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# F-actin has a very high calorimetric unfolding enthalpy

C.R. Gicquaud *, P.H. Aubin, B. Heppell, F. St-Gelais

*Département de Chimie-Biologie, Université du Québec à Trois-Rivières, CP 500 Trois-Rivières, Que., Canada G9A 5H7*

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**Abstract**

The thermal unfolding of F-actin was studied using differential scanning calorimetry. Heat denatures F-actin in two steps. The first is endothermic and corresponds to the unfolding of the peptide chain, while the second is exothermic and is due to the aggregation of the unfolded molecules. The aspect of the thermogram is influenced by the concentration of the protein. For concentrations around 1 mg/ml, the steps are superimposed, while the two steps are separated at very low concentrations. It thus becomes possible to estimate the calorimetric enthalpy for the unfolding step. The enthalpy of unfolding is 64 MJ/mol, or 1400 J/g. This value is considerably higher than those mentioned in the literature for the denaturation of actin and other proteins, which are in the range of 25–30 J/g. The large amount of energy required to unfold the molecule of F-actin could be an adaptation of its role as a protein that transmits forces, and consequently must be very resistant to mechanical constraints.


*Keywords:* F-actin; Differential scanning calorimetry; Thermal unfolding

Since the development of high sensitivity differential scanning calorimeter (DSC) by Privalov [1,2], the in vitro study of the unfolding process of proteins has been the subject of increasing interest. By this method, information can be obtained on protein stability, protein domains, and on protein–ligand interactions. Changes in conformation that result from these interactions may also be revealed [3–7].

Among these studies, several have been done with actin. By its interaction with myosin, actin plays a key role in muscular contraction and amoeboid movement. It is also a component of the cytoskeleton. Actin exists as a monomer called G-actin in a low ionic strength buffer. Increasing the ionic strength of the medium induces G-actin to polymerize into a double-stranded filament called F-actin [8]. In muscle, actin is present only in the F-actin form, while in non-muscle cells there is an equilibrium between the monomer and the polymer forms modulated by actin-binding proteins [9,10]. The actin filaments serve to transmit mechanical forces that produce movement.

The first DSC studies on actin were done by Contaxis et al. [11] and Fausnaugh et al. [12]. Later, using computer deconvolution of thermograms, Tatunashvili and Privalov [13] as well as Bertazzon et al. [14] have concluded that G-actin consists of at least two interacting domains. With the same approach, Lörinczy et al. [15] found three domains in the F form. Calorimetry also made it possible to study the interaction of actin with some ligands, nucleotides [16,17], phosphate analogues [17,18], lipids [19], myosin [20,21], phalloidin [22], and tropomyosin [23]. For a complete review, see Levitsky et al. [24].

By applying the thermodynamics of the reversible systems, thermodynamic parameters such as changes in enthalpy, entropy, and the Gibbs function have been calculated. The values obtained for actin are of the same order of magnitude as those obtained for other proteins. However, since thermal denaturation of actin is irreversible, that approach is controversial. Moreover, all the calorimetric studies of the actin were generally made

* Corresponding author. Fax: +1 819 376 5084.
*E-mail address:* claude_gicquaud@uqtr.ca (C.R. Gicquaud).

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at only one concentration and one scanning rate. Since the aspect of the thermograms changes with the scanning rate and with protein concentration, the calculation of the thermodynamic parameters is then questionable.

In the present work, we have studied the effect of the protein concentration on the thermograms of F-actin. From these thermograms, we have measured the calorimetric enthalpy for the unfolding of F-actin to be 64 MJ/mol (1400 J/g), a value much larger than those obtained for other proteins. Since denaturation and stability are closely related, this particularity of actin could be an adaptation of its role as a protein that transmits forces.

