The dipyridyls paraquat and diquat attenuate the interaction of G-actin with thymosin β_4

Thomas Huff[a], Graziella Cappelletti[b], Ewald Hannappel[a,*]

[a] *Institut für Biochemie, Medizinische Fakultät, Universität Erlangen-Nürnberg, Fahrstr. 17, D-91054 Erlangen, Germany*
[b] *Dipartimento di Biologia, Universita degli Studi di Milano, Via Celoria 26, I-20133 Milan, Italy*

Received 21 February 1998

Abstract **β-Thymosins sequester G-actin and preserve a pool of monomers of actin which constitute an important prerequisite for cellular function of the microfilament system. To study the influence of paraquat binding to G-actin on the interaction of G-actin with thymosin β_4 we determined the apparent dissociation constant of the G-actin-thymosin β_4 complex in the absence or presence of paraquat using an ultrafiltration assay. Paraquat (1,1′-dimethyl-4,4′-dipyridylium dichloride) attenuates this interaction in a concentration- and time-dependent manner. When exposed to 10 mM paraquat, the apparent dissociation constant increased 10–85-fold within 15 min to 24 h. After incubation for 24 h even a paraquat concentration as low as 100 μM increased the dissociation constant of the G-actin-thymosin β_4 complex from 0.66 μM to 0.82 μM ($P < 0.05$). Diquat (1,1′-ethylene-2,2′-dipyridylium dibromide) similarly weakens the interaction of G-actin and β-thymosins. In none of the experiments was oxidation of the methionine residue or any other modification of thymosin β_4 detected. Therefore we conclude that the dipyridyls paraquat and diquat directly interact with G-actin and thereby impede the interaction between G-actin and thymosin β_4.**

Key words: β-Thymosin; Actin; Ultrafiltration; Dissociation constant; Dipyridyl

1. Introduction

Actin is present at high concentrations in virtually all types of eukaryotic cells. About half of the intracellular actin is stabilized in its monomeric form (G-actin) by interaction with sequestering factors [1]. This monomeric actin can be used for fast generation and elongation of actin filaments after an appropriate intra- or extracellular signal [2]. Thymosin β_4 is regarded as the main intracellular G-actin sequestering peptide in most mammalian cells [3–6] and forms a 1:1 complex with G-actin, thereby inhibiting salt induced polymerization to F-actin [7–11]. Increased concentrations of β-thymosins by transient transfection of the cDNA for thymosin β_4 or thymosin β_{10} similarly led to disassembly of actin stress fibers in CV1 fibroblast cells [12] and PtK2 cells [13]. In previous reports we determined the apparent dissociation constants ($K_{d,app}$) for the interaction of G-actin with several β-thymosins and the influence of N-terminal truncation or oxidation of the methionine residue of β-thymosins using either ultracentrifugation or ultrafiltration [9,14,15].

Paraquat is a widely used and extremely effective herbicide [16], which causes irreversible damage to the human lung [17]. It is thought to generate toxic oxygen radicals in vivo and has been used as a model to elucidate cell response to oxidative stress [18–20]. Cappelletti et al. found that actin filaments are markedly affected by the action of paraquat [21,22]. Later they could also show that paraquat has an influence on the state of actin polymerization in human alveolar cells [23]. Recently, Milzani et al. published data which indicate that paraquat directly interacts with G-actin and induces actin assembly at low salt conditions in vitro [24].

Here we report the influence of paraquat and diquat, another dipyridyl, on the interaction of purified G-actin with thymosin β_4 as determined by changes in dissociation constant at low salt conditions using an ultrafiltration assay.

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: trifluoroacetic acid (Uvasol) from Merck (Darmstadt); paraquat (methyl viologen; 1,1′-dimethyl-4,4′-dipyridylium dichloride) from Sigma, fluorescamine from Serva, diquat (1,1′-ethylene-2,2′-dipyridylium dibromide) from Riedel-de-Haen. Ultrafiltration tubes were purchased from Pall Filtron.

