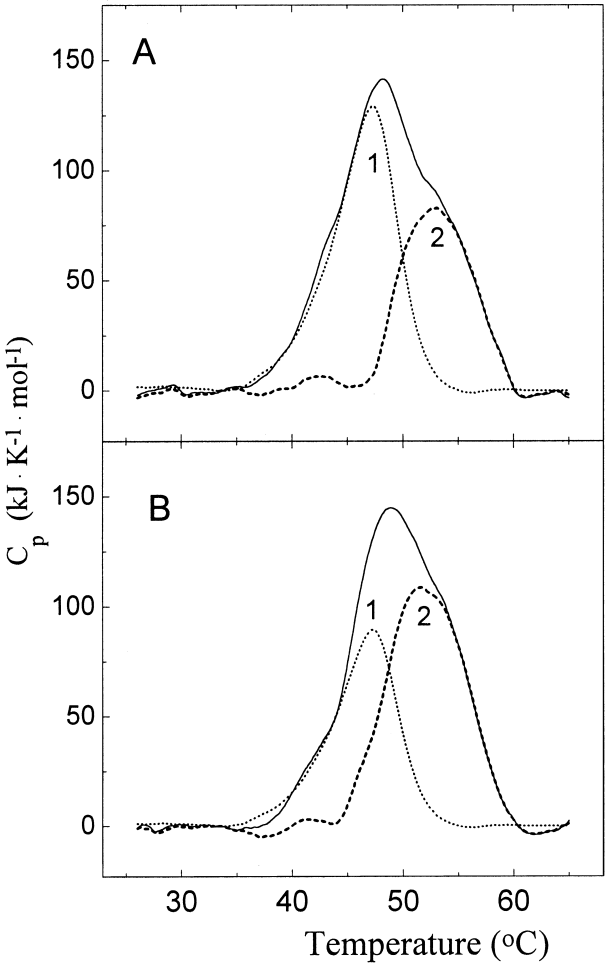

Fig. 4. DSC scans of *p*PDM-S1 in the presence of F-actin (solid line curves) obtained at molar ratios of *p*PDM-S1 to F-actin equal to 1:2.5 (A) and 1:10 (B). Other conditions were the same as in Figs. 1 and 2B. Curves 1 shown by dotted lines were obtained by decreasing the thermal transition of free *p*PDM-S1 (dotted line curve in Fig. 2B) to 60% (A) or 40% (B) of its initial size. Each dashed line curve 2, corresponding to actin-bound *p*PDM-S1, was obtained by subtraction of the dotted line curve 1 from the total heat sorption curve.

to 1:2.5, when about 40% of *p*PDM-S1 molecules were weakly bound to F-actin, the maximum of the peak of actin-bound *p*PDM-S1 was at 53°C, and its enthalpy (660 kJ/mol) was 41% of that for the total heat sorption curve (1600 kJ/mol) (Fig. 4A). The weak binding of *p*PDM-S1 to F-actin was shown to increase with actin concentration [9]. At *p*PDM-S1/actin molar ratio equal to 1:10, when more than 60% of *p*PDM-S1 was bound to F-actin, the enthalpy of the peak of actin-bound *p*PDM-S1 increased to 920 kJ/mol, i.e. to 58% of the total enthalpy of the entire heat sorption curve (Fig. 4B).

In general, these results mean that the weak binding of *p*PDM-S1 to F-actin increases the thermal stability of *p*PDM-S1 by shifting its thermal transition to a higher temperature.

4. Discussion

The data presented here show that the weak binding of *p*PDM-S1 to F-actin affects the thermal unfolding of *p*PDM-S1 by shifting the thermal transition of actin-bound *p*PDM-S1 to a higher temperature. However, because of a very low affinity of *p*PDM-S1 to F-actin (binding constant of about $4{\cdot}10^4$ M^{-1} [8,9]), not all *p*PDM-S1 molecules were bound to F-actin under conditions favoring the weak binding (Fig. 3). Therefore special treatment of DSC curves was applied to reveal the thermal transition of actin-bound *p*PDM-S1 (Fig. 4). The results suggest that the weak binding to F-actin increases the *p*PDM-S1 thermal stability by shifting the thermal transition of *p*PDM-S1 by about 5°C, from 47.3°C to 52–53°C. The actin-induced increase in T_m determined for *p*PDM-S1 appears to be even larger than that observed for unmodified S1 (4.1°C) under the same conditions (Fig. 2A).

