Biophysical Chemistry 7 (1977) 51–58

THE MECHANISM OF ATP HYDROLYSIS BY POLYMER ACTIN *

Albrecht WEGNER
Department of Biophysical Chemistry, Biozentrum, CH-4056 Basel, Switzerland

Received 21 December 1976

The rate of actin polymerization, the rate of nucleotide splitting and the rate of the nucleotide exchange have been measured simultaneously. Correlation of these three measurements demonstrated that nucleotide splitting and exchange were mainly connected with the association and dissociation reactions of actin protomers at the ends of actin filaments and were not caused by release and rebinding of nucleotide molecules at the binding sites along the filament. The observation made by others that the nucleotide exchange was accelerated in the presence of ATP was explained by the translocational head-to-tail polymerization of actin: Due to the simultaneous lengthening of the filament at one end and shortening at the other, nucleotide molecules are incorporated at one end and released at the other. In the absence of ATP, where the head-to-tail polymerization mechanism was not operative nucleotide exchange was brought about by the slow process of length fluctuation of polymers.

1. Introduction

Actin-bound nucleotide has been suggested to be involved in several functions of this protein. The nucleotide apparently plays a role in the regulation of actin polymerization. Evidence has come from the observation that actin containing ADP polymerizes at slower rate than actin containing ATP [1]. Recently it has been demonstrated that the cleavage of ATP which occurs during polymerization of actin is important in regulating the direction of growth of actin filaments. The irreversible nucleotide splitting causes the filaments to lengthen at one end and to shorten simultaneously at the other (translocational head-to-tail polymerization) [2,3]. Another function has been proposed for the nucleotide in the interaction of actin with one of the proteins to which actin is bound [4]. Investigations of the role of the actin-bound nucleotide in the interaction of actin with myosin, troponin or tropomyosin have been based on measurements of the rate of the nucleotide exchange [review: 5].

Two mechanisms of nucleotide exchange have been proposed [6]. (i) Nucleotide molecules are incorporated into the polymer solely by the association of ATP containing monomers with actin filaments and are released from the polymer solely by dissociation of subunits with bound nucleotide. (ii) Nucleotide molecules are exchanged by release from and binding to the nucleotide binding sites along the filament in addition to the exchange by association and dissociation reactions at the ends of actin filaments. Proof of one of these mechanisms has been hampered by the difficulty in separating quantitatively the contribution of nucleotide exchange at the ends of actin filaments and at the binding sites along the filaments.

For an understanding of the interactions of actin with other proteins it is important to know if or to what extent the actin-bound nucleotide is available for release from the binding sites along the filament. In the present study the contributions of the two pathways of exchange have been separated by correlating the rate of ATP splitting and of nucleotide incorporation into filaments with the kinetics of the monomer concentration.

2. Material and methods

2.1. Preparation of actin

Actin was prepared according to the method of Rees and Young [7] with the following alterations:

* Supported by Research Grant 3.183-0.73 from the Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung.

The protein was chromatographed on Bio-Gel P 150 and protected against denaturation by modification with N-ethylmaleimide [8]. Actin concentrations were determined photometrically using the biuret reaction [9].

2.2. Polymerization conditions

Solutions of monomeric actin contained 0.5 mM ATP, 0.02 mM $MgCl_2$ and 200 mg/ℓ sodium azide to prevent bacterial growth. The solution was buffered with 5 mM triethanolamine · HCl (pH 7.5). The polymerization was initiated by mixing two parts of actin solution with one part of a buffer containing sufficient $MgCl_2$ to give a final concentration of 0.5 mM.

2.3. Light scattering

All solutions were centrifuged at 100 000 g for two hours to remove dust and polymers. The scattering intensity was measured with a fluorometer (Farrand MK1) at an angle ϑ of 90 degrees and a wavelength λ of 546 nm.

For a solution of polydisperse long, thin rods like actin filaments, it has been shown [9,10] that the reduced scattering intensity R is proportional to the concentration of subunits incorporated into filaments (c_w):

$$R = \text{const.} \cdot c_w \qquad (1)$$

Eq. (1) can be applied if the length of the actin rods is greater than λ^* and the diameter is small compared to λ^*, where λ^* is $\lambda/(n\, 4\pi \sin \vartheta/2)$ and n is the refractive index.

