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Thermal unfolding of *Acanthamoeba* myosin II and skeletal muscle myosin

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Abstract

Studies on the thermal unfolding of monomeric *Acanthamoeba* myosin II and other myosins, in particular skeletal muscle myosin, using differential scanning calorimetry (DSC) are reviewed. The unfolding transitions for intact myosin or its head fragment are irreversible, whereas those of the rod part and its fragments are completely reversible. *Acanthamoeba* myosin II unfolds with a high degree of cooperativity from ca. 40–45°C at pH 7.5 in 0.6 M KCl, producing a single, sharp endotherm in DSC. In contrast, thermal transitions of rabbit skeletal muscle myosin occur over a broader temperature range (ca. 40–60°C) under the same conditions. The DSC studies on the unfolding of the myosin rod and its fragments allow identification of cooperative domains, each of which unfolds according to a two-state mechanism. Also, DSC data show the effect of the nucleotide-induced conformational changes in the myosin head on the protein stability.

Keywords: Protein unfolding; Myosin; Muscle, skeletal; Unfolding, thermal; *Acanthamoeba*

1. Preface

It is a privilege to have this opportunity to contribute to a special issue of *Biophysical Chemistry* in memory of Bill Harrington and I thank John Schellman and Pete von Hippel for making this possible. I have been fortunate throughout my scientific career to have had Bill as a friend. When hearing the music of Schubert, running the ultracentrifuge, or thinking of myosin, my thoughts turn to Bill. During the studies of Michal Zolkiewski (in collaboration with Jolanta Redowicz in Ed Korn's group) on the thermodynamics of unfolding *Acanthamoeba* myosin II and the structurally related skeletal muscle myosin that are summarized here, I missed the discussions with Bill which certainly would have taken place.

(Ann Ginsburg, April, 1995.)

2. Introduction

Myosin is one of the principal proteins responsible for motility and contractility in eukaryotic cells. There have been many reviews of the literature on myosin that focus on its structure, function and molecular mechanism of action [1–4]. A terminology has been adopted to refer to all myosins structurally similar to the one found in muscle as "conventional myosins". Conventional myosins are hexameric proteins composed of a pair of heavy chains and two

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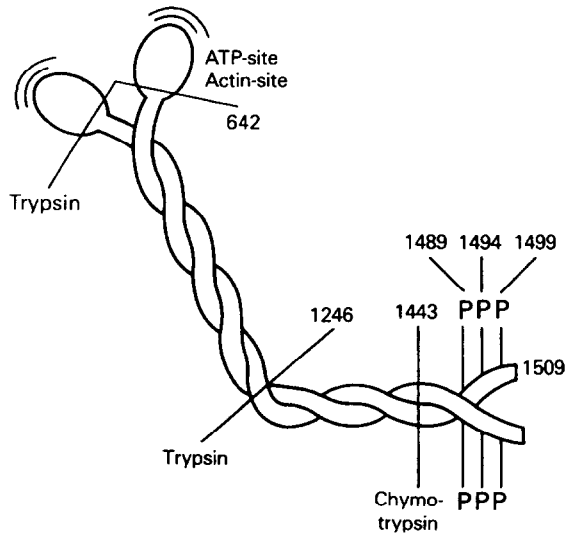

Fig. 1. Scheme of *Acanthamoeba* myosin II. Shown are the N-terminal, globular parts of the heavy chains (with associated light chains) containing actin- and ATP-binding sites and the C-terminal coiled-coil rod with an unstructured "hinge" around Lys^{1246} and serine sites of phosphorylation within the unstructured tail. Sites of protease cleavage also are indicated.

pairs of light chains. The N-terminal regions of the heavy chains form globular heads while the C-terminal parts assemble into a coiled-coil, α-helical rod. Each of the two myosin heads contains a nucleotide site for catalyzing ATP hydrolysis to ADP and phosphate and an actin binding site. The light chains are associated with the head regions (two light chains per heavy chain). The myosin rod is a super-helical structure which is stabilized by hydrophobic and ionic interactions between the non-polar and polar amino acid residues, respectively, in the C-terminal parts of the heavy chains. The amino acid sequences of myosin rods indeed display characteristic periodic repeats of non-polar and polar residues [1]. Recently, the problem of folding and assembly of multi-subunit proteins and multi-protein complexes has been attracting an increased attention of protein chemists. In this paper, we review recent advances in thermal unfolding/refolding studies of *Acanthamoeba* myosin II and other conventional myosins, in particular, skeletal muscle myosin in the monomeric (non-filamentous) state.

