THE EFFECTS OF CONCENTRATED SALT SOLUTIONS ON THE STRUCTURE AND THE ENZYMATIC ACTIVITY OF MYOSIN MOLECULES FROM SKELETAL AND CARDIAC MUSCLES

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1. Introduction

Attempts have been made by several authors [1–6] to determine the precise role of light and heavy subunits in the enzymatic activity of myosin, by the separation of myosin molecule into heavy and light subunits and the recombination of the separated subunits into an active molecule. The low level of dissociation (only about 40%) and the poor recovery of activity after this partial dissociation led to doubt about the reliability of such dissociating treatments and the conclusions drawn from recombination or hybridation experiments [7,8].

Using myosins from skeletal and cardiac muscles, we have tried: 1) to find salt treatments which induce a high and reversible dissociation of the molecule, 2) to establish the relationship between the degree of dissociation of the subunits and the loss of activity during these different salt treatments.

The results reported here show that a mixture of NH_4Cl and SH-reagent (DTNB) induces an effective and reversible dissociation of the subunits from skeletal muscle myosin while only a partial dissociation is obtained with cardiac muscle myosin. Moreover it has been shown that any dissociating treatment leads to an inactivation of the myosin molecule prior to its dissociation; recombination experiments have therefore to be re-considered in the context of the restoration of the native conformation of myosin from an inactive one.

2. Experimental

Myosin was prepared from rabbit white skeletal muscle [9] and from pig cardiac muscle as described previously [10].

The salts (urea, LiCl, NH_4Cl) were added to myosin solution according to the procedure of Dreizen et al. [4]. When other reagents like EDTA or DTNB were used, they were added to the salt–myosin mixture. The value of the pH was found to be critical in such experiments: the final pH of the mixture was determined before the experiment by a trial and error procedure, leading to the correct adjustment of the different components. All the experiments were performed at 4°C, pH 7.0 and with a final concentration 5 mg/ml myosin.

At different times of treatment, 1 to 2 ml (5 to 10 mg myosin) were sampled from the mixture. The reaction was immediately stopped either by salting out in the presence of 1.25 M potassium citrate or by lowering the ionic strength to 0.05 with water. The precipitates, collected by centrifugation at 20 000 *g* for 10 min, contained the free heavy chains and the non-dissociated myosin. They were dialysed against 0.6 M KCl–0.02 M Tris-maleate–1 mM 2-mercaptoethanol, pH 7.0 for two days. About 80% of the initial amount of sampled proteins was recovered in the precipitate. The corresponding supernatants contained all the dissociated light chains and some non precipitated heavy chains or myosins. These

last were retained by ultrafiltration on a Diaflo Membrane (Amicon Ultrafiltration Cell. Membrane Serie: XM 100A); the dissociated light chains were concentrated by a second filtration (Membrane PM 10).

In the recombination experiments, the subunits separated by 1.25 M potassium citrate and centrifuged were re-mixed and extensively dialysed as for the precipitates of heavy chains.

Ca-ATPase activity assays were performed on the preparation of native myosin, on the dialysed solutions of the precipitates obtained after salt treatment and on those of the remixed subunits. The specific activity was measured according to Gaspar-Godfroid [11] (2 mM ATP, 10 mM $CaCl_2$, 0.06 M KCl, 25°C, pH 7.0) and its reproducibility was within 5%. Ca-ATPase specific activities varied between 0.50 and 0.80 μM of P/mg/min for different preparations of rabbit skeletal muscle myosin and between 0.10 and 0.25 μM of P/mg/ml for those of pig cardiac muscle myosin. The variations in activity of the pellets according to the duration of the treatment, described as residual activities, were expressed as a percentage of the activity of native myosin.

The effects of the salt treatment on myosin structure were tested by polyacrylamide gel electrophoresis [12]. (See fig.1). The percentage of non dissociated light chains in the pellets of salt treated myosin was estimated from the results of polyacrylamide gel analysis, as previously described [13].

The variations of the ratio of hidden SH-groups and total SH-groups of myosin during salt treatments were measured by following the liberation of the thionitrophenylated anion at 412 nm [14].

