Volume 303, number 1, 59–63 FEBS 11061 May 1992

Site-specific amino-terminal mutants of yeast-expressed β-actin

Characterization of the interaction with myosin and tropomyosin

Pontus Aspenström[a], Uno Lindberg[b] and Roger Karlsson[a]

[a]*Dept. Developmental Biology, Biomedical Center, Box 587, Uppsala University, S-751 23 Uppsala, Sweden* and [b]*Dept. Zoological Cellbiology, WGI, Arrhenius Laboratories E5, University of Stockholm, S-106 91 Stockholm, Sweden*

Received 19 March 1992

Neutral or charge-shifting mutagenesis of β-actin at positions 3 and 4 strongly influenced the actomyosin interaction under non-rigor conditions. The polymerization behaviour and tropomyosin binding properties on the other hand remained unaffected.

Yeast; Heterologous expression; Actin mutant; Myosin interaction

1. INTRODUCTION

The actomyosin interaction generates the force that causes contraction in muscle cells and various forms of motility in non-muscle cells. The molecular mechanisms involved in this chemo-mechanical transduction are therefore of great interest. Recent progress in determining the 3D-structure of actin and myosin, advances in molecular genetics, and development of methods to manipulate the components of the actomyosin system at the molecular level have brought the analysis of energy transduction in the actomyosin system to a new stage [1–3]. Some of the results already attained have raised serious questions concerning the validity of the classical cross-bridge model of muscle contraction [4–9], and the possibility of an alternative mechanism based on structural transformations in the actin filaments was recently suggested [10]. With the improved techniques to produce mutated actin in amounts large enough for biochemical experiments [11], it should be possible to put the new model to critical tests.

The amino terminus of the actin molecule appears to be important for the interaction with several actin binding proteins including myosin [12–17]. In addition, myosin has been observed to interact with a carboxy-terminal segment of actin located in the proximity of the amino terminus in subdomain 1 of the actin:DNase I structure [12–18]. The location of an actomyosin interface to subdomain 1 in the actin molecule thus appears well established and seems to involve four segments of the actin sequence formed by residues 1–7, 18–28, 95–113, and 361–365 [12,18–27]. It is unclear though, which role each of these segments plays in the formation and functioning of the actomyosin complex.

In a previous report we showed that a double mutation at positions 3 and 4 of β-actin replacing the wild type aspartic acid residues with lysines (D3K,D4K) abolished the interaction of the mutant filaments with myosin subfragment 1 (S1), while a double deletion or a double replacement with alanines in the same positions (D3Δ,D4Δ and D3A,D4A respectively) only marginally affected the S1 binding [11]. Here we extend the analysis of these β-actin mutants to include their polymerizing behaviour and myosin interaction as seen by viscosity analysis, and by the S1-ATPase and myosin 'motor assays' respectively. In addition, the interaction of these mutants with skeletal muscle tropomyosin was studied.

2. MATERIALS AND METHODS

Adenosine triphosphate (ATP), dithiothreitol (DTT) and bovine pancreatic deoxyribonuclease I (DNAse I, EC 3.1.21.1) were from Boehringer, Mannheim. Hydroxyapatite (Hypatite C, lot No. 6654) was from Clarkson Chemical Company, Williamsport, PA, USA, and CNBr activated Sepharose 4B (Pharmacia-LKB, Sweden) was coupled with 500 mg DNAse I according to the instructions provided by the manufacturer. The volume of the DNase I affinity column was 80 ml.

Manipulations of DNA followed standard protocols [28]. The expression and preparation of the wild-type and mutant β-actins were performed as previously reported [11,29]. Briefly, an inoculum of *S. cerevisiae* K923 (*HMLα*, mat::*LEU2*$^+$, *hmr*::*TRP1*$^+$, *ura3*, *ade2*, *sir3*ts, MATa at 23°C, MATα at 34°C), transformed with the expression plasmid carrying either the wild-type or a mutant β-actin gene, was grown overnight at 34°C in uracil-free medium to select for plasmid retention. The culture was then diluted 1/100 into 1 liter of medium, grown for another overnight period at 34°C, and then diluted into 10 liters of yeast rich medium (YEPD) in a fermentor (Belach AB, Swe-

Correspondence address: R. Karlsson, Dept. Developmental Biology, Biomedical Center, Box 587, Uppsala University, S-751 23 Uppsala, Sweden. Fax: (46) (18) 559017.