## Materials and methods

*Actin preparation*. Rabbit striated muscle actin was purified from acetonic powder by the classical method of Pardee and Spudich [25]. The protein was obtained as a monomer and used in the following low ionic strength buffer (G-buffer): Tris–HCl 2 mM, ATP 0.2 mM, $CaCl_2$ 0.2 mM, β-mercaptoethanol 0.5 mM, and sodium azide 0.01%. The pH was adjusted to 7.5 at room temperature. However, since the Tris buffer has a pH shift with increasing temperature, the pH at the denaturation temperature of actin (around 60 °C) was 6.5. This problem could not be circumvented. Actin concentration was determined by UV absorption: $E$ (1%, 1 cm) = 6.3. The stock solution of G-actin was stored at 0 °C and discarded after a week. Purity was checked by electrophoresis.

*Differential scanning calorimetry*. DSC was performed with a Hart differential scanning calorimeter equipped with three stainless steel ampoules. In a standard preparation, an appropriately diluted G-actin solution was mixed with a stock solution of $MgCl_2$ in G-buffer to 2 mM final concentration to induce actin polymerization. Each solution was degassed for 5 min under low pressure before use to prevent any gas release artefacts or protein oxidation during heating. The calorimeter ampoules were filled with 900 ± 1 mg of this solution and allowed to stand for 1 h at 20 °C to complete actin polymerization. The baseline was determined using buffer alone. In experiments on G-actin, $MgCl_2$ was omitted.

The calorimetric enthalpy, $\Delta H_{cal}$, was determined as the area under the excess heat capacity function by using a program provided with the calorimeter.

*Size exclusion chromatography*. Size exclusion chromatography (SEC) was used to detect change in molecular size occurring during the thermal transition. It was performed using a Sephadex G 200, 30 × 1 cm column. Chromatography was done with a sample of native G-actin at 1 mg/ml and with a sample of G-actin heat-denatured in the calorimeter. G-buffer was used for elution. Absorbance was read at 290 nm.

## Results and discussion

Typical calorimetric recordings of the heat absorption for several concentrations of F-actin from 0.1 to 5 mg/ml are shown in Fig. 1, all obtained at the same scan speed of 30 °C/h. The global thermal denaturation of F-actin is irreversible since no transition was obtained in a second run of all the samples. During heating, the solution becomes turbid, which demonstrates aggregation of the unfolded molecules. Irreversibility is due to the aggregation step. Therefore, we did not attempt to evaluate equilibrium thermodynamic parameters such as entropy, Gibbs energy, and van't Hoff enthalpy [1,2,4]. Also, the kinetic model of denaturation [26–28] cannot be considered since the irreversible step takes place during the time the protein spends in the transition region, and this is strongly concentration dependent. For elevated concentrations, the rate of the irreversible step is high. Thus, the heat absorption is entirely determined by the rate of formation of the final state, and no thermodynamic information other than the total enthalpy change can be derived from the transition [29].