2.2. Proteins and peptides

Actin was prepared from bovine heart muscle by the method of Pardee and Spudich [25] and further purified by gel filtration [26] on a Sephacryl S300 column (Pharmacia) equilibrated with G-buffer (2 mM Tris, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.05% NaN_3, pH 8.0). G-actin was stored in G buffer at 0°C. Thymosin β_4 was isolated from bovine spleen as described [27]. The purity of the preparations was demonstrated by reverse phase HPLC. The concentrations of thymosin β_4 and actin were determined by amino acid analysis after acid hydrolysis (6 M HCl, 155°C, 1 h) and precolumn derivatization with *o*-phthaldialdehyde/3-mercaptopropionic acid [28].

2.3. HPLC

Chromatographic conditions were controlled by a Merck-Hitachi L-6200 system supplemented with a reaction pump for postcolumn derivatization (655A-13, Merck-Hitachi) and with a fluorometer (F-1050, Merck-Hitachi). The fluorescence signal was recorded on an integrator (D-2500, Merck-Hitachi) [29]. Analytical separations were carried out employing a linear gradient from 0.1% trifluoroacetic acid to 40% acetonitrile/0.1% trifluoroacetic acid within 30 min at a flow rate of 0.75 ml/min. Column: Beckman ODS Ultrasphere (5 μm, 4.6×250 mm); detection: postcolumn derivatization with fluorescamine.

2.4. Determination of dissociation constants by ultrafiltration

The apparent dissociation constant of the G-actin-thymosin β_4 complex was determined as described [15]. Briefly, a defined amount of the internal standard Phe-Ala was added to thymosin β_4 and diluted to a concentration of 60 μM. This solution was diluted 4-fold with either G buffer or a solution of G-actin (0.86 mg/ml). After incubation for 15 min at room temperature paraquat or diquat was added at different concentrations. The reaction mixtures were further incubated at room temperature for the time intervals indicated, transferred to the ultrafiltration tubes, and then centrifuged for 15 min at 2700×*g* in a table-top centrifuge (Eppendorf centrifuge 5417, Eppen-

*Corresponding author. Fax: (49) (9131) 852484.
E-mail: eh@biochem.uni-erlangen.de

PII S0014-5793(98)00295-6

dorf-Netheler-Hinz, Germany). 40 μl of the ultrafiltrates were analyzed by HPLC.

3. Results

3.1. Influence of paraquat on the dissociation constant at low salt conditions

To study the influence of paraquat on the interaction between G-actin and thymosin β_4 at low salt conditions, we determined the apparent dissociation constant ($K_{d,app}$) of the G-actin-thymosin β_4 complex in the absence or presence of different paraquat concentrations and after different time intervals using an ultrafiltration assay (Table 1). Due to the difference in molecular mass between thymosin β_4 (5 kDa) and its complex with actin (48 kDa), one can separate free thymosin β_4 from the G-actin-thymosin β_4 complex by means of ultrafiltration. The amount of free thymosin β_4 was determined by analyzing the ultrafiltrate by reverse phase HPLC. The amount of thymosin β_4 in the ultrafiltrate depends upon the presence of either actin alone or both actin and paraquat (Fig. 1). Panel A displays the chromatogram obtained when only Phe-Ala (internal standard, retention time 17.9 min) and 15 μM thymosin β_4 (retention time 27.4 min) are incubated. The drastic decrease of the peak area representing thymosin β_4 in the presence of equimolar amounts of G-actin is caused by the formation of the G-actin-thymosin β_4 complex (panel B). The gradual increase of this peak area in the presence of either 1 mM (panel C) or 10 mM paraquat (panel D) after a 2-h incubation period indicates a decrease in the amount of the G-actin-thymosin β_4 complex depending on the paraquat concentration. These chromatograms also demonstrate the absence of thymosin β_4-sulfoxide (the expected retention time is marked by an arrow) or other fluorescamine positive peptides. Therefore we concluded that paraquat does not oxidize thymosin β_4 under the conditions employed. Likewise we observed no oxidation of thymosin β_4 during the incubation of human alveolar cells A549 in the presence of paraquat. In the absence of paraquat the apparent dissociation constant of the G-actin-thymosin β_4 complex is not markedly changed during the prolonged incubation up to 24 h (1440 min) at room temperature (Table 1). The values observed after those times are comparable with our previously published data [14,15]. After a 15-min incubation period at paraquat concentrations between 0.5 and 10 mM a clear concentration-dependent increase (1.3–10-fold) in the dissociation constant was observed. Incubation for 2, 6 or 24 h led to a further increase of the dissociation constant (Fig. 2). After 24 h we detected a 2-fold increase in the dissociation constant in the presence of 0.25 mM paraquat. Even a paraquat concentration as low as 100 μM caused a significant increase ($P < 0.05$) from 0.66 μM in the absence of paraquat to 0.82 μM after a 24-h incubation. To exclude the possibility that the observed increase in the apparent dissociation constant was evoked by the chloride ions acting as counterion of the paraquat cation, we determined the apparent dissociation constant in the presence of 20 mM NaCl without paraquat. After a 24-h incubation period under these conditions the apparent dissociation constant increased only 2-fold ($K_{d,app} = 1.3 \pm 0.1$ μM; $n = 4$) compared with a 90-fold increase in the presence of 10 mM paraquat ($K_{d,app} = 56.3 \pm 5.9$ μM, $n = 3$).

