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CYTOSKELETON, MOTILE STRUCTURES AND MACROMOLECULAR CROWDING

Enrico Grazi

Istituto di Chimica Biologica,
Università di Ferrara,
Ferrara, Italy

INTRODUCTION

We describe the effect of macromolecular crowding on the associations of the cytoskeletal and of the motile structures. These effects are due to preferential interactions and have been treated theoretically by many authors^{1,2,3,4}. Occasionally, preferential interactions are taken into account in the study of the biological reactions, their role, however, is not yet adequately recognized.

Macromolecular crowding shifts the monomer-tetramer equilibrium of glyceraldehyde-3-phosphate dehydrogenase in favour of tetramer formation⁵; allows blunt-end ligation by DNA ligase from rabbit liver and Escherichia coli⁶; drives catenation of supercoiled and gapped DNA circles by topoisomerase I⁷; regulates the transporter-mediated ion flux across cell membranes⁸. In the specific field of the cytoskeleton preferential interactions influence the binding of glycolytic enzymes to cytoskeletal structures⁹ and myofibrils¹⁰; accelerate the rate and increase the extent of actin polymerization¹¹; increase the rate of elongation of actin filaments¹²; favour the formation of bundles of actin filaments¹³.

This last contribution stimulated our interest in the topic. We began a systematic investigation and discovered that cytoskeletal functions are widely influenced, both quantitatively and qualitatively, by large concentrations of macromolecular solutes. The influence is spanning from the reversible interconversion of actin filaments into actin bundles, to the intensification of the actin gelling activity of alpha-actinin, to the lowering of the solating activity of gelsolin, to the triggering of actomyosin retraction, supported by unphosphorylated smooth muscle myosin. All these effects are briefly presented and discussed.

EXPERIMENTAL

The effect of macromolecular crowding on the formation and the dissociation of actin bundles, as well as on the solating activity of gelsolin was studied at pH 7.0 and 37°C. Final electrolyte concentration was as follows : 107 mM K⁺, 13 mM Na⁺, 6 mM Mg²⁺, 5 mM orthophosphate, 4 mM ATP, 37 mM Cl⁻ and 70 mM propionate, ionic strength was O.145 M^{18,19,29,47}.

The actin gelling activity was studied at 37°C and pH 7.5. Final electrolyte concentration was : 0.1 M KCl, 2 mM MgCl₂, 0.5 mM ATP, 10 mM Tris-HCl⁴².

Unphosphorylated smooth muscle myosin was studied at 37°C and pH 7.0. Final electrolyte concentration was : 148 mM K⁺, 13 mM Na⁺, 15.5 mM Mg²⁺, 9.5 mM sulfate, 6 mM phosphate, 15 mM creatine phosphate, 6.5 mM ATP, 66 mM propionate⁵¹.

Macromolecular crowding was mimicked either by poly(ethylene glycol) 6000 or by serum albumin.

CELL VOLUME AND MACROMOLECULAR CROWDING

Isosmotic changes of cell volume are induced by tumor promoters and by mitotic agents, they are operated by K-Cl cotransport or by Na-H exchange, are accompanied by osmotically obliged water and, consequently, by the change of the concentration of the macromolecules¹⁴. Being produced by water fluxes, these changes propagate quite rapidly to all the cellular space, included the nuclear matrix, where they may act as signals for gene transcription.

Small fractional changes of cell volume, by generating small changes of the concentration of the macromolecules of the medium, may modify the state of aggregation of those actin populations, that are at the transition between filaments and bundles.

Conversely, changes in the state of aggregation of actin may influence the volume. In neutrophils, F-actin content undergoes cyclic variations, that correlate with periodic fluctuations in lamellipod size : actin polymerization (and possibly bundles formation) corresponds closely to lamellipod extension; actin depolymerization corresponds closely to lamellipod retraction¹⁵. The polymerization of actin is assumed to drive the extension of lamellipods as well as the extension of the acrosomal process of Thyone sperm^{15,16}.