The instrument was calibrated by measuring the scattering intensity of solutions of known c_w.

2.4. Nucleotides

^{14}C-adenosine-triphosphate was purchased from New England Nuclear Corporation. ATP and ADP were separated by ion exchange chromatography (DEAE-Sephadex A-25). The radioactivity of the fractions containing ADP and ATP was measured using Insta Gel (Packard) in a Packard model 3320/3330 scintillation counter. The ATP concentrations of actin solutions were determined by absorption at 259 nm (ϵ = 15 400 cm^{-1} M^{-1}) and enzymatically [11].

2.5. Filtration

The technique of filtration was applied for separating actin and the bound nucleotide from solutions. The filtration was carried out at a pressure difference of 300 torr. To remove unbound, radioactively labelled nucleotide the filter was washed once with 1 ml of a solution containing 2.5 mM $CaCl_2$ and 0.1 M KCl. Further washing had no significant effect on the final result. The filter was dissolved in 2 ml dimethylformamide by shaking for 2 hours. 15 ml Insta Gel and 2 ml 0.2 M $MgCl_2$ solution were added. After further shaking the radioactivity was measured. For calibration a known amount of labelled nucleotide was added to scintillation liquid treated in the same way.

2.6. Experimental procedure

A trace of radioactively labelled ATP (0.03 μCi/ml solution) was added to actin solutions. The kinetics of the formation of actin polymers was measured by light scattering. At the same time the rate of ATP splitting was followed by taking samples of actin, deprotonizing them with perchloric acid and determining the content of labelled ADP and ATP. At the final stage of polymerization where the monomer concentration had reached a constant steady-state value as indicated by the constant light scattering intensity, a second trace of radioactively labelled nucleotide (0.12 μCi/ml solution) was added. The rate of incorporation of the labelled nucleotide into the polymers was measured by separating actin from the solution and determining the labelled ADP bound to actin. The critical monomer concentration was determined by separating monomers and polymers by centrifugation and measuring the absorbance of the supernatant at 290 nm.

3. Analysis of the kinetics of nucleotide exchange and hydrolysis

3.1. Mechanisms of nucleotide exchange

The close connection of the nucleotide exchange

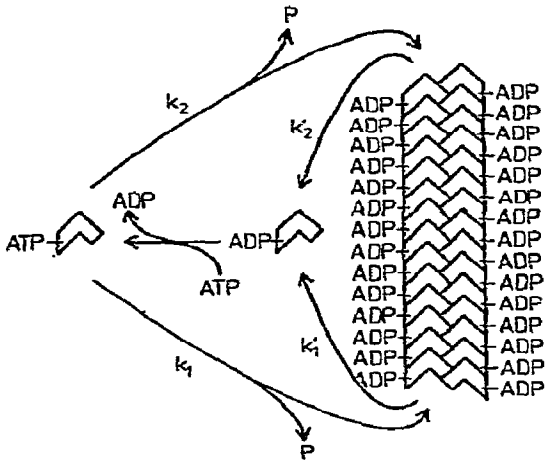

Fig. 1. Reaction scheme of actin polymerization. The chevron symbol stands for an actin protomer with bound ATP or ADP. P is inorganic phosphate.

and of the association of actin necessitates a brief consideration of the polymerization mechanism of actin [12,13,3]. Monomeric actin binds ATP strongly [14,15]. During the association of actin monomers the nucleotide is hydrolysed to ADP and phosphate. ADP is incorporated into the filament and phosphate is released. Subunits dissociating from the filament contain ADP which is replaced by ATP in a fast reaction. ATP has a much higher affinity for monomeric actin than ADP, so that in an excess of ATP nearly all monomeric actin contains ATP.

The structural polarity of actin filaments [16] requires the introduction of four rate constants for the description of the rate of actin polymerization. These are two association rate constants for the binding of actin monomers to both ends of a filament (k_1 and k_2 for end 1 or 2 respectively) and two dissociation rate constants for the release of actin protomers from both ends (k_1' and k_2' for end 1 or 2 respectively) (see fig. 1). Since the dissociation is not the reverse reaction of the association the ratios of the association and dissociation rate constants are not equilibrium constants which must be equal for both ends but are independent quantities. As a consequence of the irreversible polymerization cycles, actin filaments can grow at one end and simultaneously shorten at the other (translocational head-to-tail polymerization) [3].