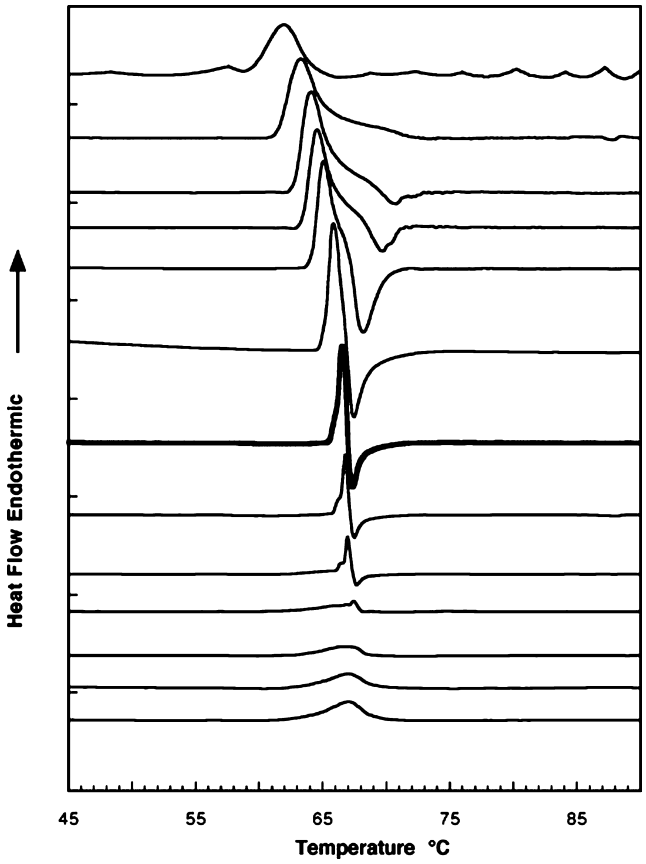


Fig. 1. The effect of concentration on the thermogram of F-actin. From top to bottom, 0.10, 0.15, 0.20, 0.25, 0.35, 0.50, 0.75 (thick line), 1.0, 1.25, 2.0, 3.0, 4.0, and 5.0 mg/ml. The scan speed was 30 °C/h. The thermograms were translated for clarity. One tick = 100 mJ/K.

One notices in Fig. 1 that the concentration of actin greatly influences the thermogram shape. For actin at 0.75 mg/ml (Fig. 1, thick line), the transition clearly has two steps. The first one is endothermic and has a $T_{max} = 66.5$ °C and a calorimetric enthalpy of $\Delta H_{cal} = 4400$ kJ/mol (98 J/g). Analysis of thermal denaturation by CD spectropolarimetry shows that a change in ellipticity at 222 nm occurs at 66.5 °C with a loss in α-helix and an augmentation in β-sheets (result not shown). This step then corresponds to the unfolding of

the peptide chain. The second step is exothermic with a $T_{max} = 67.4$ °C and a $\Delta H_{cal} = 3900$ kJ/mol (86 J/g). It is accompanied by an increase in the turbidity of the solution and precipitation of the protein. It then corresponds to the aggregation of the unfolded molecules. However, it has been reported that other factors such as deamidation, cysteine oxidation, and proline isomerization may contribute to this exothermic step [30].

Surprisingly, for concentrations inferior to 2 mg/ml, the height of the peak and the area under the curve representing the calorimetric enthalpy, $\Delta H_{cal}$, decrease with increasing concentration. At concentrations above 2 mg/ml, however, peak height and calorimetric enthalpy increase with increasing actin concentration (Fig. 2).

When the concentration of actin increases, the $T_{max}$ of the endothermic step is shifted towards higher temperatures (from 62 °C for 0.1 mg/ml to 67 °C for 2 mg/ml). On the other hand, the $T_{max}$ of the exothermic step decreases from 73 to 67 °C. The decrease in peak height with increasing actin concentration then results from the overlapping of the endothermic and exothermic phenomena. For concentrations above 2 mg/ml, the two steps completely overlap and the resultant curve is a small endothermic peak whose calorimetric enthalpy is directly proportional to the actin concentration (Fig. 2), and where $\Delta H_{cal}$ is 575 kJ/mol (12.7 J/g).

For low concentrations (around 0.2 mg/ml) the aggregation is slow and has a low cooperativity. Thus, the exothermic and endothermic steps do not superimpose. It is then possible to estimate the calorimetric enthalpy for the unfolding step. For an actin concentration of 0.2 mg/ml, the value of the calorimetric enthalpy of unfolding is 64 MJ/mol or 1400 J/g. This value is considerably higher than those mentioned in the literature for the denaturation of actin and other proteins [3,14,20,31,32].

In Fig. 3 are shown the equivalent experiments with G-actin solutions whose concentrations range from 1.0 to 6.0 mg/ml, all obtained at the same scan speed of 30 °C/h. The general shape of the thermograms is very different from those of F-actin. The thermograms of G-actin have only a single endothermic peak with a $T_{max}$ around 60 °C. We never found an exothermic step whatever the concentration of G-actin used. An important difference between the thermograms of G and F-actin is that the peak height and the calorimetric enthalpy are very small compared with the equivalent one of F-actin. The calorimetric enthalpy, $\Delta H_{cal}$, is only 200 kJ/mol and increases proportionally with actin concentration (Fig. 2). For that reason, we were not able to make thermograms with concentrations of G-actin lower than 1 mg/ml because the peak gets lost in the background noise.