3. Results

3.1. *Dissociation into light and heavy chains*

When the skeletal muscle myosin was treated either by 4 M LiCl or by 4 M urea, a fast liberation

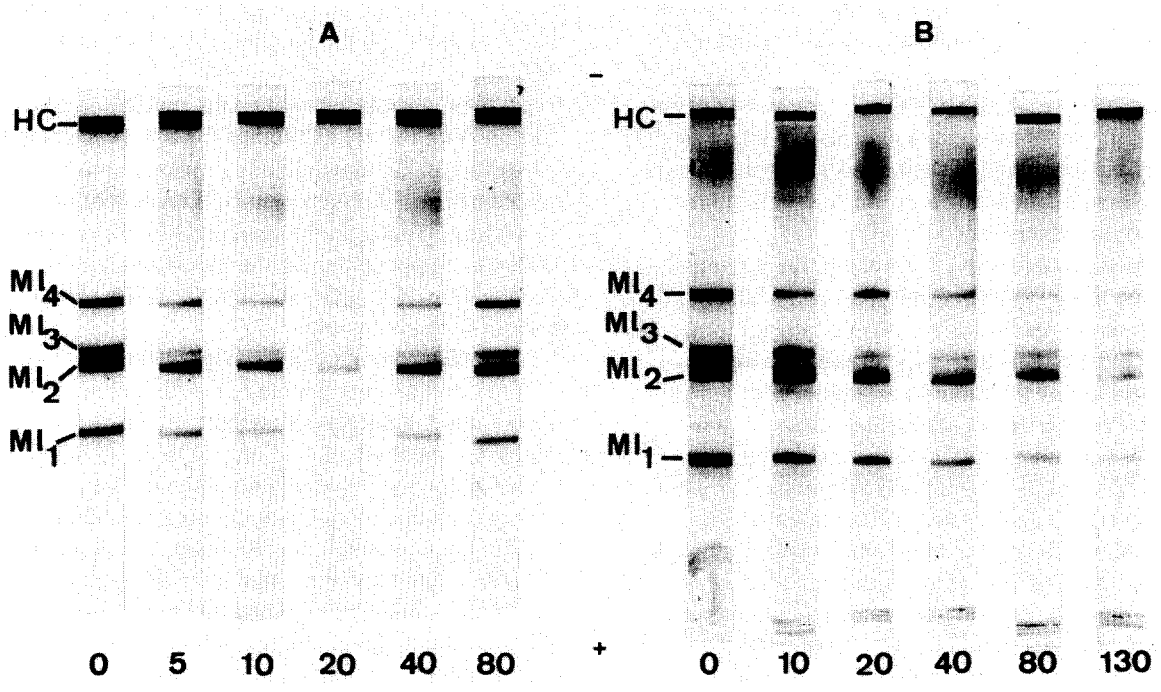

Fig.1. 10%-polyacrylamide gels in Tris–glycine at pH 8.3 [12] of the pellets obtained after salt treatment (pH 7.0, 4°C) of skeletal muscle myosin as a function of time (min). HC: heavy chains; $Ml_{1,2,3,4}$: light chains A) 4.0 M LiCl and 2 mM DTNB, sample load: 250 μg. B) 4.5 M NH_4Cl and 10 mM DTNB, sample load: 200 μg.

of light chains was observed initially; then the free light chains spontaneously reassociated with the heavy chains in the presence of the reaction mixture. For example in the treatment with 4 M urea and 10 mM EDTA (fig.2), the dissociation was maximal after 40 min but never reached more than 20–30%. Moreover the reaction was unaffected by EDTA or dithiothreitol.

The addition of a SH-reagent such as DTNB to the salt greatly accelerated the initial dissociation and the subsequent spontaneous reassociation (fig.1A). However, even in this case, the dissociation never reached more than 65%. Similar results were obtained with cardiac myosin. Sodium tetrathionate as a SH-reagent also led to the same observations.

When 4.5 M NH_4Cl was used with 9.1 mM DTNB, the dissociation of skeletal muscle myosin reached a value of (80 ± 10)% and remained constant even after a long treatment (fig.1B and fig.3). With decreasing concentrations of salt and 9.1 mM DTNB, the dissociation became slower. For a final concentration of 3.5 M NH_4Cl, no liberation of light chains could be observed even after long periods of treatment.