The total growth rate of actin filaments defined as the time derivative of the number (n) of polymer subunits is given by

$$dn/dt = (k_1 + k_2)c_1 - (k_1' + k_2'), \quad (2)$$

where c_1 is the monomer concentration.

A polymer which starts to form at time t_1 (nucleation) contains at time t_0 on the average n subunits, where

$$n = \int_{t_1}^{t_0} [(k_1 + k_2)c_1 - (k_1' + k_2')]\,dt. \quad (3)$$

Deviations from the average value n are relatively small for large n.

The rate of incorporation of monomers into polymers is the product of the rate of growth of a single filament and the concentration of all polymers (c_p):

$$\frac{dc_1}{dt} = -\frac{dn}{dt}c_p = -[k_1 + k_2)c_1 - (k_1' + k_2')]c_p. \quad (4)$$

At the final stage of polymerization the monomer concentration reaches a constant steady state value (critical monomer concentration $\bar{c}_1$). Under this condition the polymers lengthen at one end (i.e. end 1) with the same rate as they shorten at the other (end 2). The average length remains unchanged

$$\frac{dn}{dt} = \frac{dn_1}{dt} + \frac{dn_2}{dt} = 0, \quad (5)$$

where n_1 or n_2 are the numbers of subunits by which the polymers are lengthened or shortened.

In the following treatment the exchange of nucleotide molecules bound to actin filaments is discussed on the basis of two mechanisms proposed by Kasai and Oosawa [6]. In the first model it is assumed that the nucleotide is incorporated into and released from the filament solely by association or dissociation of protomers at the ends of filaments. In the second model an exchange and splitting of nucleotides at the binding sites along the filament as well as by association and dissociation of subunits at the ends is postulated.

3.2. First model

3.2.1. Rate of incorporation of labelled nucleotide

If at time t_0 when the monomer concentration is near the critical monomer concentration $\bar{c}_1$ radioactively labelled ATP is added to a solution of poly-

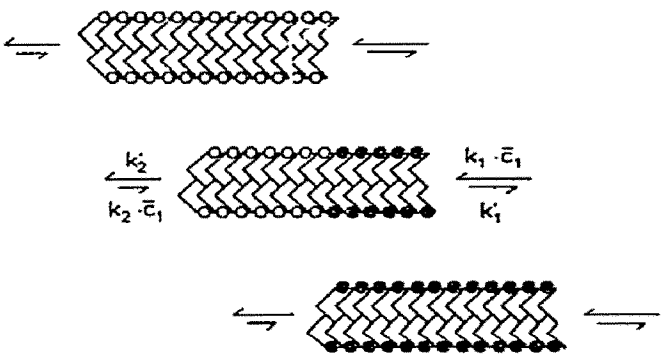

Fig. 2. Mechanism of nucleotide exchange at the ends of actin filament. The chevron symbol stands for an actin protomer. ADP is drawn as circles and radioactively labelled ADP as filled circles. Top: State of a filament at time t_0. Middle: State of a filament at a time t where $t_0 < t < t_0 + n/(k_1\bar{c}_1 - k'_1)$. Bottom: State of a filament at time $t_0 + n/(k_1\bar{c}_1 - k'_1)$.

meric actin labelled nucleotide starts to be incorporated at the lengthening end whereas unlabelled nucleotide is released from the shortening end. The rate of nucleotide exchange is governed by the rate of lengthening or shortening of a polymer (see fig. 2). After some time the filament is completely saturated with labelled nucleotide. The time ($t(n)$) needed for a saturation of filaments with n subunits is equal to the time needed for a lengthening of the filaments by n subunits at end 1 or a shortening by n subunits at end 2

$$t(n) = n/(k_1\bar{c}_1 - k'_1) = -n(k_2\bar{c}_1 - k'_2). \tag{6}$$

Consequently for unsaturated filaments (concentration c_p), the rate of incorporation of labelled nucleotide (concentration *ADP) is

$$\frac{\mathrm{d}\,{*\mathrm{ADP}}}{\mathrm{d}t} = (k_1\bar{c}_1 - k'_1)c_p$$

$$t_0 < t < t_0 + n/(k_1\bar{c}_1 - k'_1)$$

and for saturated filament is

$$\frac{\mathrm{d}\,{*\mathrm{ADP}}}{\mathrm{d}t} = 0$$

$$t > t_0 + n/(k_1\bar{c}_1 - k'_1). \tag{7}$$

The determination of c_p, the number of subunits n, and the various rate constants is extremely difficult. The problem can be avoided by correlating the rate of nucleotide incorporation with the kinetics of monomer concentration in the following way.