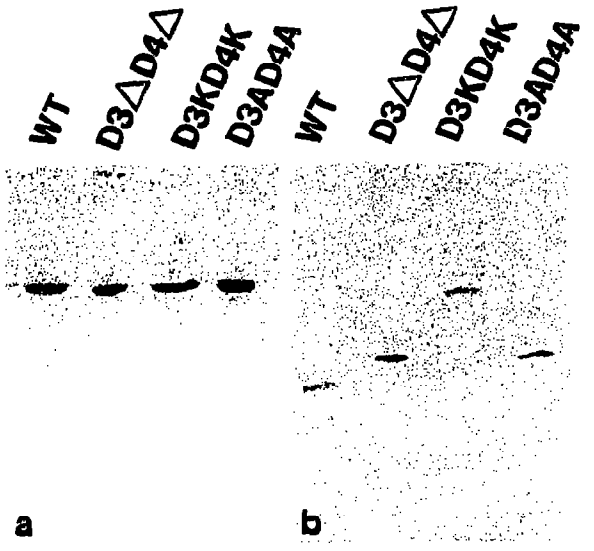

Fig. 1. The homogeneity of the yeast expressed actin after DNase I affinity and hydroxyapatite chromatography. Panel (a) shows SDS-PAGE and panel (b) isoelectric focusing analysis of the wild-type and mutant actins. Wild-type β-actin is denoted WT while the mutants are labeled as in the text. The IEF gel is oriented with the alkaline side facing the top of the figure.

den). Expression of the heterologous actin was induced by shifting the temperature to 23°C at the beginning of the log phase. The cells were collected by centrifugation after 18–20 h at the end of the log phase and were stored at −70°C. After suspension in 5 mM Tris-HCl, pH 7.6, 0.1 mM $CaCl_2$, 0.5 mM ATP, 1 mM NaN_3, containing protease inhibitors as described earlier [11], the cells were disintegrated in a beadmill (Innomed-Konsult, Täby, Sweden). After the addition of RNAse A and DTT to the final concentrations of 10 μg/ml and 0.5 mM respectively, the resulting lysate was clarified by centrifugation and applied to the DNAse I–Sepharose column which was equilibrated with 5 mM Tris-HCl, pH 7.6, 0.1 mM $CaCl_2$, 0.5 mM ATP, 0.5 mM DTT, 1 mM NaN_3 (G-buffer). After washing, total actin was eluted with 40% (v/v) formamide and 10% (v/v) glycerol in G-buffer, concentrated on an Amicon YM-10 membrane and applied to a hydroxyapatite column (diameter; 1×23 cm) equilibrated with 5 mM KPO_4, pH 7.6, 0.5 mM DTT. The β-actin was separated from the endogenous yeast actin by elution with a linear gradient formed by 60 ml of 5 mM KPO_4, pH 7.6, 0.5 mM DTT, and 60 ml of 40 mM KPO_4, pH 7.6, 1.5 mM glycine, 0.5 mM DTT. Each fraction (1.2 ml) was collected into tubes containing 60 μl of 10 mM ATP. Fractions containing β-actin were combined and dialyzed overnight against G-buffer.

Myosin subfragment (S1) was prepared by chymotryptic cleavage of rabbit skeletal muscle myosin [30] and stored at −20°C in a buffer containing 5 mM imidazole, pH 7.0, 15 mM NaCl, 0.1 mM PMSF and 50% glycerol. Rabbit skeletal muscle tropomyosin was purified according to Smillie [31] and stored at −20°C.

Protein concentrations were determined by the method of Bradford [32] or spectrophotometrically at 290 nm for actin using an extinction coefficient of 0.63, and at 280 nm for S1 and tropomyosin using extinction coefficients of 0.75 and 0.3 respectively.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 10% acrylamide was performed according to Laemmli [33], or in the case of the tropomyosin assay on 15% acrylamide minigels as described by Matsudaira and Burgess [34]. Flat bed isoelectric gels with a pH range of 5.0–7.0 were run as previously described [11].