Many animal cell types respond to swelling or shrinkage by activating membrane transporters : the swelling-induced K-Cl cotransport, the shrinkage-induced Na-H exchange. The small fractional changes in cell volume are sensed as small fractional changes in the cytoplasmic macromolecular concentration. These, in turn, elicit an amplified change of the thermodynamic activity of proteins, that regulate membrane transport⁸. The scaled particle theory¹⁷ predicts, in fact, that, in a highly crowded solution, the activity coefficient of each macromolecular species is a function of the total concentration of the macromolecules.

ANCILLARY CYTOSKELETAL PROTEINS AND THE CONVERSION OF ACTIN FILAMENTS INTO

ACTINS BUNDLES

The ancillary cytoskeletal proteins modulate the concentration of the macromolecules required to promote the transition of actin filaments into actin bundles. As an example, in our experimental conditions, the formation of bundles of actin filaments occurs at 3% poly(ethylene glycol) for caldesmon-decorated actin, at 4-5% poly(ethylene glycol) for filamin-decorated actin, at 5-7% poly(ethylene glycol) for caldesmon-tropomyosin-decorated actin, at 6-7% poly(ethylene glycol) for F-actin and at 9-10% poly(ethylene glycol) for tropomyosin-decorated actin^{18,19}.

A finer regulation is achieved by changing the ratios of the ancillary proteins with respect to actin. Filamin-decorated actin undergoes bundling, even in the absence of poly(ethylene glycol), when the filamin to actin molar ratio is increased to 1:8²⁰. The concentration of poly(ethylene glycol), required to induce the transition of F-actin filaments into actin bundles,

decreases with the increase of the caldesmon to actin molar ratio, but, even at a caldesmon to actin molar ratio of 1:3, poly(ethylene glycol) is required for bundling¹⁹.

The intracellular distribution of ancillary cytoskeletal proteins may determine the state of aggregation of actin. Filamin, that favours actin bundling, is present in the leading edge and membrane ruffles^{20,21}, while tropomyosin, that hinders actin bundling, is confined to the internal part of the cultured cells²².

In some cases the interplay between macromolecular concentration, state of aggregation of actin and distribution of the ancillary cytoskeletal proteins is quite complex. Caldesmon is reported to bind tighter to tropomyosin-decorated F-actin than to F-actin²³, as a consequence, a system composed by tropomyosin-decorated F-actin and by caldesmon-decorated F-actin evolves toward a system composed by F-actin and by tropomyosin-caldesmon-decorated F-actin. This has a profound effect on the state of aggregation of actin. In 3% poly(ethylene glycol) solutions, the progression of the system from the first to the second state increases the proportion of actin filaments over actin bundles. In more concentrated poly(ethylene glycol) solutions (6-7%), the same progression of the system increases the proportion of actin bundles over actin filaments¹⁹.

MECHANISMS FOR THE DISSOCIATION OF ACTIN BUNDLES

Many scientists, being used to work in solutions of small electrolytes, may consider the filamentous form as the ground state of aggregation of actin in the cell. In this respect, the report of Suzuki et al.¹³ is quite striking. These authors show that actin bundles represent the ground state of aggregation of actin, when the osmolarity of the macromolecules of the medium is close to that of the cell sap. The question to ask, therefore, is how, in the cell, actin bundles can be forced to dissociate into actin filaments.

Many actin bundling proteins are proposed to regulate the rapid formation and dissociation of actin bundles.

Human erythrocyte protein 4.9 is reported to display an actin-bundling activity, which is abolished when the protein, in preformed actin bundles, is phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase²⁴.

Lipocortin 85 is reported to display a Ca^{2+} -dependent actin-bundling activity²⁵.

The actin-bundling activity of synapsin I is reported to be abolished, when the protein is phosphorylated by Ca^{2+} -calmodulin-dependent protein kinase II²⁶.

The Ca^{2+} -calmodulin-caldesmon complex is reported to dissociate at low Ca^{2+} concentration; in turn, the released caldesmon is reported to displace filamin from filamin-actin bundles, which then dissociate into filaments^{27,28}.

In no cases, however, it was checked whether preformed actin bundles actually dissociate, when the concentration of the macromolecules is close to that present in the cytosol. As an example, we found that, in 3% poly(ethylene glycol), Ca^{2+} -calmodulin prevents the binding of caldesmon to F-actin but fails to dissociate caldesmon-F-actin bundles, that are formed at that concentration of poly(ethylene glycol) (unpublished results). This is at variance with the data of Sobue et al.²⁷, obtained in solutions of small electrolytes.

