Similar affinities of ADP and ATP for G-actin at physiological salt concentrations

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The equilibrium constant for the exchange of ATP and ADP at G-actin was determined by fluorimetric titration of G-actin-bound ϵ-ATP by ATP or ADP. The affinity of ATP for G-actin was found to be only about 3-fold higher than the affinity of ADP for G-actin at 37°C, pH 7.5 and physiologically relevant salt concentrations (100 mmol K^+/l, 0.8 mmol Mg^{2+}/l, <0.01 mmol Ca^{2+}/l).

Actin *1-N^6-Ethenoadenosine 5'-triphosphate* *Fluorescence* *Nucleotide exchange* *Equilibrium constant*

1. INTRODUCTION

G-actin-bound ATP prevents denaturation of G-actin [1,2]. During polymerization of G-actin, ATP is hydrolyzed to form ADP [3]. The hydrolysis of ATP is a prerequisite for the treadmilling process of actin filaments [4]. Given the importance of the nucleotides for the function of actin, the exchange of ADP and ATP at G-actin was investigated in [5–9]. These studies have been performed at low salt concentrations to avoid polymerization of actin. At these conditions, ATP has been found to bind considerably more strongly to G-actin than ADP. Here, we investigate nucleotide exchange at physiologically relevant salt concentrations by using a sensitive fluorescence assay.

2. MATERIALS AND METHODS

2.1. Preparation and purification of the nucleotides

ϵ-ATP was prepared as in [10] with the modification that the crude product was applied to a DEAE–Sephadex A25 column (2.5 × 40 cm) and eluted with a linear NH_4HCO_3-gradient (0.15–0.33 mol/l). The main peak was pooled. The salt was removed by using vacuum. The ϵ-ATP was precipitated with ethanol. Thin-layer chromatography using *i*-butyric acid–NH_4OH–water (75:1:24, by vol.) on Merck-cellulose F sheets yielded a single spot. ADP was purchased from Boehringer (Mannheim) and purified as described for ϵ-ATP. Thin-layer chromatography on polyethyleneimine-cellulose (PEI-cellulose) sheets (Merck) using 1 mol HCOOH/l and 0.3 mol LiCl/l [11] yielded a single spot. The absence of ATP was demonstrated enzymatically as in [12]. ATP was purchased from Merck and used without further purification. Concentrations of nucleotides were based on the following molar absorption coefficients: ϵ-ATP (275 nm), 5.6×10^3 l.mol^{-1}.cm^{-1} [10]; ATP,ADP (259 nm), 15.4×10^3 l.mol^{-1}.cm^{-1} [13].

2.2. Preparation of actin

ϵ-ATP–G-actin was prepared as in [14,15] with the following alterations: The exposed SH-group of actin was protected against oxidation by modification with *N*-ethylmaleimide (NEM) [16]. Chroma-

Abbreviations: ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; ϵ-ATP, 1-N^6-ethenoadenosine 5'-triphosphate; NEM, *N*-ethylmaleimide; PEI, polyethylenimine; Tris, tris(hydroxymethyl)-aminomethane; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid

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tography of the protein was performed on a Sephacryl S-200 superfine column (2.5 × 90 cm). Actin was shown to be free of adenylate kinase by incubating 1 μmol G-actin/l with 1 mmol ADP/l, 100 mmol K^+/l and 0.8 mmol Mg^{2+}/l at 37°C for 30 min. After trichloroacetic acid precipitation, thin-layer chromatography on PEI-cellulose sheets yielded a single spot ($R_F = 0.6$) [11]. ε-ATP concentration of the actin solutions were adjusted by chromatography of G-actin on a Sephadex G-25 column equilibrated with the desired nucleotide concentration. Protein concentrations were measured as in [17] or by absorption ($\epsilon_{290} = 24.9 \times 10^3$ l.mol^{-1}.cm^{-1} [4]).

2.3. Fluorescence

A Jobin Yvon 3D spectrofluorimeter equipped with a mercury–xenon lamp was used for the measurements. The excitation wavelength was 365 nm and emission was recorded at 410 nm.

All samples contained 20 mmol Tris–HCl/l (pH 7.5), 3 mmol NaN_3/l and various concentrations of nucleotides, potassium, magnesium and EGTA or calcium. Immediately before measuring ε-ATP–G-actin solution was added to reach a final actin concentration of 1 μmol/l and a total volume of 2.5 ml. Change of fluorescence was followed until the equilibrium was reached. All experiments were done at 37°C.