Myosin II from *Acanthamoeba castellanii* (Fig. 1) is composed of a pair of heavy chains of $M_r \approx$ 172 000 and two pairs of light chains of $M_r \approx$ 17 500 and 17 000 [5,6]. The amino acid sequence of the myosin II head is very similar to the corresponding sequences of muscle myosins [7]. However, the amino acid sequence of the myosin II rod cannot be aligned with the rod sequences of other myosins, although it does show similar periodic repeats of hydrophobic and charged residues found in all conventional myosins and other coiled-coil structures [7]. The myosin II rod is significantly shorter than that of skeletal muscle myosin (ca. 90 nm vs. ca. 160 nm [8,9]). Also, in contrast to muscle myosins, the 29 C-terminal residues of each heavy chain of myosin II comprise an unstructured tail-piece containing three serines that can be phosphorylated in vivo [10,11] and in vitro [12,13] (Fig. 1). Phosphorylation inhibits both the actin-activated Mg–ATPase activity of filamentous myosin II [10–14] and actin filament movement in an in vitro motility assay of filamentous and monomeric myosin II [14]. Phosphorylation of the carboxyl termini also produces conformational changes in myosin II, as shown by proteolytic studies [15,16] and differences in sedimentation velocity coefficients [16].

Limited proteolysis of myosins has been a useful tool for studying isolated fragments which are usually referred to as structural domains [17]. The domain structure of skeletal muscle myosin has been extensively reviewed before (see [1,3] and references therein). Briefly, rabbit skeletal muscle myosin can be split with trypsin within the rod segment into heavy meromyosin (HMM) and light meromyosin (LMM). Papain cleaves HMM into subfragment 1 (S1) which corresponds to the globular head and subfragment 2 (S2) corresponding to the N-terminal portion of the rod. The LMM can be cleaved by further trypsin treatment into three discrete fragments. The myosin head (S1) can be further divided proteolytically into three fragments. It should be mentioned also that the X-ray crystal structure of S1, including the head subdomains, has been recently solved [18].

Proteolytic cleavage of *Acanthamoeba* myosin II is significantly different from that of skeletal muscle myosin. For example, it has not been possible to cleave myosin II in the region of the head–rod junction (Fig. 1). Consequently, the N-terminal tryptic fragment of myosin II lacks the C-terminal part of

the head (ca. 25%) that is present in muscle myosin S1 [19]. Treatment with trypsin at pH 6.3 also splits myosin II rod at the unstructured "hinge" region containing a proline residue at position 1245 [20]. The 66-residue, unstructured C-terminal tail-pieces of each heavy chain (containing phosphorylation sites) can be removed with chymotrypsin [21].

The problem of determining protein stability can be approached most rigorously by using a thermodynamic description of the folding/unfolding processes. Different physical and chemical parameters of the protein structure are usually measured during unfolding reactions which can be driven by increasing the temperature or the concentration of denaturants. Differential scanning calorimetry (DSC) offers several important advantages in the study of the thermal unfolding of proteins when compared to other methods. Unlike spectral parameters which follow the progress of the macromolecular rearrangements as a function of temperature, DSC measures directly the temperature dependence of a thermodynamic function, i.e., the heat capacity (C_p) of protein solutions [22]. Also, since C_p is the first derivative of the enthalpy, DSC usually gives a better resolution of the progress of unfolding reactions than any of the spectral methods [23]. DSC measurements often indicate that proteins have more than one cooperative domain, each of which unfolds independently according to a two-state mechanism [22–24]. Such domains are not necessarily identical to protein structural domains and their thermodynamic properties can be affected by the interactions between domains as well as by the binding of ligands [25]. It must be stressed, however, that in order to obtain a reliable mechanism for protein unfolding, especially in the case of multi-subunit proteins, DSC results should be supported by other types of measurements.

