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Nucleotide Hydrolysis Regulates the Dynamics of Actin Filaments and Microtubules [and Discussion]

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Nucleotide hydrolysis regulates the dynamics of actin filaments and microtubules

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SUMMARY

Actin filaments and microtubules are major dynamic components of the cytoskeleton of eukaryotic cells. Assembly of these polymers from monomeric actin or tubulin occurs with expenditure of energy, because ATP (or GTP) tightly bound to actin (or tubulin) is irreversibly hydrolysed during polymerization. Therefore, actin filaments an microtubules are dissipative structures. Our purpose has been to understand how the dissipation of chemical energy perturbs the laws of reversible helical polymerization defined by Oosawa, and affects the dynamics of these polymers. A kinetic study has shown that nucleotide is hydrolysed on the polymer within at least two steps consecutive to the incorporation of the monomer: cleavage of the γ-phosphoester bond followed by the slower release of P_i; only the second reaction appears reversible. P_i release, and not cleavage of the γ-phosphate, is linked to the destabilization of protein–protein interactions in the polymer, and therefore plays the role of a conformational switch. The dynamic properties of the polymer in the NTP- and NDP-P_i intermediate states of the assembly process have been investigated using non-hydrolysable analogues of nucleotides and structural analogues of P_i, AlF_4^- and (BeF_3^-, H_2O). Because nucleotide hydrolysis is uncoupled from polymerization, actin filaments and microtubules grow with a 'cap' of terminal NTP- and NDP-P_i-subunits that interact strongly, and prevent the rapid depolymerization of the unstable core of the polymer formed of NDP-subunits. The fact that the dynamic properties of the polymer are affected by bound nucleotide results in a nonlinear dependence of the rate of elongation on monomer concentration. This nonlinearity accounts for the dynamic instability behaviour of microtubules, which is an important feature of their function, and explains the oscillatory polymerization kinetics in a population of synchronized microtubules.

The above analysis provides the basis for anticipating possible modes of regulation of cytoskeletal assembly via modulation of the rate of nucleotide hydrolysis. The role of the metal ion (Ca^{2+}, Mg^{2+}) chelated to the β- and γ-phosphates of ATP (or GTP) and the stereochemistry of nucleotide binding to actin and tubulin have been studied using the CrATP and CrGTP β γ-bidentate analogues of MgATP and MgGTP. The changes in the environment of the triphosphate moiety of the nucleotide following the release of P_i on F-actin, and of Mg^{2+} and P_i on microtubules, is proposed as being part of the conformational switch leading to the destabilization of the polymers.

1. INTRODUCTION

In eukaryotes, cellular space is organized by an ensemble of fibrous polymers called cytoskeleton. Actin filaments and microtubules, which are major components of the cytoskeleton, are dynamic structures, in contrast to most structural macromolecular assemblies, such as viruses. Their assembly and disassembly processes account for many motile activities of the cell. In addition, these helical polymers are dissipative structures: assembly of the polymer is coupled to energy consumption, in the form of ATP or GTP hydrolysis.

Monomeric actin binds ATP very tightly ($K_A = 10^{10}\ M^{-1}$); conversely tubulin binds GTP ($K_A \approx 10^8\ M^{-1}$). A polymerization cycle involves addition of the NTP-monomer to the polymer end, hydrolysis of NTP on the incorporated subunit, liberation of P_i in solution, and dissociation of the NDP-monomer. Exchange of NTP for bound NDP occurs on the monomer only, and precedes its being committed to another polymerization cycle. Therefore monomer-polymer exchange reactions are performed with expenditure of energy, exactly one mol of ATP (or GTP) per mol of actin (or tubulin) incorporated in actin filaments (or microtubules); 40% of the ATP consumed in the living cell is used to maintain the dynamic state of actin. It is therefore important to understand how the free energy of nucleotide hydrolysis is utilized in cytoskeleton assembly.