3. RESULTS

3.1. Experimental design

The exchange of ATP (t) for ADP (d) at G-actin (G) was measured by using the fluorescent ATP-derivative ε-ATP (ε). The exchange of ATP for ADP could not be followed directly because of the lack of an appropriate measuring signal. The exchange of ε-ATP was monitored by means of the great ratio of the relative molar fluorescence coefficients $i_{\epsilon-G}/i_\epsilon$ of G-actin-bound ε-ATP and free ε-ATP [15,18]. This ratio was found to be 42. In all assays, ε-ATP–G-actin with a defined excess of ε-ATP was added to buffers containing various concentrations of ε-ATP and either ATP or ADP. The initial fluorescence intensity (I_s) and the fluorescence intensity of a blank containing only the excess concentration of ε-ATP (I_b) were measured. Furthermore the fluorescence intensity after equilibration (I) was determined. The required concentration of ε-ATP–G-actin was calculated according to [2,15]:

$$[\epsilon G] = [G]_0 \left(\frac{i_{\epsilon G}}{i_\epsilon} \cdot \frac{I - I_b}{I_s - I_b} - 1 \right) \cdot \left(\frac{i_{\epsilon G}}{i_\epsilon} - 1 \right)^{-1} \quad (1)$$

where $[G]_0$ is the total actin concentration. The equilibrium constant of the exchange of ATP for ADP ($K_{t/d}$) was determined by an analysis of the following two reactions (2,3):

(i) ε-ATP–ATP exchange at G-actin:

$$t - G + \epsilon \overset{K_{t/\epsilon}}{\rightleftharpoons} \epsilon - G + t$$

$$K_{t/\epsilon} = \frac{[t] \cdot [\epsilon - G]}{[\epsilon] \cdot [t - G]} = \frac{([t]_0 - [G]_0 + [\epsilon - G]) \cdot [\epsilon - G]}{([\epsilon]_0 - [\epsilon - G]) \cdot ([G]_0 - [\epsilon - G])} \quad (2)$$

(ii) ε-ATP–ADP exchange at G-actin:

$$\epsilon - G + d \overset{K_{\epsilon/d}}{\rightleftharpoons} d - G + \epsilon$$

$$K_{\epsilon/d} = \frac{[\epsilon] \cdot [d - G]}{[d] \cdot [\epsilon - G]} = \frac{([\epsilon]_0 - [\epsilon - G]) \cdot ([G]_0 - [\epsilon - G])}{([d]_0 - [G]_0 + [d - G]) \cdot [\epsilon - G]} \quad (3)$$

The equilibrium constant of the exchange of ATP for ADP was calculated by combining $K_{t/\epsilon}$ and $K_{\epsilon/d}$:

$$K_{t/d} = \frac{[t] \cdot [d - G]}{[d] \cdot [t - G]} = K_{t/\epsilon} \cdot K_{\epsilon/d} \quad (4)$$

The subscript zero denotes total concentrations.

3.2. Determination of the equilibrium constants

The time course of the exchange of actin-bound ε-ATP for ATP is depicted in fig.1. The half-time of the exchange reaction was about 25 s at the conditions of this experiment (100 mmol KCl/l, 0.8 mmol $MgCl_2$/l, 0.04 mmol EGTA/l). However, in buffers containing only 0.8 mmol $CaCl_2$/l the half-time was found to be about 10-fold longer (250 s) in agreement with the values in [2,9]. In all experiments the G-actin concentration was above the critical monomer concentration. However, at 1 μmol G-actin/l polymerization is so slow that only a negligible proportion of actin undergoes assembly during the time of the experiment (1200 s) [19]. At low total nucleotide concentrations the initial exchange phase was followed by a slow continuous decrease of the fluorescence intensity (up-