3. Thermal unfolding of *Acanthamoeba* myosin II

The DSC profile for *Acanthamoeba* myosin II shows a single, sharp endothermic transition with a maximum (T_{max}) at ca. 42°C (Fig. 2). The thermally induced reaction is partially reversible (ca. 50% of the endotherm area is observed in a re-scan of the

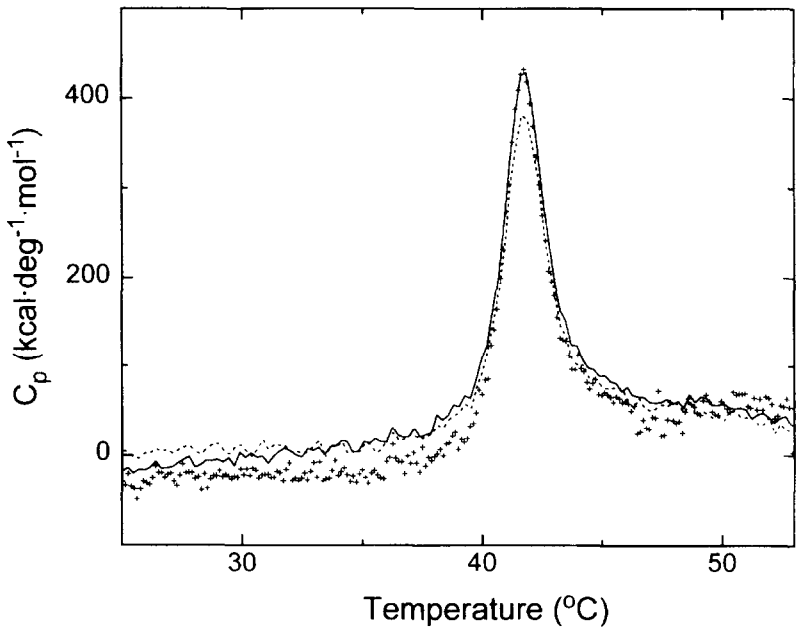

Fig. 2. DSC scans for (solid line) dephospho-myosin II (1.23 mg/ml), (dotted line) phospho-myosin II (1.11 mg/ml) and (crosses) myosin II after removal of C-terminal 66 amino acids from each heavy chain (0.92 mg/ml) in 10 mM imidazole–HCl, pH 7.5, 0.6 M KCl and 1 mM dithiothreitol. The data have been obtained with a MicroCal MC-2 scanning calorimeter (sample volume 1.2 ml) using 60°C/h scan rate and are normalized for concentration and scan rate after subtraction of the instrument base line [26].

same sample [26]). The DSC transition corresponds to a temperature-induced, partial unfolding of myosin II, as confirmed by circular dichroism (CD) measurements in the far UV [26]. CD spectra show that a decrease in the secondary structure of myosin II occurs in the temperature region of the DSC transition (Fig. 2). However, the high-temperature form of myosin II is not equivalent to the random-coil conformation obtained in 6 M guanidine · HCl [26]. Light scattering increases have indicated that the thermally induced unfolding of myosin II is followed by protein self-association which can be reversed by rapid cooling of the sample [26]. No separation of the heavy and light chains of myosin II has been observed.