2. HOW NTP HYDROLYSIS ASSOCIATED TO POLYMERIZATION PERTURBS THE THERMODYNAMICS OF REVERSIBLE POLYMERIZATION

(*a*) *Nucleotide hydrolysis is linked to the destabilization of actin filaments and microtubules*

The most striking observation concerning the role of nucleotide hydrolysis is that the critical concentration

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(that is the monomer⇌polymer equilibrium dissociation constant) for polymerization of ADP-actin is 25-fold larger than for polymerization of ATP-actin. However, in both cases the filament is made of F-ADP subunits, and the rate constant for association of ADP-actin to filament ends is only 2.5 fold lower than the rate constant for association of ATP-actin. Similarly microtubules, although made of GDP-subunits, cannot be assembled from GDP-tubulin. GTP is necessary for assembly and to maintain the polymer in a steady state. In the absence of free NTP, the 1:1 complex of either ATP-actin or GTP-tubulin can polymerize, but the polymer once formed spontaneously depolymerizes. In the case of microtubules, depolymerization is complete, while in the case of actin, depolymerization stops when the concentration of ADP-monomer in the medium reaches the value of the critical concentration for polymerization of ADP-actin (for review see Carlier (1989)).

Clearly the above observations are inconsistent with a simple two-state polymerization model within which only two species, NTP-monomer and NDP-polymer, coexist in solution.

(*b*) *Thermodynamic and kinetic parameters for reversible polymerization (Oosawa's law)*

The theory of reversible helical polymerization of proteins has been fully described by Oosawa (1975). The following equation describes polymer growth:

$$J(c) = \mathrm{d}c/\mathrm{d}t = k_+[P]c - k_-[P], \qquad (1)$$

where $J(c)$ is the rate of polymer growth, c and P represent the concentrations of monomer and polymer elongating sites respectively; k_+ and k_- are the rate constants for monomer association to and dissociation from polymer ends. According to equation (1), k_+ and k_- can easily be derived from the linear dependence of $J(c)$ on c, and the critical concentration $c_c = k_-/k_+$ defined as the monomer concentration at which $J(c) = 0$.

(*c*) *Thermodynamic and kinetic parameters for ATP-actin and GTP-tubulin polymerization*

The polymer growth, $J(c)$, for both F-actin and microtubules showed nonlinear monomer concentration dependence in the presence of NTP (see Carlier (1989) for review). However, in the presence of ADP, the plot of $J(c)$ versus monomer concentration for actin was a straight line, as expected for a reversible polymerization. Therefore nucleotide hydrolysis affects the rate parameters for polymerization. The data imply that newly incorporated subunits dissociate from the polymer at a slower rate than internal NDP-subunits; in other words a double conclusion can be derived: (i) the effect of nucleotide hydrolysis is to decrease the stability of the polymer by increasing k_-; (ii) nucleotide hydrolysis is uncoupled from polymerization and occurs in a step that follows incorporation of a NTP-subunit in the polymer. Newly incorporated, slowly dissociating, terminal NTP-subunits form a stable 'cap' at the ends of F-actin and microtubules. Stochastic loss of this cap accounts for the 'dynamic instability' of microtubules (Mitchison & Kirschner 1984).

(*d*) *Oscillatory polymerization of microtubules as an example of dynamic instability*

At steady state, individual microtubules observed in real time alternate between long periods of slow growth and short periods of rapid depolymerization (Horio & Hotani, 1986), resulting in zero growth rate on average. This behaviour contrasts with the very small fluctuations exhibited by reversible polymers at equilibrium and results in a rapid turnover of microtubules at steady state. This behaviour can be described by a model (Hill & Chen 1984; Bayley *et al.* 1990) within which microtubules undergo transitions between two states: a growing state in which microtubules have a cap of terminal GTP-subunits, and a depolymerizing state in which microtubules having terminal GDP-subunits exposed to the solvent depolymerize rapidly. The transitions between these two states are linked, within this model, to the stochastic gain and loss of terminal GTP-subunits. This same model can account quantitatively for the oscillatory polymerization kinetics observed with crowded populations of microtubules (Carlier *et al.* 1987; Melki *et al.* 1988; Mandelkow *et al.* 1988). Such solutions of microtubules at steady state develop a stationary, striped, periodic spatial pattern that can be observed by light birefringence (Hitt *et al.* 1989) and neutron scattering (Job & Tabony 1990), and shows similarity with other dissipative spatial patterns. All these behaviours arise from the strong nonlinearity of $J(c)$ for microtubules.