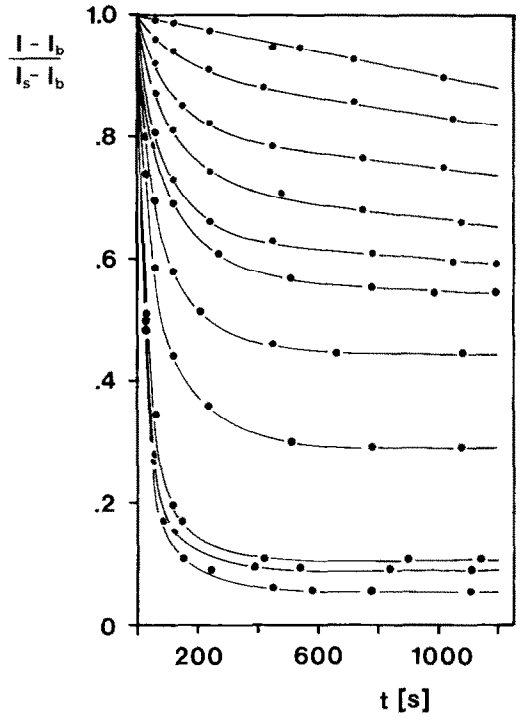

Fig.1. Time course of the reduced fluorescence intensity $(I - I_b)/(I_s - I_b)$ (I, fluorescence intensity at equilibrium; I_b, f.i. of excess ϵ-ATP; I_s, f.i. at the beginning of the exchange). The decrease is caused by the exchange of G-actin-bound ϵ-ATP for ATP. Total actin concentration, 1 μmol/l; initial ϵ-ATP concentrations, 1 μmol/l G-actin-bound ϵ-ATP, 12.8 μmol/l excess ϵ-ATP; ATP concentrations (from top to bottom), 0, 1, 3, 5, 7, 9, 15, 30, 65, 100, 500 μmol/l. All samples contained 100 mmol/l KCl, 0.8 mmol/l $MgCl_2$, 0.04 mmol/l EGTA.

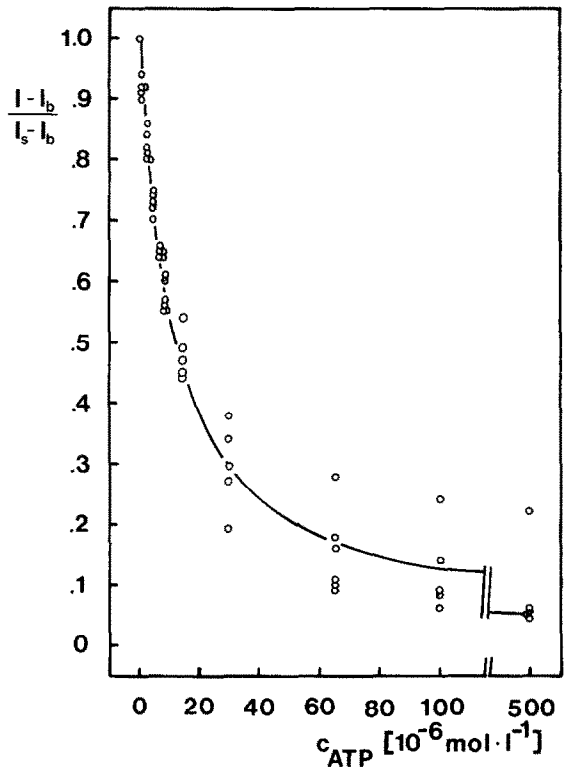

Fig.2. Fluorescence titration of G-actin-bound ϵ-ATP by ATP. Total actin concentration, 1 μmol/l; initial ϵ-ATP, concentrations, 1 μmol I G-actin-bound ϵ-ATP, 12.8 μmol/l excess ϵ-ATP. The continuous line represents an equilibrium curve calculated for $K_{t/\epsilon} = 1.15$. All samples contained 100 mmol/l KCl, 0.8 mmol/l $MgCl_2$ and 0.04 mmol/l EGTA.

per curves in fig.1). This slow decrease is to be attributed to an irreversible denaturation of G-actin occuring at low nucleotide concentrations [1,2]. A little correction for denaturation was applied by linear extrapolation of the fluorescence intensity of the second slow phase to time zero.

Fluorimetric titration curves of ϵ-ATP–G-actin by ATP or ADP are depicted in fig. 2 and 3. The titration curves were measured at physiologically relevant salt concentrations (100 mmol KCl/l and 0.8 mmol $MgCl_2$/l). Curves calculated for the best fitting exchange constants $K_{t/\epsilon}$ and $K_{\epsilon/d}$ are displayed. Exchange constants obtained at different salt conditions are summarized in table 1.