A solution of F-actin heated beyond the temperature of denaturation becomes turbid and precipitates, which shows that the unfolded molecules of F-actin associate to form large aggregates. On the other hand, G-actin's solutions remain translucent: its unfolded molecules did not form large aggregates. To see if the unfolded molecules of G-actin are monomeric or aggregated, we used size exclusion chromatography (SEC) to detect changes in molecular size. The result presented in Fig. 4 shows that heat-denatured G-actin molecules associate as dimers and trimers. It correlates with the literature. Kuznetsova et al. [33,34] showed by ultracentrifugation that G-actin has a homogenous supramolecular structure with a sedimentation coefficient of 20 S after thermal denaturation.

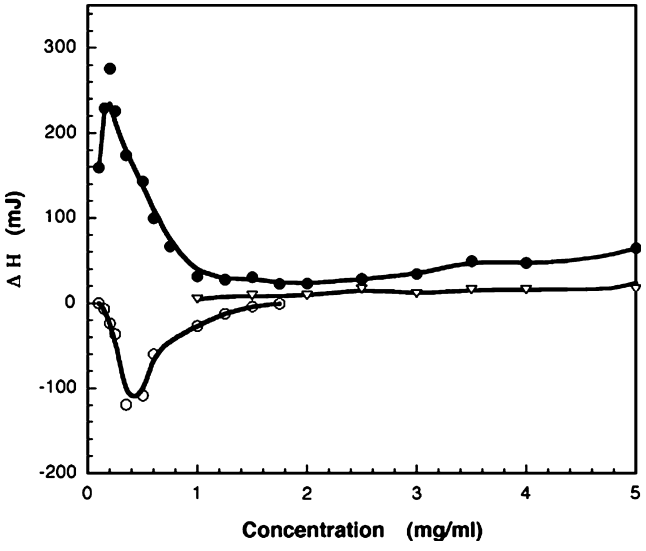


Fig. 2. The effect of concentration on the calorimetric enthalpy ($\Delta H_{cal}$) of the heat denaturation of actin. Filled circles, $\Delta H_{cal}$ of the endothermic step of F-actin; open circles, $\Delta H_{cal}$ of the exothermic step of F-actin; and triangles, $\Delta H_{cal}$ of the G-actin.

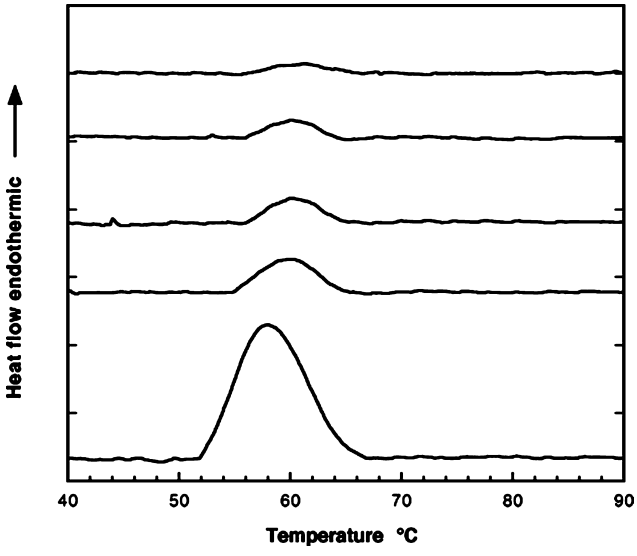


Fig. 3. The effect of concentration on the thermogram of G-actin. From top to bottom, 1.0, 2.0, 3.0, 4.0, and 6.0 mg/ml. The scan speed was 30 °C/h. The thermograms were translated for clarity. One tick = 20 mJ/K (five times smaller than the tick in Fig. 1).