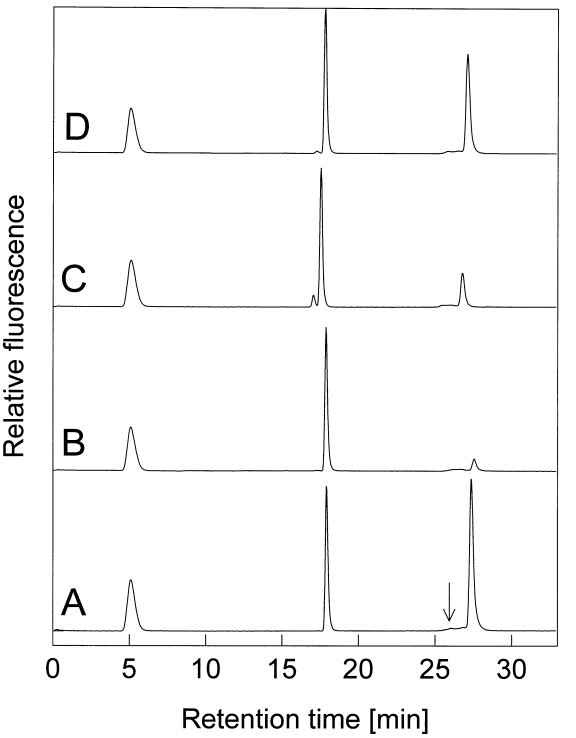

Fig. 1. Influence of actin and paraquat on the thymosin β_4 amount in the ultrafiltrate. 40 μl of the ultrafiltrate obtained after incubation of internal standard and thymosin β_4 (15 μM) were analyzed by HPLC. Incubation (2 h) (A) without actin and paraquat; (B) in the presence of 15 μM actin; (C) in the presence of 15 μM actin and 1 mM paraquat; (D) in the presence of 15 μM actin and 10 mM paraquat. Chromatographic conditions are as described in Section 2. Retention times (min): Phe-Ala (17.9); thymosin β_4 (27.4). The arrow indicates the retention time of oxidized thymosin β_4.