The cardiac muscle myosin did not react as the

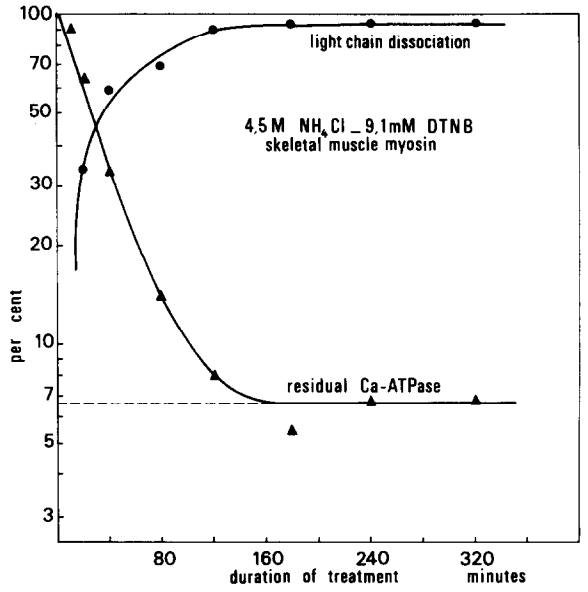

Fig.3. Residual Ca-ATPase activity (▲) and amount of dissociated light chains (●) of skeletal muscle myosin (5 mg/ml) treated with 4.5 M NH_4Cl and 9.1 mM DTNB, pH 7.0, 4°C as a function of time. The same profile for residual Ca-ATPase activity was observed with 3.5 M NH_4Cl and 9.1 mM DTNB.

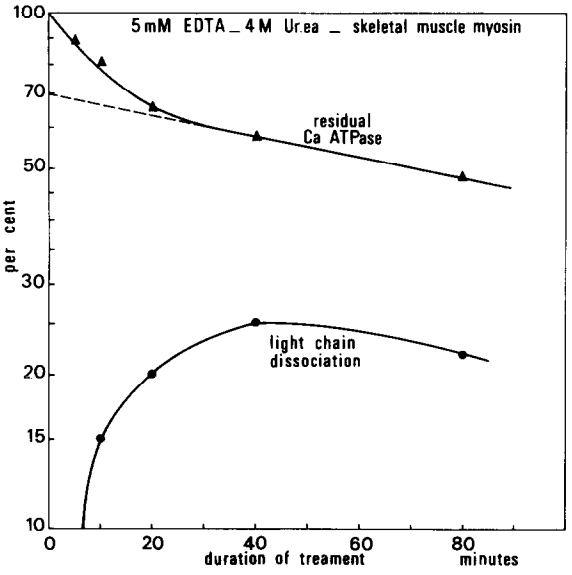

Fig.2. Residual Ca-ATPase activity (▲) and amount of dissociated light chains (●) of skeletal muscle myosin (5 mg/ml) treated with 5 mM EDTA and 4 M urea, pH 7.0, 4°C as a function of time.

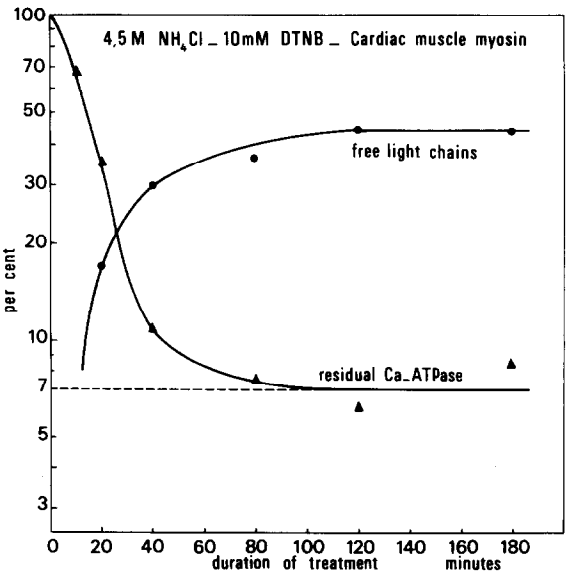

Fig.4. Residual Ca-ATPase activity (▲) and amount of dissociated light chains (●) of cardiac muscle myosin (5 mg/ml) treated with 4.5 M NH_4Cl and 10 mM DTNB, pH 7.0, 4°C as a function of time.

skeletal one when treated with 4.5 M NH_4Cl and 10 mM DTNB. Only the 26 000 daltons light chain, containing cysteine residues [15], was distinctly dissociated; the other smallest 18 000 daltons light chain, which lacks cysteine residues, always remained bound to the heavy chains (fig.4).