Rigorous analysis of DSC data requires that the system being investigated is in thermodynamic equilibrium during the scans. It has been observed earlier that the thermal unfolding of many small, globular proteins is completely reversible and DSC data cor-

Table 1
Thermodynamic parameters for the thermally induced unfolding of myosins and their fragments

Sample [a]	Buffer conditions [b]	T_{max} (°C) [c]	ΔH (kcal/mol)	Ref.
Acanthamoeba myosin II (413 000 M_r)	10 mM imidazole, pH 7.5	41.7	1080	[26]
Rabbit skeletal muscle myosin (470 000 M_r)	10 mM imidazole, pH 7.5	46.3; 53.7	2500	[26]
Rabbit skeletal muscle myosin (470 000 M_r)	20 mM phosphate, pH 7.0	ca. 46; ca. 54	1715	[29]
Bovine heart myosin	25 mM Hepes, pH 7.0	ca. 45; ca. 54	1930	[30]
Carp myosin [d] (470 000 M_r) A	20 mM Tris, pH 8.0	34; 47	380	[31]
Carp myosin [d] (470 000 M_r) B		39; 49	550	
Myosin II head fragment (68 000 M_r)	10 mM imidazole, pH 7.5	41.1	170	[26]
Chymotryptic S1 (87 000 M_r + light chains)	20 mM phosphate, pH 7.0	46.3	255	[29]
Chymotryptic S1	50 mM Tris, pH 7.9	45	300	[32]
Chymotryptic S1 (95 000 M_r + light chains)	10 mM Hepes, pH 7.3	47.2	270	[33]
Skeletal muscle myosin rod (252 000 M_r)	20 mM phosphate, pH 7.0	ca. 43; ca. 54	1058	[29]
Skeletal muscle myosin rod (200 000 M_r)	25 mM phosphate, pH 6.5	ca. 43; ca. 52	930	[23]
Skeletal muscle myosin rod	50 mM phosphate, pH 7.0	47; 53	– [e]	[34]
Skeletal muscle myosin rod (220 000 M_r)	0.2 M phosphate, pH 7.0	46; ca. 57	1040	[35]
HMM (355 000 M_r)	50 mM Tris, pH 7.9	41; 48	– [f]	[32]
LMM (130 000 M_r)	0.2 M phosphate, pH 7.0	45; 55	740	[35]
S2 (90 000 M_r)	0.2 M phosphate, pH 7.0	46; 57	185	[35]
S2 (60 000 M_r)	50 mM Tris, pH 7.9	40.3	350	[32]
Rabbit skeletal muscle myosin light chains (37 000 M_r) [g]	20 mM phosphate, pH 7.0	51.5	85	[29]

[a] The molecular weights of myosin preparations are listed as reported by the referenced authors.
[b] The buffer conditions also usually include 0.5 M or 0.6 M KCl and 1 mM dithiothreitol and/or 1 mM EDTA.
[c] For skeletal muscle myosin T_{max} corresponds to visible C_p maxima of broad thermal transitions (see Fig. 3B).
[d] The forms A and B of myosin were obtained from carp acclimated at 10°C and 30°C, respectively.
[e] Circular dichroism measurements.
[f] Not determined, due to a strong influence of protein aggregation on the DSC data.
[g] Average molecular weight of two light chains in the preparation.

respond to an equilibrium process [22,23]. Conversely, the unfolding of larger, oligomeric proteins is usually irreversible. The presence of irreversible processes, such as protein aggregation, poses a great difficulty since $C_p(T)$ is affected by all thermally induced reactions. If an irreversible step dominates the kinetics and the heat of the complete process, the position and shape of a DSC endotherm will strongly depend on the rate of scanning [27,28]. For myosin II, however, scan rate dependence of the DSC data is limited to the post-transition region [26]. This indicates that the DSC transition showed in Fig. 2 corresponds mainly to reversible steps in the unfolding reaction. The enthalpy of thermal unfolding, as estimated by the area of the DSC transition, is given in Table 1. Current studies in progress show that the myosin II rod (beginning at residue 849, expressed in *E. coli*) unfolds reversibly with a T_{max} value that is similar to that of the whole myosin II molecule. The unfolding of the rod is coupled to the dissociation of the heavy chains, as evidenced by a light scattering decrease. This suggests that it is the myosin II head that self-associates and is responsible for the lack of full reversibility in the thermal unfolding of the intact protein.