However, we also observed a significant increase, up to 7–8°C, of the actin-induced shift of S1 thermal transition for S1 specifically modified at the SH1 or SH2 group as well as for S1 in which both SH1 and SH2 groups were modified by *N*-phenylmaleimide, a monofunctional reagent similar to *p*PDM (Levitsky et al., unpublished data). It has been shown that these modified S1 preparations demonstrate, unlike *p*PDM-S1, the ATP-sensitive strong binding to F-actin [8,23]. Thus, the increased actin-induced shift to higher temperature of the maximum of *p*PDM-S1 thermal transition is most likely caused by modification of the SH1 and SH2 groups themselves rather than by cross-linking of these groups leading to the weak binding of S1 to F-actin.

It should be noted that at elevated temperature during the DSC experiment the fraction of actin-bound *p*PDM-S1 may be different from that estimated by cosedimentation measurements performed at room temperature. For example, one can propose that the heating leads to the loss of ADP trapped on *p*PDM-S1 thus affecting the affinity of *p*PDM-S1 for F-actin. Direct estimation of the fraction of actin-bound *p*PDM-S1 by cosedimentation methods is inapplicable under conditions of the DSC experiment (i.e. at 40°C or above) because of aggregation which accompanies the thermal denaturation of the protein. However, there are some literature data suggesting that the heating during the DSC experiment should not significantly alter the weak binding of *p*PDM-S1 to F-actin. It has been shown that removal of the trapped nucleotide from *p*PDM-S1 causes a very small increase in the affinity of *p*PDM-S1 for actin (binding constant increases from $3.5{\cdot}10^4$ M^{-1} to $5.5{\cdot}10^4$ M^{-1}) [24]. This effect of the bound nucleotide on the binding of *p*PDM-S1 to F-actin is too small in comparison with the difference by several orders of magnitude between the weak and strong binding of S1 to F-actin. Furthermore, according to the three-step docking model of formation and stabilization of the AM [25], the initial step (formation of a collision complex which corresponds in many respects to the binding of *p*PDM-S1 to actin) is strongly dependent on ionic strength but independent of temperature. These data allow to suppose that *p*PDM-S1 remains weakly bound to F-actin at elevated temperature during the DSC experiment.

Previous DSC studies have shown that DSC is useful for probing global conformational changes in the myosin motor caused by ligand-binding [12,13,20,22]. The formation of stable ternary complexes of S1 with ADP and P_i analogues such as orthovanadate (V_i) or beryllium fluoride (BeF_x) caused a significant increase of S1 thermal stability, as judged by the values measured for T_m and ΔH_{cal} [12,20,22,26] (Table 1). In contrast, the thermal unfolding of *p*PDM-S1 was not affected

by the addition of ADP and P_i analogues (Table 1). In agreement with recently published data [22], these results mean that *p*PDM-S1 is unable, unlike S1, to undergo nucleotide-induced structural changes. However, it is able, like S1, to undergo structural changes induced by interaction with F-actin.

Overall, this work presents new data suggesting that structural changes revealed by DSC in the myosin head, that are due to interaction with F-actin, occur not only upon strong binding but also on the weak binding of the head to F-actin. This assumption is corroborated by previous DSC data demonstrating that these structural changes are strongly affected by charge changes in loop 2, which is the site mainly responsible for the weak binding of the myosin head to F-actin [13]. We may speculate that since these structural changes occur in the myosin head on forming the weak binding state they must occur during the initial steps of actin–myosin interaction and before the power-stroke. They therefore may play an important role for the transition of the AM from the weakly bound state to the strongly bound state.

However, it is important to note that *p*PDM-S1 is a functional but not a structural analogue of the weakly bound state of S1. The conformational state of *p*PDM-S1 is different from that of the weakly bound states of S1 (S1·ATP and S1·ADP·P_i) [27,28] or their structural analogues [20,22] and, furthermore, *p*PDM-S1 is unable to undergo nucleotide-induced structural changes [22] (Table 1). To obtain more reliable structural information about the weakly bound states it is necessary to investigate by DSC the weak binding to F-actin of stable S1 ternary complexes with ADP and P_i analogues (S1·ADP·V_i, S1·ADP·BeF_x, and S1·ADP·AlF_4^-). These complexes bind weakly to F-actin [21,29] and structurally resemble the weakly bound states of S1, S1·ATP and S1·ADP·P_i [24,28]. However, these complexes are only stable in the absence of actin and they decompose rapidly after actin addition. Therefore the interaction of these complexes with actin can only be studied by special approaches. Work in this direction is now in progress in our group.

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