The time interval $t(n)$ can be expressed in terms of the time $(t_0 - t_1)$ needed to build up a filament combining eqs. (2), (3), (5) and (6)

$$t(n) = \frac{1}{s}\int_{t_1}^{t_0} (c_1/\bar{c}_1 - 1)\mathrm{d}t, \tag{8}$$

where

$$s = (k_1\bar{c}_1 - k'_1)/(k'_1 + k'_2).$$

The parameter s gives the ratio between the rate of lengthening at end 1 and the frequency of dissociation or association steps at both ends. This parameter was determined experimentally by measurement of the exchange of covalently labelled actin as described elsewhere (3).

In actin solutions nucleation occurs continuously so that the time of nucleation t_1 is different for each filament. The concentration of filaments (Δc_p) which start to form in a small interval of time (Δt) around t, is given by [cf. eq. (4)]

$$\Delta c_p = -\frac{\mathrm{d}}{\mathrm{d}t}\left\{\frac{1}{[(k_1+k_2)c_1 - (k'_1+k'_2)]}\frac{\mathrm{d}c_1}{\mathrm{d}t}\right\}\Bigg|_{t=t_1}\Delta t. \tag{9}$$

The rate of nucleotide incorporation into those filaments at time t_2 is

$$\frac{\mathrm{d}\,{*\mathrm{ADP}}}{\mathrm{d}t} = -s\frac{\mathrm{d}}{\mathrm{d}t}\left\{\frac{1}{c_1/\bar{c}_1 - 1}\frac{\mathrm{d}c_1}{\mathrm{d}t}\right\}\Bigg|_{t=t_2}\Delta t,$$

$$t_0 < t_2 < t_0 + 1/s\int_{t_1}^{t_0}(c_1/\bar{c}_1 - 1)\mathrm{d}t \tag{10}$$

and

$$\frac{\mathrm{d}\,{*\mathrm{ADP}}}{\mathrm{d}t} = 0, \quad t_2 > t_0 + \frac{1}{s}\int_{t_1}^{t_0}(c_1/\bar{c}_1)\mathrm{d}t.$$

The rate of incorporation into all filaments is obtained by summing up the contributions of all unsaturated polymers

$$\frac{\mathrm{d}\,{*\mathrm{ADP}}}{\mathrm{d}t}\Bigg|_{t=t_2} = -s\int_0^{t_1}\frac{\mathrm{d}}{\mathrm{d}t}\left\{\frac{1}{[c_1/\bar{c}_1 - 1]}\frac{\mathrm{d}c_1}{\mathrm{d}t}\right\}\mathrm{d}t$$

$$= -s\frac{1}{c_1/\bar{c}_1 - 1}\frac{\mathrm{d}c_1}{\mathrm{d}t}\Bigg|_{t=t_1}, \tag{11}$$

where

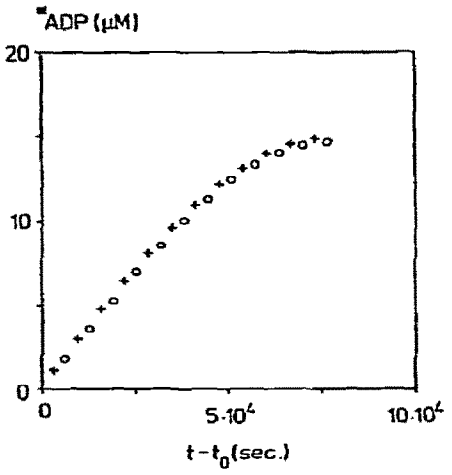

Fig. 3. Simulations of nucleotide exchange as calculated by the approximate eq. (11) (dots) and by a numerical simulation (crosses). The curves have been computed for the following parameters: $k_1 = 5 \times 10^3\ M^{-1}\ s^{-1}$, $k_2 = 5 \times 10^3\ M^{-1}\ s^{-1}$, $k'_1 = 1.25 \times 10^{-2}\ s^{-1}$, $k'_2 = 3.75 \times 10^{-2}\ s^{-1}$, $s = 0.25$. It has been assumed that the polymerization commenced with monomers only. The continuous nucleation has been introduced by assuming that the monomers and dimers (c_2) are in a fast preequilibrium ($K = c_1^2/c_2 = 10^{-1}$ M) (see ref. [9]). The time of addition of labelled ATP has been taken to be 20 000 s.

