The Enhanced ATPase Activity of Glutathione-substituted Actin Provides a Quantitative Approach to Filament Stabilization*

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[Cys374]glutathionyl-actin was prepared by isolation of the reaction product of G-actin with Ellman’s reagent (5,5’-dithiobis-(2-nitrobenzoic acid)), followed by reaction with glutathione. Filaments of this actin disulfide are susceptible to even weak shearing stress as exerted, for example, by heating to 37 °C. This treatment produces a 25-fold enhanced steady-state ATPase activity as compared to unmodified F-actin at room temperature. Monitoring the reduction of this enhanced ATPase activity is a reliable method for quantifying the effectiveness of filament-stabilizing agents and for determining their apparent dissociation constants. A detailed comparative study of filament-stabilizing agents was performed, and some hitherto unknown filament-protection effects were revealed. Inorganic phosphate provides stabilization only to a maximum of 45% ATPase inhibition, but reaches this effect already at cytoplasmic P concentration (~4 mM). Arsenate seems to bind with similar affinity, but with distinctly less protective activity (maximum of 16%). High concentrations of alkali ions provide a more effective protection (maximum of 95%), Li⁺ being more efficient than Na⁺ and K⁺. Divalent cations (Ca²⁺, Mg²⁺) had a strong stabilizing effect on KC₁-polymerized actin. The stabilizing effect of KCl and P is independent and additive. Correspondingly, at K₂HPO₄ concentrations greater than 4 mM, K⁺ ions contribute considerably to stabilization. In the presence of 100 mM KCl plus 4 mM P, conditions which mimic the physiological environment, filament protection is nearly as effective as with the mushroom toxin phalloidin. The strong stabilizing effect of phalloidin occurred at concentrations far below those required for toxicity in its interaction with actin filaments.

MATERIALS AND METHODS

Chemicals—All reagents were analytical grade, and all solutions were prepared with doubly distilled water. 5,5’-Dithiobis-(2-nitrobenzoic acid), glutathione, and phalloidin were from Sigma; ATP from Boehringer Mannheim; radioactive ATP from Amersham Corp.; and MgCl₂ and CaCl₂ (ultrapure) from Alfa (Ventron, Karlsruhe, Federal Republic of Germany). Sephadex G-25 and Sephacryl S-200 HR were from Pharmacia LKB Biotechnology Inc.

Actin Purification and Derivatization—Actin from rabbit skeletal muscle was prepared from acetone powder and purified through two cycles of polymerization essentially as described (11), with the exception that β-mercaptoethanol was omitted from all buffer solutions. Protein concentrations were determined spectrophotometrically using εmax = 25,480 M⁻¹ cm⁻¹ or by the Bradford dye-binding procedure (12). [Cys374]glutathionyl-actin was prepared as described previously (13) or as follows.1 G-actin (4·10⁻⁵ M) in 2 mM Tris-HCl (pH 7.5) containing 0.5 mM ATP, 0.1 mM CaCl₂, 2 mM NaN₃ (buffer G) was reacted with a 20-fold excess of 5,5’-dithiobis-(2-nitrobenzoic acid) (20 mM in 1% NaHCO₃) at 4 °C until 1 eq of nitrosothiolactone acid (εmax = 13,900 M⁻¹ cm⁻¹) was released. Excess of reagent was separated by running the reaction mixture through a Sephadex G-25 column (60 x 1 cm) equilibrated and eluted with buffer G. The resulting “activated” [Cys374]actin disulfide was found to be stable for at least 1 h at 4 °C at pH 8. It was reacted with a 50-fold excess of glutathione, again under spectrophotometric control as described above. The final product was purified by passing the mixture over Sephadex G-25 or a similar column of Sephacryl S-200. Polymerization was induced by addition of MgCl₂ to a final concentration of 2 mM or, for studying the effects of Ca²⁺ and Mg²⁺, by addition of KCl (final concentration

1 P. Tews, unpublished data.
of 100 mM) to a G-actin solution briefly treated with 0.2 mM EGTA. In all cases, the ATP concentration was 0.5 mM.