Table 1
Influence of different paraquat concentrations and incubation times on the apparent dissociation constant of the actin-thymosin β_4 interaction

Paraquat (mM)	Incubation time (min)			
	15	120	360	1440
0	0.55 ± 0.04 (6)	0.63 ± 0.07 (9)	0.72 ± 0.09 (3)	0.66 ± 0.10 (8)
0.1	n.d.[a]	0.69 ± 0.03 (3)	n.d.	0.82 ± 0.04 (3)
0.25	0.66 ± 0.04 (3)	0.77 ± 0.08 (3)	n.d.	1.38 ± 0.14 (3)
0.5	0.73 ± 0.04 (3)	1.15 ± 0.07 (6)	1.62 ± 0.11 (6)	1.36 ± 0.18 (3)
1.0	1.34 ± 0.16 (3)	1.91 ± 0.21 (9)	n.d.	2.47 ± 0.48 (6)
10	5.59 ± 1.25 (5)	20.3 ± 3.7 (5)	33.2 ± 5.0 (3)	56.3 ± 5.9 (3)

The apparent dissociation constants ($K_{d,app}$, μM) for the interaction between G-actin and thymosin β_4 were determined by ultrafiltration assays after different incubation times at 25°C in the presence or absence of indicated paraquat concentrations. Values given are means ± S.D. and number of independent experiments in parentheses. For calculation of the apparent dissociation constants see [14,15].
[a]Not determined.

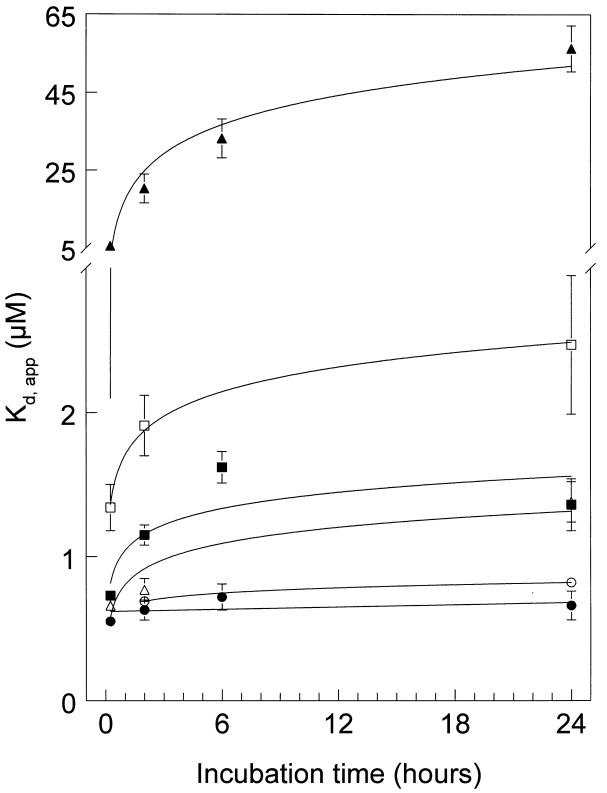

Fig. 2. Time-dependent influence of increasing paraquat concentrations on the apparent dissociation constant. ●, control; ○, 100 μM; △, 250 μM; ■, 500 μM; □, 1 mM; ▲, 10 mM paraquat.

3.2. Influence of diquat on the dissociation constant at low salt conditions

To find out if the G-actin-thymosin β_4 interaction was also attenuated by other dipyridyls, we performed similar incubations using diquat, another well established dipyridyl pesticide. Diquat attenuated the interaction between G-actin and thymosin β_4 comparably to paraquat (Table 2). The presence of 1 mM diquat caused a 1.7- or 3-fold increase after 15 min or 24 h, respectively. After a 24-h incubation a roughly 80-fold increase was observed with 10 mM diquat.

4. Discussion

The pool of monomeric actin inside cells is important for the function of the cytoskeletal system, which plays a significant role in the response to intra- or extracellular signals [2]. It is stabilized by the interaction of actin monomers with sequestering proteins like β-thymosins [7,8,10]. In most mammalian cells thymosin β_4 was found to be the main G-actin sequestering peptide. The dissociation constant for the stoichiometric complex was determined by various groups using different methods to be in the range of 0.5–2.0 μM [9,11,14,15,30–33]. This value is well within the range of the critical concentration for actin polymerization, i.e. 0.1 μM for the pointed end and 0.7–1.2 μM for the barbed end [30,34,35]. This indicates that a small attenuation of the stability of the G-actin-thymosin β_4 complex ($K_{d,app}$) leads to a disturbance of the monomeric actin pool thus interfering with the equilibrium of G- and F-actin. Our results show that in vitro paraquat lowers the affinity of G-actin for thymosin β_4 in a concentration- and time-dependent manner.