$$t_2 - t_0 = \frac{1}{s} \int_{t_1}^{t_0} (c_1/\bar{c}_1 - 1)\mathrm{d}t.$$

$\mathrm{d}c_1/\mathrm{d}t$ at time $t = 0$ is zero since the experimental conditions are chosen so that at the beginning of the polymerization no polymers are present.

The derivation of this equation is based on the approximation that the degree of polymerization of a filament is equal to the average value which is given by an integration of the rate of growth over the time (eq. (3)). Proof for the validity of this assumption comes from a comparison of the approximation with a numerical calculation for a set of kinetic parameters which is realistic for actin polymerization [9] (see fig. 3).

3.2.2. Rate of ATP splitting

The rate of ATP splitting or ADP production is determined by the frequency of monomer addition to the filament

$$\frac{\mathrm{d}[\mathrm{ATP}]}{\mathrm{d}t} = -\frac{\mathrm{d}[\mathrm{ADP}]}{\mathrm{d}t} = -(k_1 + k_2)c_1 c_p. \tag{12}$$

Substituting eqs. (2), (4) and (5) we arrive at

$$\frac{\mathrm{d}[\mathrm{ATP}]}{\mathrm{d}t} = -\frac{\mathrm{d}[\mathrm{ADP}]}{\mathrm{d}t} = \frac{c_1}{c_1 - \bar{c}_1}\frac{\mathrm{d}c_1}{\mathrm{d}t}. \tag{13}$$

Eqs. (11) and (13) correlate the kinetics of the monomer concentration with the rate of ATP splitting or nucleotide incorporation respectively. The parameter s can be determined independently. By a comparison of the time dependence of the monomer concentration with the rate of ATP splitting and nucleotide incorporation, it can be proved if the kinetic behaviour of the nucleotide-actin system is compatible with the proposed model.

3.3. Second model

3.3.1. Rate of incorporation of radioactively labelled nucleotide

Actin polymers bind ADP in a molar ratio of one molecule per subunit. In view of the saturation of the nucleotide binding sites it is a reasonable assumption that the rate of a nucleotide exchange and splitting at the binding sites along the filament is governed by the rate of dissociation of an ADP molecule from the polymer (rate constant k'_3) (see fig. 4).

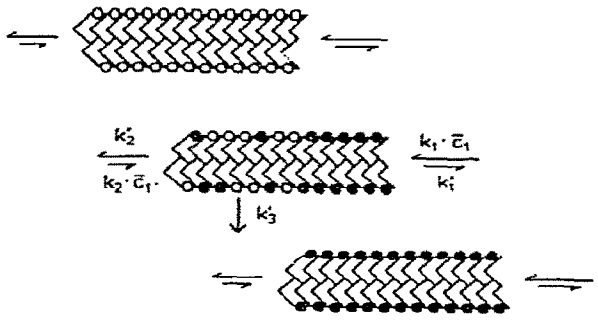

Fig. 4. Mechanism of nucleotide exchange at the ends and at the binding sites along the filament. The chevron symbol stands for an actin protomer. ADP is drawn as circles and radioactively labelled ADP as filled circles. Top: State of a filament at time t_0. Midlde: State of a filament at time t where $t_0 < t < t_0 + n/(k_1\bar{c}_1 - k'_1)$; m ADP molecules have been inrorporated at the lengthening end and $(n - m)$ ADP molecules have been partially exchanged at the binding sites of the filament. Bottom: State of a filament at time $t_0 + n/k_1\bar{c}_1 - k'_1)$.