**ATPase Measurements**—When polymerization was complete, [γ-32P]ATP (100 nCi/mg of actin, 0.5% of total ATP) was added, and aliquots of this solution were transferred to reaction vials containing the agents to be measured. During incubation at 37 °C, aliquots were taken at 30-min intervals and analyzed for 32P, essentially as described by Martin and Doty (14), but modified as follows. Aliquots (100 μl) were pipetted into reaction vials containing 400 μl of a 1:3 mixture of 8% sodium silicotungstate in 0.2 N HSO₄, 0.2 mM H₂PO₄ and 4% ammonium heptamolybdate in 4 N H₂SO₄ and vortexed for 30 s. Phosphomolybdate was extracted into 0.5 ml of a 1:1 mixture of toluene and isobutyl alcohol (v/v) by vortexing for 5 min. Aliquots of the upper layer were added to 5 ml of Zinsser Gel (Roth, Karlsruhe, FRG) and counted in a Kontron MR300 liquid scintillation system. Concentrations of up to 20 mM unlabeled P₃ did not interfere with the determination of 32Pγ released during the experiment.

**Data Analysis**—The data relating ATPase activity to the concentration of the stabilizing agent were analyzed by a least-squares fit to a binding isotherm of the form

\[ y = 1 - C_1 \frac{x/K_{m1}^{0}}{1 + x/K_{m1}^{0}} - C_2 \frac{x/K_{m2}^{0}}{1 + x/K_{m2}^{0}} \]

where

- \( y \) is ATPase activity at free stabilizer concentration
- \( x \) is ATPase activity at \( x = 0 \)
- \( K_{m1}^{0} \) and \( K_{m2}^{0} \) are apparent dissociation constants for the binding of stabilizers 1 and 2 to F-actin, respectively;
- \( C_1 \) and \( C_2 \) are the maximum ATPase inhibition attainable with stabilizers 1 and 2, respectively. The second term is only used in cases where binding of two ligands (for example, K⁺ and anions) occurs, as with \( K^+HPO_4 \) or \( K^+HAsO_4 \).

**RESULTS**

When Mg²⁺-polymerized [Cys³⁷⁴]glutathionyl-actin is stored at 37 °C in 0.5 mM ATP in the presence of [γ-32P]ATP, hydrolysis occurs at a rate of 5 eq of 32Pi/mol of actin/h. This is 5–6 times higher than that of unsubstituted actin at room temperature. On addition of 4 mM K₂HPO₄ or 0.1 M KCl, the rate of ATP hydrolysis was reduced to 45 or 25%, respectively. Since these values were in good agreement with the stabilization effects determined by other methods under these conditions (6,8,9) and highly reproducible in a series of experiments with different actin preparations, we investigated whether the rate of P₃ release expressed as moles of P₃/moles of glutathionyl-actin/hour could be a suitable parameter for measuring the effectiveness of filament-stabilizing agents.

In typical experiments of this kind, we determined the rate of P₃ production by glutathionyl-actin between 15 and 90 min of incubation at 37 °C. One of the advantages of the glutathionyl-actin ATPase assay is ease of comparison of the effects of various filament-protecting agents such as P₃, KCl, and phalloidin within one system. As shown in Fig. 1, we confirmed that 100 mM KCl gives strong filament protection. The stabilizing effect of 4 mM P₃ is distinctly weaker. In a mixture of 4 mM P₃ and 100 mM KCl, the stability of filaments was considerably higher than with each of the agents alone, indicating that the stabilization effects of the two agents are additive. In the presence of 100 mM KCl and 4 mM P₃, filaments were clearly seen to be nearly as stable as in the presence of phalloidin (Fig. 1).