Understanding how dynamic instability is regulated *in vivo* is now one of the most challenging issues in the microtubule field. Microtubule dynamics vary through the cell cycle and change abruptly upon entry into mitosis, this effect being mediated by cdc2 kinase (Verde *et al.* 1990). The large increase in microtubule turnover in mitosis appears to be solely due to an increase in the frequency of transition from the growing (putatively GTP-capped) state to the rapidly depolymerizing state (Belmont *et al.* 1990). How this effect is exerted remains to be understood.

(*e*) *Conclusions*

The above results demonstrate that NTP hydrolysis associated with actin and tubulin polymerization acts as a regulatory switch affecting the strength of protein–protein interactions. In this respect, this biological system appears similar to the G-proteins or other regulatory nucleoside triphosphatases. Indeed, one can consider that actin and tubulin exist in two states: a 'non-interacting' state, in which ATP-G-actin or GTP-tubulin do not hydrolyse nucleotide, and an 'interacting' state in which F-actin and microtubular tubulin hydrolyse nucleotide in a single turnover reaction, and nucleotide hydrolysis is linked to a

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weakening of actin–actin or tubulin–tubulin interactions in the polymer lattice.

In order to anticipate possible modes of regulation of cytoskeleton dynamics *in vivo*, it is necessary: (i) to identify the kinetic intermediates involved in the polymerization process and to characterize their structural and functional properties; (ii) to define the essential elementary steps in the hydrolysis process.

3. KINETIC STEPS IN NUCLEOTIDE HYDROLYSIS ON F-ACTIN AND MICROTUBULES

(*a*) *Kinetics of ATP hydrolysis on F-actin*

ATP is hydrolysed in at least two consecutive steps on F-actin, cleavage of the γ-phosphoester bond, followed by P_i release, according to the following scheme (for review, see Korn *et al.* (1987)).

$$\text{F-ATP} \rightleftarrows \text{F-ADP-P}_i \rightleftarrows \text{F-ADP} + P_i$$

P_i release occurs at a relatively slow rate ($k_{-P_i} = 0.005\ s^{-1}$), so that the transient intermediate F-ADP-P_i has a life time of 2–3 min. While the γ-phosphate cleavage step is irreversible as assessed by ^{18}O exchange studies (Carlier *et al.* 1987), the release of P_i is reversible. Binding of $H_2PO_4^-$ ($K_D \approx 10^{-3}$ M) causes the stabilization of actin filaments and the rate of filament growth varies linearly with the concentration of actin monomer in the presence of P_i (Carlier & Pantaloni 1988). Therefore P_i release appears as the elementary step responsible for the destabilization of actin–actin interactions in the filament.

(*b*) *Kinetics of GTP hydrolysis in microtubule assembly*

The kinetic scheme I proposed for ATP hydrolysis on F-actin also applies to GTP hydrolysis on microtubules, however both cleavage of the γ-phosphate bond and liberation of P_i are one order of magnitude faster than for actin (Melki *et al.* 1990; Burns 1991). As a result, only under conditions of rapid polymerization can appreciable amounts of GTP and GDP-P_i transiently bound to microtubules be detected, and these transients have life times of a few seconds. At steady state, the GTP/GDP-P_i cap must be smaller than on F-actin, which partly explains the accelerated dynamics of microtubules.

(*c*) *Probing the intermediate NDP-P state on F-actin and microtubules using structural analogues of P_i: AlF_4^- and BeF_3^-, H_2O*