The number (m) of radioactively labelled nucleotide molecules which are incorporated into a filament within an interval of time $t_2 - t_0$ by lengthening of end 1 is given by

$$m = (k_1\bar{c}_1 - k'_1)(t_2 - t_0). \quad (14)$$

The probability that one of the remaining $n - m$ subunits at the shortening end has exchanged its nucleotide molecule by release of ADP from the binding sites along the filament is $1 - \exp(-k'_3(t_2 - t_0))$. The time course of labelled-nucleotide exchange is given by the sum of the rates of nucleotide incorporation at the lenthening end, release at the shortening end and exchange at the binding sites along the filament:

$$\frac{d\,{}^*\mathrm{ADP}}{dt} = [(k_1\bar{c}_1 - k'_1) + \{1 - \exp(-k'_3(t_2 - t_0))\}\ (k_2\bar{c}_1 - k'_2) + k'_3\{1 - \exp(-k'_3(t_2 - t_0))\}(n - m)]c_p \quad (15)$$

The continuous nucleation is taken into consideration by summing up the contributions of all filaments as described in section 3.2.

$$\left.\frac{d\,{}^*\mathrm{ADP}}{dt}\right|_{t=t_2} = -s\,\frac{\exp(-k'_3(t_2 - t_0))}{c_1(t_1)/\bar{c}_1 - 1}\left.\frac{dc_1}{dt}\right|_{t=t_1} + k'_3\{1 - \exp(-k'_3(t_2 - t_0))\}(c_1|_{t=0} - c_1|_{t=t_1}). \quad (16)$$

The difference between the monomer concentration at the initiation of the polymerization ($c_1|_{t=0}$) and at the time t_1 ($c_1|_{t=t_1}$) is equal to the concentration of subunits incorporated into polymers (c_w) at the time $t = t_1 : c_w = c_{tot} - c_1$. Eq. (16) now becomes

$$\left.\frac{d\,{}^*\mathrm{ADP}}{dt}\right|_{t=t_2} = -s\,\frac{\exp(-k'_3(t_2 - t_0))}{c_1(t_1)/\bar{c}_1 - 1}\left.\frac{dc_1}{dt}\right|_{t=t_1} + k'_3\{1 - \exp(-k'_3(t_2 - t_0))\}c_w(t_1). \quad (17)$$

3.3.2. Rate of ATP splitting

The rate of ATP splitting or ADP production is determined by the rate of release of ADP from the polymer subunits and the frequency of association reactions at the ends of the polymers [see eq. (13)]

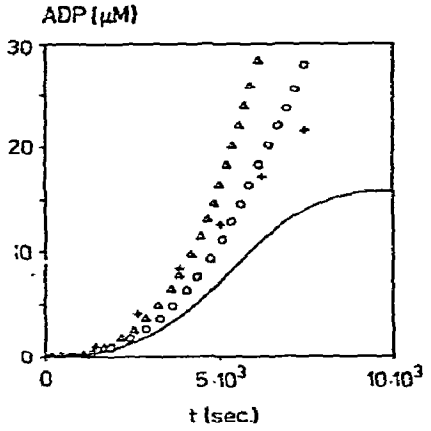

Fig. 5. Continuous line: Time course of the subunit incorporation into polymers c_w. Crosses: Time course of the formation of ADP. Dots: Time course of ADP derived according to eq. (13). Triangles: Time course of ADP derived according to eq. (18) with $k'_3 = 5 \times 10^{-4}\ s^{-1}$.

$$\frac{d[\mathrm{ATP}]}{dt} = \frac{d[\mathrm{ADP}]}{dt} = \frac{c_1}{c_1 - \bar{c}_1}\frac{dc_1}{dt} + k'_3 c_w. \quad (18)$$

Nucleotide exchange at the binding sites along the filament is indicated by an acceleration of ATP splitting and of nucleotide incorporation compared to an exclusive splitting and exchange at the ends of the filaments.

4. Measurements

4.1. Kinetics of ATP splitting

The kinetics of the actin-subunit incorporation into polymers (c_w) and of the formation of ADP is shown in fig. 5. The total actin concentration (c_{tot}) was 22 μM, the critical monomer concentration ($\bar{c}_1$) was 6.3 μM and the total nucleotide concentration was 470 μM. The time course of nucleotide splitting which can be derived from the kinetics of the monomer concentration (eqs. (13), (18)) has been calculated by replacing the integrals by a sum of intervals of 120 s width. The curves have been computed for the case of exclusive ATP splitting at the ends of actin filaments and for a slow additional ATP splitting at the binding sites along the filament.