The assay allowed us to study filament stabilization as a function of the concentration of a stabilizing agent. Hyperbolic curves were obtained for inhibition by LiCl, NaCl, and KCl (Fig. 2), MgCl₂, and CaCl₂ (Fig. 3), and phalloidin (Fig. 4). Assuming that binding of a stabilizing agent to F-actin is a prerequisite for ATPase inhibition, the data were fitted to simple binding isotherms. Apparent dissociation constants and values for maximum stabilizing capacity (C) were obtained from curve fits and have been compiled in Table I. The assay also allowed us to easily screen other possible filament-stabilizing agents. Thus, we found that the stabilization effect exerted by phosphate is shown also by the anion of another

![Fig. 1. Release of 32P from [γ-32P]ATP by steady-state ATPase of Mg²⁺-polymerized glutathionyl-actin (2·10⁻⁶ m) at 37 °C in presence of various filament-stabilizing agents. [] 2 mM Tris-HCl (pH 7.8), 0.5 mM ATP; 2 mM NaN₃, 2 mM MgCl₂; O, with 4 mM K₂HPO₄ added; with 100 mM KCl added; with 100 mM KCl + 4 mM K₂HPO₄ added; with 1 eq. of phalloidin added.](http://www.jbc.org/)

![Fig. 2. Decrease of [γ-32P]ATP hydrolysis by steady-state ATPase of glutathionyl-F-actin (2·10⁻⁶ m) at 37 °C as dependent on concentrations of KCl (O), NaCl (O), and LiCl (A). The curves were derived by fitting the data to a binding isotherm for one single binding site.](http://www.jbc.org/)
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tribasic acid, arsenate. As already known, sulfate showed no stabilizing effect on actin filaments.

In the experiments with K$_2$HPO$_4$ and K$_2$HAsO$_4$, fitting the data to a simple binding isotherm was not possible (Fig. 5), probably due to the fact that both K$^+$ and Pi bind to the filament. Assuming that two distinct binding sites exist independently of each other, we used in these cases a binding isotherm with a second term as described under “Materials and Methods.” This allowed us to calculate both an apparent dissociation constant for Pi (K$^{app}_P$ = 0.16 mM) and an apparent dissociation constant for K$^+$ (K$^{app}_K$ = 27 mM), which corresponds, within the limits of error, to the values calculated from our measurements with KCl (K$^{app}_K$ = 27 mM) and K$_2$SO$_4$ (K$^{app}_K$ = 31 mM). From a plot corrected for the stabilization effect due to K$^+$ ions introduced together with Pi, the added concentration of Pi was plotted rather than free Pi, which will be lower due to complexation with Mg$^{2+}$. From the known Mg-Pi dissociation constant, this effect can be calculated to be negligible under our conditions (5).

Of the monovalent cations (Fig. 2), Li$^+$ possesses the highest affinity for F-actin (K$^{app}_K$ = 4.4 mM). The value for K$^+$ (K$^{app}_K$ = 27 mM) is in the same range as the apparent dissociation constants for K$^+$ binding to G-actin evaluated by others from fluorescence data (K$^{app}_K$ = 15 mM (15), 41 mM (16)). The stabilizing capacities of the three alkali ions are similar, showing saturation at ~5% residual ATPase activity. Fig. 2 further indicates that 100 mM KCl, the concentration commonly used in actin preparations, gives ~75% of the maximum stabilization effect attainable with this salt.

The filament-stabilizing effects of divalent cations have

**TABLE I**

Values of apparent dissociation constants and stabilizing capacity for various stabilizing agents, evaluated from inhibition of [Cys$^{374}$]glutathionyl-actin ATPase activity at 37°C and pH 7.8 as described under “Materials and Methods.”