Fluoroaluminate and fluoroberyllate have a tetrahedral configuration in solution with the bond lengths similar to those of inorganic phosphate, and have been shown to restore the functional properties of GTP-transducin when added to GDP-transducin (Bigay *et al.* 1987). These phosphate analogues bind to F-ADP-actin and to GDP-microtubules in competition with P_i, but with an affinity three orders of magnitude higher than P_i (Carlier *et al.* 1988; Carlier & Combeau 1988). The F-ADP-BeF_3 filaments and GDP-BeF_3-microtubules are extremely stable, the rate of dissociation of NDP-BeF_3 subunits from polymer ends is very low, actually even lower than the rate of dissociation of NDP-P_i subunits (Carlier *et al.* 1989). In addition, some evidence can be found that the conformation of the NDP-BeF_3- state is different from that of the NDP-P_i state. BeF_3^- and AlF_4^- bind to and dissociate from the NDP-polymer at very slow rates. All the above properties of BeF_3^- and AlF_4^- are very similar to those of vanadate binding to e.g. ADP-myosin (Goodno 1979) and led to the suggestion that BeF_3^- and AlF_4^- could mimic the NDP-P* transition state, or at least adopt a configuration closer to that of bound NTP than to bound NDP-P_i. The fact that AlF_4^-, and not AlF_3^- as proposed by Bigay *et al.* (1987) is actually bound to the NDP-polymer (Combeau & Carlier 1989) brings more support to this view, since Al then interacts with the bridging oxygen of NDP and with four fluorides, a total of five coordination bonds. Similar results have been obtained for binding of BeF_3^- and AlF_4^- to the bacterial F_1-ATPase (Dupuis *et al.* 1989). Further experiments should be aimed at understanding the structure of bound NDP-AlF_4, for example using the superhyperfine coupling of Mn ESR signal with ^{17}O labeled NDP, or using NMR of ^{19}F. Interestingly, AlF_4^- and BeF_3^- do not bind to monomeric G-ADP-actin, nor to monomeric GDP-tubulin, nor to ring oligomers of GDP-tubulin which are not able to hydrolyse the nucleotide. This observation is consistent with AlF_4^- and BeF_3^- acting as analogs of the transition state, and also indicates that the environment of the γ-phosphoester bond of the nucleotide is not the same in the monomer and in the polymerized states of actin and tubulin.

The results of the experiments using phosphate analogues lead to add another step in the kinetic scheme for hydrolysis of ATP or GTP on F-actin or microtubules:

$$\text{F-ATP} \rightarrow \text{F-ADP-P*} \rightleftarrows \text{F-ADP-P}_i \rightleftarrows \text{F-ADP} + P_i$$

In the above scheme, F-ADP-P* represents the transition state energetically identical to the F-ADP BeF_3^- state. The transition from F-ADP-P* to F-ADP-P_i would be slow and rate limiting for P_i release. In this scheme, which resembles the one proposed for ATP hydrolysis on myosin for example (Hibberd & Trentham 1986), P_i binds to F-ADP in rapid equilibrium, while dissociation of P_i following cleavage of ATP is slow.

4. STEREOCHEMISTRY OF NUCLEOTIDE BINDING TO ACTIN AND TUBULIN: ROLE OF DIVALENT METAL ION IN NUCLEOTIDE BINDING AND HYDROLYSIS

(*a*) *ATP binding to actin*

Actin binds ATP very tightly in the presence of a divalent metal ion that can be either Ca^{2+} or Mg^{2+}. The exchange inert analogue of Mg-ATP, β,γ-Cr ATP can displace both tightly bound nucleotide and

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divalent metal ion from G-actin, leading to the conclusion that the tightly bound metal ion interacts with the β- and γ-phosphate of ATP in the nucleotide site (Valentin-Ranc & Carlier 1989). The conformation, ability to polymerize and rate of ATP hydrolysis differ when CaATP or MgATP is bound to actin (see Carlier (1991) for a review). In particular, the hydrolysis of MgATP is fast, whereas the hydrolysis of CaATP is slow (Carlier *et al.* 1986) and corresponds to ATP hydrolysis on divalent cation-free actin (Valentin-Ranc & Carlier 1991). It appears that only Mg^{2+} is able to play an effective role in catalysis. CrATP has also been useful for probing the stereochemistry of ATP binding. The data showed that the metal-ATP chelate was bound in the Λ configuration, which is confirmed by the three-dimensional structure of actin at atomic resolution (Kabsch *et al.* 1990). CrATP is hydrolysed on F-actin upon polymerization; the hydrolysis product is Cr-ADP-P_i that remains bound to F-actin and P_i is not released. The resulting F-Cr-ADP-P_i-actin filament shows a high stability, as expected.

After metal-ATP hydrolysis on F-actin, only P_i is released in solution, and the β-monodentate metal-ADP remains bound to F-actin.