3.2. *Variations of the Ca-ATPase activity of treated myosin*

When the Ca-ATPase activity of the pellets obtained after any salt treatment of skeletal and cardiac muscle myosins was plotted against the time of treatment, a rapid fall of the activity was initially observed but the corresponding percentage of inactivation was always higher than that of the eventual inactivation as a result of the dissociation. For example on fig.2 (4 M urea, 10 mM EDTA), after 40 min the liberation of light chains was maximal and equal to 25% whereas the percentage of lost activity was higher than 40%. In the treatment with 3.5 M NH_4Cl and 9.1 mM DTNB (fig.3), no light chains were liberated but the percentage of lost activity reached more than 90% after 160 min as in the treatment with 4.5 M NH_4Cl and 9.1 mM DTNB. Similar results were observed with cardiac muscle myosin (fig.4).

With longer times of urea of LiCl treatment, the residual activity continued to decrease but less rapidly than during the initial dissociating period. This continuous decrease, associated with the spontaneous reassociation of separated subunits, suggested the non specific and irreversible reassociation of the myosin subunits. No protecting effect of dithiothreitol on the inactivation was observed.

With longer times of NH_4Cl-DTNB treatments, the residual activity remained constant and equal to (8 ± 3)% (fig.3 and fig.4). This last observation implied the reversibility of the whole process.

3.3. *Measurements of thionitrophenylation during salt treatment*

Without any salt treatment, only (17.6 ± 1) SH-groups per skeletal muscle myosin molecule were thiophenylated. During salt treatments, a variation in the number of the thiophenylated SH-groups of myosin was observed. For example, after treatment with NH_4Cl for times during which the residual activity remained constant, (22 ± 1) SH-groups per myosin molecule were thiophenylated. When considering the cysteine content of the myosin molecule (higher than 40 cysteine residues/mol), the observed labelling is rather low in spite of a molar ratio DTNB/myosin higher than 1000. Nevertheless it should be pointed out that all SH-containing light chains from skeletal or cardiac muscle myosins were labelled after salt treatment.

3.4. *Reassociation experiments*

Whatever the muscle type, the salt treatment and the duration of the experiment, the separated light and heavy chains completely reassociated after mixing and dialyzing. In the time range corresponding to the rapid decrease of activity and to dissociation, a partial recovery of the initial enzymatic activity was observed. This recovery varied with the experimental conditions of reassociation (pH, time of dialysis, . . .) and never exceeded 50% of the lost activity after any salt treatment. In the time range in which either spontaneous reassociation occurs (LiCl or urea treatments) or the residual activity remains constant (NH_4Cl-DTNB treatment), no significant activity was recovered.

4. Discussion

The variation in the number of the accessible SH-groups of myosin during salt treatments suggest the occurence of conformational changes of the myosin molecule. The concomitant measurement of the decrease in activity and of the degree of dissociation confirms that these conformational changes simultaneously induce inactivation and dissociation as suggested by various authors [1,2,4]. However the extent of inactivation on dissociation, observed after any salt treatment, together with the experimental conditions for which there is the same sharp fall in activity with or without liberation of free subunits (4.5 M and 3.5 M NH_4Cl, 10 mM DTNB), indicate that the inactivation always precedes any dissociation. The low recovery of activity after brief salt treatment, in previously published recombination or hybridation experiments [4–6], is due rather to the partial reversibility of this initial step of inactivation than to the ability of the separated subunits to reassociate. Therefore the important point in further recombination experiments

after salt treatment is to find experimental conditions for which not only a high dissociation is initially obtained but where the initial step of inactivation is completely reversible. Finally, if no direct information concerning the eventual role of the different subunits in the catalytic properties of the myosin molecule can be obtained from salt treatments, the effects reported here of SH-reagents on the binding of heavy and light chains from skeletal and cardiac muscle myosins emphasize the role of the SH-groups in the quaternary structure of the myosin molecule. In particular these observations extend the known effects of DTNB alone on the myosin from skeletal muscle [16]. The stability of the light chain, which lacks thiol and is unique to cardiac muscle, suggest the existence of different binding forces specific to this last type of myosin.

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