<table>
<thead>
<tr>
<th>Added agent</th>
<th>Proposed stabilizer</th>
<th>K$^{app}_P$ (mM)</th>
<th>C$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$SO$_4$</td>
<td>K$^+$</td>
<td>31 ± 5</td>
<td>92 ± 3</td>
</tr>
<tr>
<td></td>
<td>SO$_4^-$</td>
<td>≈ 0</td>
<td></td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>K$^+$</td>
<td>43 ± 12</td>
<td>93 ± 3</td>
</tr>
<tr>
<td></td>
<td>HPO$_4^-$</td>
<td>0.16 ± 0.04</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>K$_2$HAsO$_4$</td>
<td>HAsO$_4^-$</td>
<td>0.20 ± 0.07</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>KCl</td>
<td>K$^+$</td>
<td>27 ± 3</td>
<td>95 ± 3</td>
</tr>
<tr>
<td></td>
<td>Cl$^-$</td>
<td>≈ 0</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>Na$^+$</td>
<td>21 ± 2</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>LiCl</td>
<td>Li$^+$</td>
<td>4.4 ± 0.7</td>
<td>96 ± 1</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Ca$^{2+}$</td>
<td>0.13 ± 0.07</td>
<td>74 ± 3</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>Mg$^{2+}$</td>
<td>1.4 ± 0.3</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>Phalloidin</td>
<td>ND</td>
<td>100 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

* C$^*$, stabilizing capacity (maximum attainable ATPase inhibition).
* Total stabilizing capacity (both sites occupied).
* ND, not determined.
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been studied by incubating G-actin with EGTA and subsequent polymerization with KCl to obtain F-actin free from divalent cations. Strong stabilizing effects of up to 98% inhibition of steady-state ATPase for Ca\(^{2+}\) and 78% inhibition for Mg\(^{2+}\) were found. Curve fitting required the assumption of two binding sites for divalent cations, as previously reported (15, 17), yielding apparent dissociation constants for a moderate affinity site with a $K_D$ of ~0.1 mM and a low affinity site with a $K'_D$ of ~2 mM (Fig. 3 and Table I). The values for the low affinity sites were determined by another experiment in which CaCl\(_2\) and MgCl\(_2\) were added to Mg\(^{2+}\)-polymerized actin. In this case, the moderate affinity sites are already occupied by Mg\(^{2+}\) ions, and the stabilizing effect induced by binding to only low affinity sites was observable. The values obtained were very similar to those of the first experiment (data not shown).

The most potent filament-protecting agent known is phalloidin. We found that binding to 5% of all actin subunits is sufficient to achieve 50% inhibition of steady-state ATPase (Fig. 4). Thus, filament stabilization by phalloidin is greater than reported so far. In contrast to previous studies (4, 21), we detected a residual ATPase activity (~5%) in the presence of an equimolar amount of phalloidin that only disappeared on the addition of excess phalloidin.

**DISCUSSION**

Solutions of F-actin containing ATP exhibit a low ATP-hydrolyzing activity known as steady-state ATPase (18). Recent work on the molecular mechanism of actin polymerization relates this ATPase activity to a dissociation of ADP-bearing subunits from filament ends, followed by an ADP/ATP exchange and the release of P$_i$, on repolymerization (for review, see Refs. 19 and 20). In addition, it has been suggested that nucleotide exchange, caused by a kind of "loosening" of filament structure under mechanical stress, can also occur in integrated actin subunits (21). When filaments are exposed to shear stress, as, for example, by ultrasonication (4, 22) or heating (23), ATPase activity is enhanced. This is a consequence of an increase in the number of filament ends due to an enhanced fragmentation rate (24). Since fragmentation depends on filament stability, the amount of ATP hydrolysis can be used as a measure of filament stabilization, although a stoichiometric dependence of $P_i$ release on filament breakage has not been reported as yet.