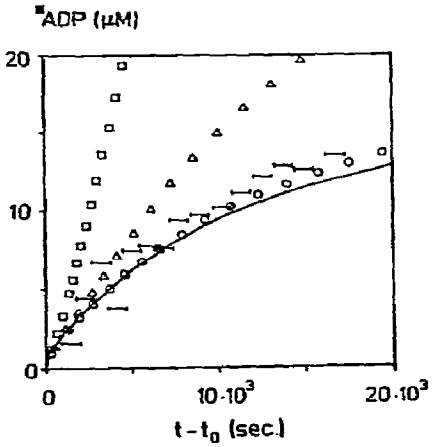

Fig. 6. Bars: Time course of the labelled ADP incorporated into polymers. Continuous line: Time course of incorporated ADP derived according to eq. (11). Time course of incorporated ADP derived according to eq. (17) with $k'_3 = 10^{-4}$ (dots), with $k'_3 = 2 \times 10^{-4}$ (triangles) and with $k'_3 = 5 \times 10^{-4}$ (squares).

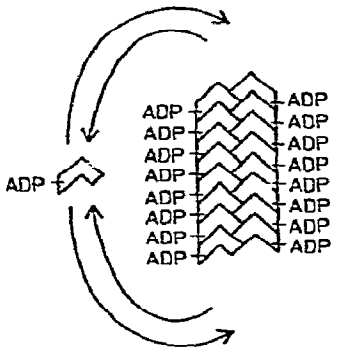

Fig. 7. Reaction scheme of reversible polymerization.

4.2. Rate of nucleotide incorporation

Fig. 6 shows the kinetics of the incorporation of labelled nucleotide into actin filaments. The time course of incorporation has been derived from the kinetics of the monomer concentration according to eqs. (11) and (17) for the two models. The parameter s, which has been determined independently (3), was 0.25.

5. Discussion

The kinetics of nucleotide incorporation is fitted well by the model of exchange exclusively due to binding of monomers at the ends of actin filaments. It seems not to be justified to base a proof for an exchange at the binding sites along the filament on the observed slightly higher rate of incorporation, as the difference between the measured and the calculated curve is a small difference of two large quantities. The kinetics of ATP splitting shows some systematic deviations from the time course derived for the mechanism of splitting solely at the ends of filaments. A more refined treatment which includes additional reaction steps for the association of monomers to the filaments could explain these deviations. However in general the measured and the calculated curves are in agreement. A slow exchange of nucleotide at the binding sites along the filament can not be excluded by means of these experiments. The rate of such an exchange would be certainly slower than 2×10^{-4} s^{-1} (see figs. 5, 6) under the experimental conditions.

Studies on the nucleotide exchange have been reported for pure F-actin and for actin with other bound muscle proteins (5, 17, 18, 19, 20, 21, 22, 23). It has been found that in the isolated system the nucleotide is quickly exchanged in the presence of magnesium and ATP. Lack of one of these components leads to a slow exchange. Also replacement of magnesium by calcium or replacement of ATP by ADP or pyrophosphate causes the nucleotide to be more slowly released or exchanged (6). The different effects of ATP and ADP can be interpreted in the following way: As pointed out in 3.1 and ref. [3] the translocational head-to-tail polymerization is rendered possible by the irreversible splitting of ATP which occurs during the polymerization cycles of actin. In the absence of ATP, however, the actin protomers which associate with the end of a filament or dissociate from the end, bind the same nucleotide, namely ADP, so that the dissociation is the reverse reaction of the association (see fig. 7). In the case of reversible polymerization the growth of a filament is either outwards from or inwards to the centre at both ends of a filament or if the monomer concentration is equal to the critical monomer concentration, at either end of the filament occur as many association reactions as dissociation reactions. In

the latter case the exchange of subunits is brought about by the fluctuation of the length of filaments. Exchange of subunits due to fluctuations of the length is a slow process, especially for long filaments. compared to the directed exchange of the translocational head-to-tail polymerization (3, 6).

The reason for the acceleration of nucleotide exchange in the presence of ATP may be that the reversible polymerization, which occurs in the presence of ADP, is switched by ATP into translocational head-to-tail polymerization.

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