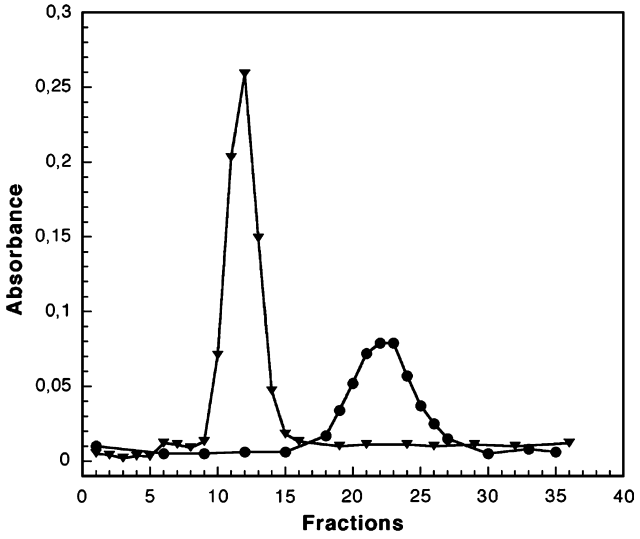


Fig. 4. Size exclusion chromatography on a Sephadex G 200 column; for native G-actin (circles) and for heat-denatured G-actin (triangles).

Thus, upon heating, the G-actin molecules unfold and immediately form small aggregates. Consequently, the unfolding and aggregation steps could not be separated whatever the DSC conditions used, and only the global phenomenon can be observed by calorimetry.

An important result of our DSC experiments is the extremely high value of the enthalpy change involved in the unfolding of F-actin. Some high values of calorimetric enthalpy have been reported: 5.3 MJ/mol for *Escherichia coli* glucosamine-6-phosphate deaminase [35], 9.8 MJ/mol for β-glycosidase from *Sulfolobus solfataricus* [36,37], and a 30 MJ/mol for the Tarantula hemocyanin [38]. *Sulfolobus* and Tarantula are organisms living in extreme temperature conditions. However, if one considers the specific enthalpy, they are in the range of 25–30 J/g, comparable with those of monomeric globular proteins [3,32].

Since the values of $\Delta H_{cal}$ deviate completely from the values reported in the literature for other proteins, we carefully checked the operation of our calorimeter and the software to calculate the enthalpy, using naphthalene and pure phospholipids as standards whose calorimetric enthalpies are known. Moreover, the values of $\Delta H_{cal} = 575$ kJ/mol for F-actin at concentrations above 2 mg/ml and 200 kJ/mol for G-actin are of the same order of magnitude as those reported by others [13–15,18,31]. This eliminates any instrumental errors.

The exothermic reaction produced by the aggregation of the denatured protein was often considered as an artefact because it interferes with the endothermic reaction and does not permit the treatment of the calorimetric traces in terms of equilibrium thermodynamics [24,39]. Therefore, conditions have been developed to eliminate the exothermic reaction. Some authors [36,40] used extreme pH values, where precipitation does not occur, used relatively rapid scans to minimize aggregation problems [41], or used capillary design cells to eliminate the exothermic reaction due to the precipitation of proteins [24,39,42]. Moreover, the concentrations of protein that are generally used (between 1 and 70 mg/ml) are high. We found no work in the literature with the very low concentrations of protein that we used here. It is probable that the reactions of unfolding and aggregation occur simultaneously under these conditions. The calorimetric enthalpy reported in the literature for actin, and for proteins generally, is probably the sum of the unfolding and the aggregation steps.

Actin is a protein that interacts with myosin to produce movement. At the molecular level, the actin molecule is submitted to important forces of traction. Thus, the forces of cohesion that maintain the molecules in the unfolded state must be very strong. These forces can be characterized by the energy required for (cooperative) macro unfolding of the structure, i.e., by calorimetric enthalpy. The high stability of the actin molecule would thus be an adaptation of its functions as a protein involved in movement.

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