Steady-state ATPase activity of normal Mg\(^{2+}\)-polymerized actin at room temperature is relatively low (in 0.5 mM ATP: ~0.2 nol of P$_i$/nol of actin/h). Thus, studying the effects of filament-stabilizing agents on ATPase activity of unsubstituted F-actin would require unfavorably long measuring times (6) or the enhancement of ATPase activity by ultrasonication (10), a procedure that may cause problems of standardization. We show here that substitution of Cys\(^{374}\) with a bulky and negatively charged residue causes destabilization of actin filaments and a corresponding enhancement of the steady-state ATPase activity. Through this destabilization, one can obtain (without any extrinsic mechanical stress) significant and reproducible values for ATP hydrolysis within 1 h or less. Corresponding experiments with unsubstituted F-actin showed that, for each of the protecting agents studied here, the values of $P_i$ production paralleled those obtained with glutathionyl-actin, but were lower, requiring extended measuring times and showing considerable scatter (data not shown). This observation confirms that the conclusions drawn from experiments with the modified protein will apply to unsubstituted actin as well.

Hitherto, studies on filament stabilization have in most cases used critical concentration data as determined from the enhanced fluorescence of [Cys\(^{374}\)]pyrene-labeled actin upon polymerization (25). Results obtained with this method. for example, for the action of KCl and LiCl (9) or P$_i$, (8) were in principle confirmed by this study; but our results suggest that measurement of enhanced steady-state ATPase activity may be advantageous in some cases. For example, the assay does not interfere with the stabilizing effect of ATP itself, which, due to the formation of an "ATP cap" at least at one of the filament ends (26), prevents the observation of a significant stabilization effect by P$_i$, on unperturbed F-actin. In agreement with this, the effect of P$_i$, on the critical concentration of ATP-G actin was found to be small and had to be enhanced by ultrasonication or dilution-induced depolymerization (8). Whereas most of the results obtained by ATPase measurements were in good agreement with those of other methods, we were able to show that the maximum stabilization effect of P$_i$, is already reached at ~4 mM as compared to 20 mM K$_2$HPO$_4$ reported by Rickard and Skaterline (8).

As seen in Fig. 5, concentrations introduced together with P$_i$, contribute considerably to stabilization, particularly at higher concentrations. After correction for this effect, we found that maximum inhibition of ATPase activity by P$_i$, amounts to ~45%, which is in line with the finding of Carlier and Panta-loni (6), who reported a 35% more effective ATPase inhibition by 75 mM Na$_2$HPO$_4$ as compared to 75 mM Na$_2$SO$_4$. Previously determined dissociation constants for P$_i$, are 1 or 2 orders of magnitude higher than those based on ATPase measurements, suggesting that ATPase inhibition by P$_i$, is not a linear function of P$_i$, binding. In addition, it is worth noting that P$_i$, does not inhibit the single turnover ATP hydrolysis on F-actin, as was shown by Rickard and Sheterline (5).

The sensitivity of the enhanced ATPase assay system also allowed us to determine filament stabilization by a combination of stabilizing agents. Such experiments showed, for example, that the protecting activities of KCl and P$_i$, are additive. This result argues for different and independent modes of interaction of the two agents. Since the combination of P$_i$ and KCl is nearly as effective in protecting filaments as is phalloidin and the concentrations of ATP, P$_i$, and KCI assayed here are close to physiological, actin filaments in myofibrils may be nearly as stable as when complexed with phalloidin. We believe that this high degree of stabilization may be significant for the function of thin filaments under forces involved in muscle contraction.

This study also confirmed previous observations that phal-loidin is the most potent stabilizing agent known so far. A high degree of stabilization (~50%) is achieved when only 1 in ~20 subunits is complexed with the toxin. This argues for a much higher degree of cooperativity than believed so far (4). Our experiments did not allow us to test exactly for the stoichiometry of the phalloidin-actin complexation. Whereas a close to maximum stabilization was achieved by 0.5 eq of phalloidin, the addition of a further 0.5 eq, or excess of phalloidin, enhanced stability only by a few percent, although significantly. Therefore, the formation of a 1:1 complex, as postulated by our binding studies with [H]$\Delta$demethylphal-loidin (28), is possible. The high cooperativity of the effect is under further investigation.

In conclusion, we believe that the enhanced ATPase activity associated with the low mechanical stability of glutathionyl-actin provides a convenient tool for detecting and quantifying the effectiveness of actin filament-stabilizing agents and for investigating their mechanisms of action.
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