When the dissociation of actin bundles is operated by a protein, the main drawback is the steric hindrance, due to the dense packing of actin bundles. As an example, in resting solutions, tropomyosin does not dissociate F-actin bundles in 7% poly(ethylene glycol), even though tropomyosin-decorated F-actin forms bundles only at 9-10% poly(ethylene glycol)^{18,19}. To achieve the dissociation of the bundles the solution must be constantly mixed²⁹.

On the contrary, even in resting solutions, tropomyosin promotes the dissociation of caldesmon-decorated F-actin bundles in 3% poly(ethylene glycol). Probably, in this case, actin bundles are less densely packed¹⁹, because of the lower osmotic stress, caused by the lower concentration of poly(ethylene glycol).

It is unlikely that cytoplasmic streaming may overcome the steric constraints, imposed by the densely packed bundles of actin filaments : it thus appears that dissociation of actin bundles is best triggered by small molecules. A potential candidate to this role is Mg^{2+} . In vitro, the increase of free Mg^{2+} concentration favours bundling of actin, while the decrease of free Mg^{2+} concentration favours the dissociation, into filaments, of the tropomyosin-decorated F-actin system. F-actin alone is insensitive to this mechanism of regulation²⁹.

At 37°C, pH 7.14, in 7.2% poly(ethylene glycol), the transition between filaments and bundles occurs at 1.7 - 2.0 mM free Mg^{2+} . These concentrations are quite larger than 0.6 mM, that is the estimated value for free Mg^{2+} concentration in mammalian tissues³⁰. Nevertheless, because of the multiplicity of the factors that regulate the transition between actin filaments and actin bundles and because of the practical impossibility to match cell conditions, the free Mg^{2+} concentration cannot be excluded as a potential candidate for regulation. Moreover, it must be taken into account that, in the cell, the concentration of free Mg^{2+} may be increased by lowered energy charge of the ATP system³¹, by altered intracellular Mg^{2+} binding, secondary to changes in Ca^{2+} influx, or pH or to cell shrinkage³². In our experimental conditions a shift from 1.7 to 2 mM free Mg^{2+} , which produces a substantial increase in the amount of tropomyosin-decorated actin bundles over tropomyosin-decorated actin filaments, is brought about by a 0.5 mM decrease in ATP concentration. Stimulation by ADP of human platelets induces a similar decrease in "metabolic" ATP concentration³². These changes in ATP concentration are accompanied by the formation of actin bundles in the course of the aggregation reaction^{33,34}.

THE ACTIN GELLING ACTIVITY OF ALPHA-ACTININS AND MACROMOLECULAR CROWDING

It is a common notion that the actin gelling activity of alpha-actinins becomes almost undetectable at 37°C^{35,36,37,38,39}. This behaviour, apparently, casts doubt on the functioning of these proteins *in vivo*³⁷. As a matter of fact, alpha-actinin from chicken gizzard, at nM concentrations, increases significantly the rigidity of actin gel, even at 37°C, provided that the concentration of actin is low (2-3 μ M)^{40,41}. This finding, that may be regarded as a curiosity, indicates that, only at low concentrations, actin filaments are free to diffuse and to be optimally crosslinked by alpha-actinin. More significantly, at 37°C, in 6% poly(ethylene glycol), addition of 30 nM alpha-actinin to 12 μ M actin, induces the gelation of the system and increases the rigidity from 23.5 to 54 dynes/cm². The onset of gelation is concomitant with the transition of actin filaments into actin bundles⁴². The binding isotherm of alpha-actinin changes from the anomalous binding isotherm with filamentous actin^{40,41} to the hyperbolic binding isotherm ($K_{diss} = 11.3 \mu M$) with actin bundles⁴³. It is likely that the parallel arrays of filaments, in actin bundles, offer an ordered matrix of sites, which favour the bidentate binding of alpha-actinin. The crosslinking by alpha-actinin prevents the filaments from sliding in actin bundles. As a result, since the network of actin bundles is largely anastomosed, the rigidity of the system is increased by alpha-actinin, even at 37°C. These observations support the view that, *in vivo*, alpha-actinin functions are mostly carried on by interaction with actin bundles, in agreement with the finding that, in the cell, alpha-actinin is mostly associated with actin bundles⁴⁴.