At physiologically relevant salt concentrations

Table 1

All values were obtained at 37°C and at pH 7.5

[Salt] (mol/l)	$K_{t/\epsilon}$	$K_{\epsilon/d}$	$K_{t/d}$
100 KCl 0.8 $MgCl_2$ 0.04 EGTA	1.15	3.1	3.5
100 KCl 0.8 $MgCl_2$ 0.8 $CaCl_2$	1.20	7.5	9.0
0.8 $CaCl_2$	1.55	70	110

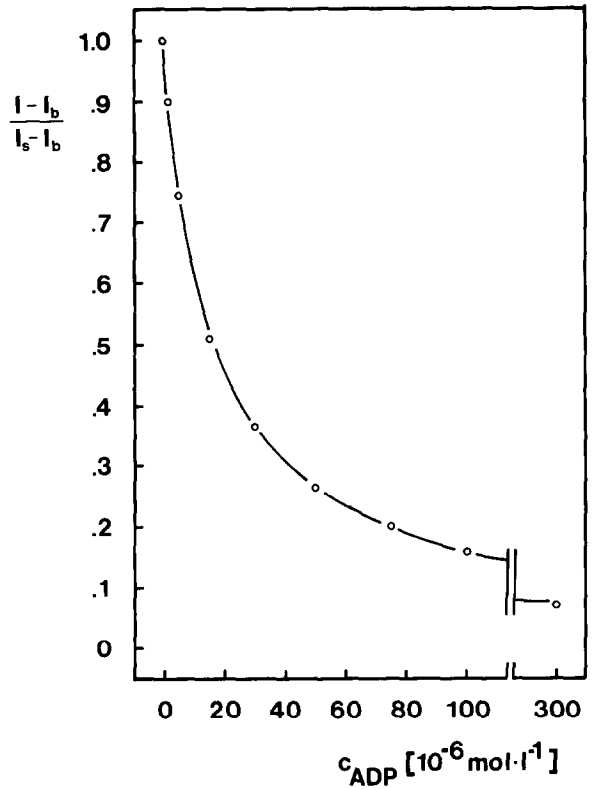

Fig.3. Fluorescence titration of G-actin-bound ϵ-ATP by ADP. Total actin concentration, 1 μmol/l; initial ϵ-ATP concentrations, 1 μmol/l G-actin-bound ϵ-ATP, 3.7 μmol/l excess ϵ-ATP. The continuous line represents an equilibrium curve calculated for $K_{\epsilon/d} = 3.1$. All samples contained 100 mmol/l KCl, 0.8 mmol/l $MgCl_2$ and 0.04 mmol/l EGTA.

the affinity of ATP for G-actin was found to be about 3-fold higher than the affinity of ADP for G-actin. The exchange constant $K_{t/d}$ appeared to be independent of traces of calcium because the exchange was not affected by the presence or absence of EGTA. When the [Ca^{2+}] was increased to unphysiologically high concentrations (0.8 mmol Ca^{2+}/l, 0.8 mmol Mg^{2+}/l, 100 mmol K^+/l) the exchange constant increased continuously to a value of about 9. If calcium was the only cation present, ATP bound more than 100-fold stronger than ADP.

4. DISCUSSION

Previously reported exchange constants have been determined at low salt concentrations. The values of the exchange constants range from 25 [5]–62 [6] and 100 [7]–175 in 0.8 mmol Ca^{2+}/l [9]. In [8], an exchange constant $K_{t/d} > 8$ in 0.1 mmol Mg^{2+}/l was evaluated. Our results demonstrate that the exchange constant depends strongly on the salt conditions. Calcium ions seem to cause a great difference in the affinities of the two nucleotides for G-actin whereas in physiologically relevant salt concentrations the affinities are similar.

The mechanism of actin assembly shares many features with tubulin polymerization. Tubulin binds GTP which is hydrolyzed during microtubule formation to yield GDP [20]. The affinity of tubulin for GTP has been reported to be 1–4-fold higher than the affinity for GDP [21,22]. Actin and tubulin appear to resemble one another as regards the exchange of nucleotide triphosphates and diphosphates.

In the presence of high concentrations of calcium ions ATP binds about 100-fold more strongly to G-actin than ADP [9]. The absolute binding constant of ADP to G-actin has been reported to be quite independent of the calcium concentration [23]. This suggests that calcium increases the affinity of G-actin for ATP. As G-actin-bound ATP prevents irreversible denaturation of actin, calcium stabilizes G-actin. Rees and Young [14] reported that a homogeneous monomeric actin solution can be prepared if 0.2 mmol Ca^{2+}/l is included in all purification steps. They found that in the absence of Ca^{2+}, actin easily forms oligomers of denaturated actin.

Our results were obtained in physiologically relevant salt concentrations. The observed moderate difference between the affinities of ATP and ADP for G-actin may be pertinent to free monomeric actin in vivo. In some types of cells large amounts of unpolymerized actin are associated with proteins which inhibit actin assembly (e.g., profilin [24]). It would be interesting to know whether these profilin-like proteins modulate the equilibrium of the exchange of ATP and ADP at G-actin.

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