(*b*) *GTP binding to tubulin*

It is known that MgGTP or unchelated GDP are bound to tubulin (Correia *et al.* 1987, 1988). The situation is therefore different from actin: At some point in the polymerization cycle, Mg must dissociate from tubulin. CrGTP has been used to examine the role and fate of GTP-bound metal ion in tubulin polymerization (Carlier *et al.* 1991). Tubulin binds Cr-GTP β, γ bidentate preferentially in the Δ pseudo-axial configuration (like ras p21). CrGTP is actively hydrolysed during tubulin polymerization, and both Cr and P_i are liberated in solution, leaving GDP bound to microtubules. Tubulin appears to be a unique example of a nucleoside triphosphatase able to break the very stable Cr–phosphate bonds and liberate the products Cr and P_i in solution; in most cases in enzymology, Cr-nucleotides are used as dead-end inhibitors, with Cr-NDP-P_i remaining tightly bound to the enzyme after a single turnover reaction. Therefore the environment of the metal-β-γ phosphate moiety of GTP bound to tubulin must be unique.

5. STRUCTURAL CHANGE OF F-ACTIN AND MICROTUBULES ASSOCIATED TO NUCLEOTIDE HYDROLYSIS

The results from thermodynamic and kinetic studies on actin and tubulin polymerization indicate that a structural change of the polymer is linked to P_i release. The nature of this change is still an open issue. Evidence for different structural states of the filament in the F-ADP, F-ADP-P_i and F-ADP-BeF_3 states can be obtained by a combination of cryoelectromicroscopy and solution low angle X-ray scattering techniques (Lepault *et al.*, submitted). This structural change is expected to be less spectacular than in the case of ras p21, because the conformation of the subunit is somewhat constrained in the NTP state by the structure of the polymer itself. The change in the coordination of the divalent metal ion following the release of P_i is likely to trigger this structural change.

In the three-dimensional structure of actin, the environment of the phosphate moiety of the nucleotide appears roughly the same whether CaADP or Ca ATP is bound, whereas one would expect to observe two different conformations. The reason for this finding is unclear, however it must be stressed that the three-dimensional structure is derived from X-ray diffraction of crystals of the DNaseI–actin complex, which is, like G-actin, unable to hydrolyse ATP. The conformation obtained may therefore correspond to G-actin frozen in the G-ATP state independently of the bound nucleotide. Structural studies in conjunction with site directed mutagenesis experiments should eventually solve the issue.

The three-dimensional structure of tubulin is not known, and tubulin does not display the consensus sequences of the GTP binding site of G-proteins (Dever *et al.* 1987). Therefore nothing is known at present about the change in structure linked to GTP hydrolysis. Several lines of evidence however indicate that lateral rather than longitudinal tubulin–tubulin interactions are weakened following GTP hydrolysis on microtubules (Melki *et al.* 1989).

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Discussion

H. Gutfreund (*Department of Biochemistry, University of Bristol, U.K.*). Do I understand correctly that although F-actin-ADP-P_i is a very stable intermediate during the process, there is no oxygen exchange, i.e. the hydrolysis is irreversible?

M.-F. Carlier. Martin Webb saw very little P_i-water oxygen exchange. Intermediate ^{18}O-exchange experiments starting with (^{18}O)ATP showed no exchange but it is possible that this is due to P_i being unable to rotate in the catalytic site. However, the lack of exchange, and the fact that there is no net synthesis of bound ATP, means that the hydrolysis step is likely to be close to irreversible. I think that similar experiments have been done on microtubules and the same was found.

P. M. Bayley (*National Institute for Medical Research, Mill Hill, London, U.K.*). This interesting result of a small percentage of the chromium nucleotide stabilizing the microtubule, could that possibly be one of the other isomers which is being produced or is present in the original preparation?

M.-F. Carlier. We have performed high performance liquid chromatography (HPLC) analysis of the bound chromium GTP and it seems to be only the delta pseudo-axial isomer.

P. M. Bayley. Does inorganic phosphate promote the nucleation and polymerization of actin-ADP, which would be consistent with the linearization of the assembly kinetics?

M.-F. Carlier. Yes, we observed that.

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