GELSOLIN AND MACROMOLECULAR CROWDING

The gelsolin:actin molar ratio approaches 1:100 in the cell⁴⁵, a value adequate, *in vitro*, to support nucleation, capping and cutting of actin filaments. Moreover, since these functions are

regulated by Ca^{2+} and by phosphatidylinositol 4,5-bisphosphate⁴⁶, gelsolin becomes an attractive candidate to promote gel-sol transition *in vivo*.

The functions of gelsolin, *in vitro*, are usually tested on filamentous actin. It is known, however, that, owing to the large concentration of the macromolecules, in the cell actin is often present as bundles of filaments. In these structures, the strong latero-lateral interactions between actin filaments could counteract the effect of filament cutting by gelsolin. This occurs indeed⁴⁷. In the presence of 15 nM gelsolin, rigidity of 12 μM F-actin drops from 3.9 to 0.27 dynes/cm², while the rigidity of actin bundles, formed in 6% poly(ethylene glycol), is unaffected, being 29 and 27.5 dynes/cm², respectively, in the absence and in the presence of 30 nM gelsolin. Protection against the action of gelsolin is complete also when the system, in poly(ethylene glycol), is supplemented with 1.5 μM tropomyosin, a condition in which actin bundles are not formed. Perhaps, also in this case, the extent of the latero-lateral association of the filaments increases, even though not to such a level to be unambiguously detected at the electron microscope. The action of gelsolin could also be hampered by the strengthening of monomer-monomer interactions, that occurs in the presence of high concentrations of macromolecules⁴¹.

UNPHOSPHORYLATED SMOOTH MUSCLE MYOSIN AND MACROMOLECULAR CROWDING

It is known that, *in vitro*, MgATP dissociates unphosphorylated smooth muscle myosin filaments, by promoting the transition of monomeric myosin from the extended (6S) to the folded (11S) shape⁴⁸. *In vivo*, however, unphosphorylated smooth muscle myosin filaments are stable^{49,50}, as are stable *in vitro*, provided that the solutions are supplemented with large concentrations of macromolecules : 6% poly(ethylene glycol) or 25% serum albumin. Thus macromolecular crowding, by driving the association of myosin monomers, displaces the 6S - 11S equilibrium⁵¹.

Ca^{2+} -dependent phosphorylation of the 20 kDa light chain subunit of myosin clearly represents a major pathway for the regulation of contractile force in smooth muscle. As an example, light chain phosphorylation has been shown to initiate both short and sustained arterial contraction⁵². However, many examples of dissociation between force levels, myosin light chain phosphorylation levels, intracellular free Ca^{2+} concentration levels and cross-bridge cycling rates have been reported, and the possible existence of a second regulatory mechanism for smooth muscle tone is difficult to negate. The observation that, *in vitro*, in the presence of a large concentration of macromolecules, unphosphorylated smooth muscle myosin supports actomyosin retraction, shows that the basic machinery of smooth muscle does not require myosin light chain phosphorylation to initiate contraction⁵¹. It is very likely that myosin light chain phosphorylation is required only by the fully regulated contractile machinery⁵¹.

Caldesmon may play a relevant role in regulation. The addition of supraphysiological concentrations of caldesmon to skinned gizzard smooth muscle fibers, induces relaxation of submaximal contraction, in the absence of changes in myosin light chain phosphorylation⁵³. Conversely, a caldesmon proteolytic fragment, that contains the residues from Gly⁶⁵¹ to Ser⁶⁶⁷ plus an added cysteine at the C terminus and that does not inhibit actomyosin ATPase activity, induces contraction in vascular smooth muscle cells of ferret aorta, even at a pCa = 9. The contraction is induced by displacing the inhibitory region of endogenous caldesmon. These results suggest that caldesmon regulates contraction by providing a basal resting inhibition of muscular tone⁵⁴.

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