Fig. 2 also illustrates that the DSC profiles of phospho-myosin II, dephospho-myosin II and chymotrypsin-treated myosin II, which lacks the 66-residue C-terminal tail-pieces, are very similar. Thus, phosphorylation-induced conformational changes, as revealed by other methods [15,16], must involve intramolecular rearrangements that do not substantially change the exposure of the hydrophobic protein core, as this would affect the stability of myosin II and the unfolding enthalpy.

4. Thermal unfolding of skeletal muscle myosin and its fragments

For brevity, we will concentrate on DSC studies of the thermal unfolding of skeletal myosin (Table 1). It should be noted, however, that guanidine · HCl-induced denaturation of S1 and myosin rod have also been studied [36,37]. The thermal reactions for the whole myosin or the fragments containing the myosin head (HMM, S1) are irreversible [29,32,33]. In contrast, almost complete reversibility is observed

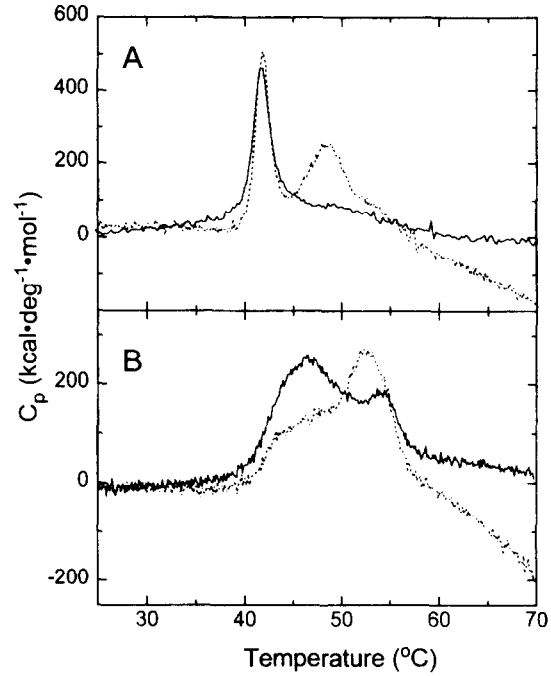

Fig. 3. DSC data for (A) dephospho-myosin II (1.23 mg/ml) and (B) rabbit skeletal muscle myosin (1.0 mg/ml) in the (solid lines) absence and (dotted lines) presence of 5 mM Mg·AMPPNP obtained at a scan rate of (A) 60°C/h or (B) 90°C/h, after subtracting buffer base lines and normalization for protein concentration and scan rate [26]. The buffer was the same as that given in the legend to Fig. 2.

for myosin rod and its fragments [23,29,32,34,35,38]. This reversibility is abolished when isolated S1 is present in solutions of myosin rod [29]. These findings are supported by the electron microscopic observations of the heat-induced aggregation of myosin heads with a simultaneous partial dissociation of the heavy chains within the rod [39].

Fig. 3 illustrates substantial differences between DSC profiles for *Acanthamoeba* myosin II and rabbit skeletal muscle myosin [26]. The latter unfolds less cooperatively than the former (i.e., over a wider temperature range), which suggests that there are differences in both the number and properties of thermodynamic domains of these myosins. Thermodynamic parameters for the thermal unfolding of myosins and their fragments are given in Table 1. For comparison with DSC studies, T_m values from CD measurements of the myosin rod unfolding [34] are included also.

The data in Table 1 show that the specific enthalpy for the thermal unfolding of mammalian muscle myosins is ca. 4–5 cal/g, which corresponds to ca. 70% of the values reported for the complete unfolding of smaller, monomeric proteins [22]. This agrees with CD results which indicate that ca. 70% of the secondary structure of myosin is thermally labile [26]. The myosins from fish appear to be less stable than mammalian ones, since these unfold at lower temperatures with a lower overall enthalpy change [31,40].