Table 2
Influence of different diquat concentrations and incubation times on the apparent dissociation constant of the actin-thymosin β_4 interaction

Diquat (mM)	Incubation time (min)	
	15	1440
0	0.60 ± 0.05 (3)	0.62 ± 0.07 (3)
1	1.04 ± 0.09 (3)	1.83 ± 0.36 (3)
10	4.45 ± 0.98 (3)	47.3 ± 4.2 (3)

For legend see Table 1.

The cytotoxicity of paraquat and diquat has been attributed in most publications to their action on cell redox systems by the generation of reactive oxygen species [18–20,36–38]. On the other hand, there are also reports about the influence of paraquat on the cytoskeletal system. Li et al. [39] and Cappelletti et al. [21,22] reported on the disruption of actin filaments in cells exposed to paraquat. Later, Cappelletti et al. [23] suggested that paraquat does not act by depolymerizing actin filaments, but rather by increasing intracellular F-actin content. Because we had previously shown [9,15] that the methionine group of thymosin β_4 can be oxidized by millimolar H_2O_2 concentrations and that this oxidation decreases its affinity to G-actin about 20-fold, we speculated that paraquat may act by oxidation of thymosin β_4. However, our experiments show that in vitro there is no oxidation of thymosin β_4 even after incubation with 10 mM paraquat for 24 h. Recently, Milzani et al. [24] reported a direct interaction of paraquat with G-actin and its ability to induce actin polymerization at low salt conditions. From their report and our results it can be concluded that one mechanism of paraquat cytotoxicity is based on its direct interaction with G-actin. Actin in turn is prevented from interacting properly with thymosin β_4 resulting in a decrease in the monomeric actin pool and a shift in the G-actin/F-actin ratio. Thus the dipyridyls paraquat and diquat have profound influence on a cell parameter important for the dynamic behavior of the microfilament system.

Acknowledgements: We thank Doris Jaegers for her technical assistance and Dr. Rainer Bläsius for critically reading the manuscript.

References

[1] Pollard, T.D. and Cooper, J.A. (1986) Annu. Rev. Biochem. 55, 987–1035.
[2] Carlier, M.F. and Pantaloni, D. (1994) Semin. Cell Biol. 5, 183–191.
[3] Hannappel, E., Xu, G.-J., Morgan, J., Hempstead, J. and Horecker, B.L. (1982) Proc. Natl. Acad. Sci. USA 79, 2172–2175.
[4] Xu, G.J., Hannappel, E., Morgan, J., Hempstead, J. and Horecker, B.L. (1982) Proc. Natl. Acad. Sci. USA 79, 4006–4009.
[5] Hannappel, E. and Leibold, W. (1985) Arch. Biochem. Biophys. 240, 236–241.
[6] Hannappel, E. and van Kampen, M. (1987) J. Chromatogr. 397, 279–285.
[7] Safer, D. and Nachmias, V.T. (1994) BioEssays 16, 473–479.
[8] Hannappel, E. and Wartenberg, F. (1993) Biol. Chem. Hoppe-Seyler 374, 117–122.
[9] Huff, T., Zerzawy, D. and Hannappel, E. (1995) Eur. J. Biochem. 230, 650–657.
[10] Yu, F.-X., Lin, S.-C., Morrison-Bogorad, M., Atkinson, M.A.L. and Yin, H.L. (1993) J. Biol. Chem. 268, 502–509.
[11] Heintz, D., Reichert, A., Mihelic, M., Voelter, W. and Faulstich, H. (1993) FEBS Lett. 329, 9–12.