The shape of DSC endotherms for skeletal muscle myosin (Fig. 3B) suggests that the thermal reaction is not a simple two-state process, but involves the sequential or concerted unfolding of multiple cooperative domains. However, due to the irreversibility of reactions and the possible influence of aggregation, rigorous deconvolution of the DSC data for intact myosin, HMM and S1 is unreliable [26,29,32]. An exception is the study by Levitsky et al. [33], in which advantage has been taken of the irreversibility of DSC scans. This approach has identified three discrete steps responsible for irreversibility of thermal reactions in S1 [33]. It is not clear, however, if and how the described "aggregation" domains are related to the above-mentioned structural or thermodynamic domains in a protein.

Pioneer studies of Privalov et al. [23] on the stability of the myosin rod and its proteolytic fragments have demonstrated the presence of six cooperative unfolding domains in the rod. It also has been found that the two polypeptide chains of the rod separate during the unfolding and form two random coils. Since all six DSC domains within the rod correspond to the unfolding of six isolated, double-stranded fragments, dissociation of the two heavy chains does not appear to produce an identifiable intermediate or a two-state transition. Privalov's results have been confirmed by Bertazzon and Tsong [29,38] and Lopez-Lacomba et al. [35]. The latter papers include detailed studies on the pH dependence of the properties of cooperative domains in the myosin rod.

Studies on the unfolding of the rod fragments show that LMM contains five cooperative domains [35,38]. However, deconvolution of DSC transitions for S2 gives one [32], two [35] or three [38] two-state transitions. This variation can be attributed to differences in the preparations of S2 used in those studies (see Table 1).

5. Nucleotide effects on the unfolding of *Acanthamoeba* myosin II and skeletal muscle myosin

The function of myosin is to convert the energy of ATP hydrolysis into a directed movement of myosin filaments against actin filaments. This must involve a conformational change in the myosin molecule which is coupled to ATP binding and the ATP hydrolysis cycle. According to a model of Harrington and coworkers (for review see [1,3]) partial unfolding (helix–coil transition) of the "hinge" region in the myosin rod (between HMM and LMM), which leads to a shortening of the rod, is responsible for movement generation. Another model, which is supported by recent X-ray crystallographic data [18,41], postulates that the crucial conformational change occurs within S1. The most elegant demonstration of this comes from the in vitro motility assay (for review see [3]), in which S1 alone promotes the movement of actin filaments.

Large conformational changes in proteins may influence stability as well as domain interactions and, thus, influence the mechanism of protein unfolding. In the presence of nucleotides, thermal stabilization has been observed for the skeletal muscle myosin head region in either the intact molecule (Fig. 3B) [26], HMM [32] or isolated S1 [32,33] and for *Acanthamoeba* myosin II (intact molecule or head fragment) (Fig. 3A) [26]. Shifts in T_{max} for DSC endotherms which occur on binding ligands can be caused by differences in binding affinities of a ligand to native and unfolded forms of a protein [42,43], without any changes in protein conformation. However, different saturating nucleotide concentrations give the same T_{max} for the myosin II head [26], which indicates that this is not a simple case of preferential interaction of the nucleotide with the folded form of myosin II [42,43]. The insensitivity of T_{max} to increasing concentrations of saturating nucleotide suggests that the unfolding reactions are coupled to a nucleotide-induced conformational change. The T_{max} shift of ca. +7°C, which is observed in DSC for the myosin II head fragment on the addition of nucleotide, is not as apparent in CD

measurements of intact myosin II at 222 nm [26], presumably because the CD spectrum is dominated by the ellipticity arising from the α-helical rod structures.

The thermal stabilization of myosin II heads does not depend on the state of phosphorylation of the tail and is greatest with AMPPNP (a non-hydrolyzable ATP analogue), less with ADP + phosphate and still smaller with ADP alone [26]. The thermal stabilization of S1 is greatest in the presence of ADP · vanadate and smaller with AMPPNP or ADP [32,33]. These data are consistent with a model in which unfolding reactions of myosin are coupled to an equilibrium between different conformations of the myosin head. Existence of the temperature-modulated conformational equilibrium is supported by spectroscopic and thermodynamic data [2,44,45]. Conformational changes in the myosin head which are coupled to ATP binding and the release of both ADP and phosphate are a source of the power stroke.

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