[12] Yu, F.X., Lin, S.C., Morrison-Bogorad, M. and Yin, H.L. (1994) Cell Motil. Cytoskel. 27, 13–25.
[13] Sanger, J.M., Golla, R., Safer, D., Choi, J.K., Yu, K.R., Sanger, J.W. and Nachmias, V.T. (1995) Cell Motil. Cytoskel. 31, 307–322.
[14] Huff, T., Müller, C.S.G. and Hannappel, E. (1997) FEBS Lett. 414, 39–44.
[15] Huff, T. and Hannappel, E. (1997) Anal. Chim. Acta 352, 249–255.
[16] Calderbank, A. (1968) Adv. Pesticide Contr. Res. 8, 127–235.
[17] Smith, P. and Heath, D. (1976) CRC Crit. Rev. Toxicol. 4, 411–445.
[18] Speranza, M.J., Bagley, A.C. and Lynch, R.E. (1993) J. Biol. Chem. 268, 19039–19043.
[19] Grune, T., Reinheckel, T., Joshi, M. and Davies, K.J. (1995) J. Biol. Chem. 270, 2344–2351.
[20] Kazzaz, J.A., Xu, J., Palaia, T.A., Mantell, L., Fein, A.M. and Horowitz, S. (1996) J. Biol. Chem. 271, 15182–15186.
[21] Cappelletti, G. and Maci, R. (1993) Bull. Environ. Contam. Toxicol. 50, 717–723.
[22] Cappelletti, G., Incani, C. and Maci, R. (1994) Cell Biol. Toxicol. 10, 255–263.
[23] Cappelletti, G., Maggioni, M.G., Carnevali, M.D.C., Bonasoro, F. and Maci, R. (1996) Eur. J. Cell Biol. 71, 293–302.
[24] Milzani, A., Dalledonne, I., Vailati, G. and Colombo, R. (1997) FASEB J. 11, 261–270.
[25] Pardee, J.D. and Spudich, J.A. (1982) Methods Enzymol. 85, 164–181.
[26] MacLean-Fletcher, S. and Pollard, T.D. (1980) Biochem. Biophys. Res. Commun. 96, 18–27.
[27] Hannappel, E., Wartenberg, F. and Bustelo, X.R. (1989) Arch. Biochem. Biophys. 273, 396–402.
[28] Hannappel, E., Kalbacher, H. and Voelter, W. (1988) Arch. Biochem. Biophys. 260, 546–551.
[29] Huff, T., Müller, C.S.G. and Hannappel, E. (1997) Anal. Chim. Acta 352, 239–248.
[30] Weber, A., Nachmias, V.T., Pring, M., Pennise, C.R. and Safer, D. (1992) Biochemistry 31, 6179–6185.
[31] Jean, C., Rieger, K., Blanchoin, L., Carlier, M.F., Lenfant, M. and Pantaloni, D. (1994) J. Muscle Res. Cell Motil. 15, 278–286.
[32] Carlier, M.-F., Jean, C., Rieger, K.J., Lenfant, M. and Pantaloni, D. (1993) Proc. Natl. Acad. Sci. USA 90, 5034–5038.
[33] Heintz, D., Reichert, A., Mihelic-Rapp, M., Stoeva, S., Voelter, W. and Faulstich, H. (1994) Eur. J. Biochem. 223, 345–350.
[34] Wegner, A. and Isenberg, G. (1983) Proc. Natl. Acad. Sci. USA 80, 4922–4925.
[35] Pantaloni, D. and Carlier, M.-F. (1993) Cell 75, 1007–1014.
[36] Bus, J.S., Aust, S.D. and Gibson, J.E. (1974) Biochem. Biophys. Res. Commun. 58, 749–755.
[37] Bus, J.S. and Gibson, J.E. (1984) Environ. Health Perspect. 55, 37–46.
[38] Baldwin, R.C., Pasi, A., MacGregor, J.T. and Hine, C.H. (1975) Toxicol. Appl. Pharmacol. 32, 298–304.
[39] Li, W.D., Zhao, Y.Z. and Chou, I.N. (1987) Toxicol. Appl. Pharmacol. 91, 96–106.