The viscosity analysis was performed at 25°C using Cannon-Manning viscometers and 0.7 ml samples containing 0.5 mg/ml actin. The actin-activated S1 ATPase activity was determined at 25°C in G-buffer containing 2 mM $MgCl_2$, 25 μg/ml S1, and varying concentrations of filamentous actin in a total volume of 0.5 ml to which 1 μCi of [γ-^{32}P]ATP was added. Aliquots of 100 μl were withdrawn at 5 min intervals and hydrolyzed phosphate was determined by treating samples according to Seals et al. [35] for scintillation counting.

For the tropomyosin co-sedimentation assay, samples of tropomyosin were mixed in G buffer containing 2 mM $MgCl_2$ with actin in increasing amounts resulting in tropomyosin/actin ratios of 1:1.8, 1:7 and 1:28. After incubation for 20 min at room temperature, the samples were centrifuged at 30 psi for 20 min in a Beckman airfuge. The resulting pellets were washed once in G-buffer containing 2 mM $MgCl_2$, re-centrifuged and the two supernatants were combined and subjected to SDS-PAGE as was the pelleted material.

The motility assay was performed as described by Kron and Spudich [36] using rabbit skeletal muscle myosin 100–300 μg/ml in 25 mM imidazole, pH 7.0, 25 mM KCl, 5 mM $MgCl_2$, 100 mM DTT (assay-buffer) and wild type and mutant actin labeled with tetramethylrhodamine phalloidin (Molecular Probes, Oregon, USA). After addition of assay buffer containing 1 mM ATP, filament movement was followed using a Zeiss photomicroscope III equipped for epifluorescence, and with a CCD camera (C2400), an image intensifier and a processor (Argus-10) from Hamamatsu Photonics, and a super-VHS video from Panasonic. For velocity determinations of video recorded filaments the video signal was time-base corrected with a 780 Synchronizer-TBC (BLT). The velocity of an individual filament was determined from the video recordings by 4–5 measurements of the distance translocated per unit time when performing a smooth movement.

3. RESULTS

Yeast-expressed β-actin can be isolated by a combination of DNase I affinity and hydroxyapatite chromatography. The SDS-PAGE and IEF analysis of the product is seen in Fig. 1. The charge-changing mutations introduced at the actin amino terminus (D3Δ,D4Δ, D3K,D4K and D3A,D4A actin) led as expected to

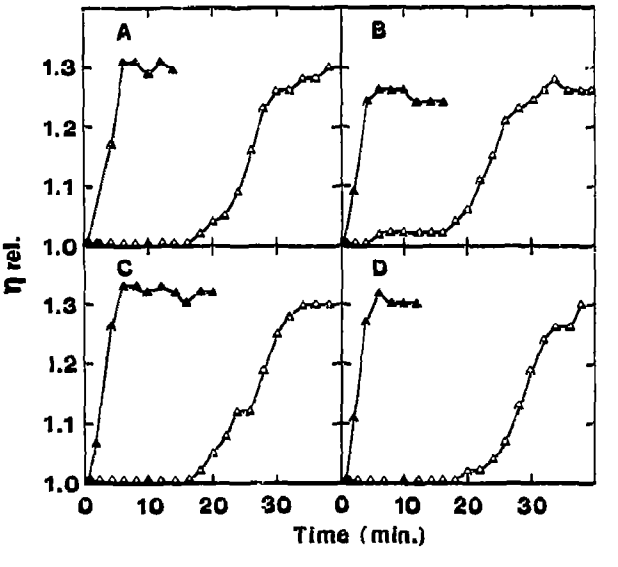

Fig. 2. Viscosity analysis of wild-type and mutant β-actins. Polymerization was induced by addition of either 2 mM $MgCl_2$ (▲) or 0.1 M KCl (△) to 0.5 mg/ml of actin in G-buffer at 25°C. The viscosity was measured at 2 min intervals. Note that the *D3Δ,D4Δ* mutant actin reaches a somewhat lower steady-state viscosity compared to the other actins.

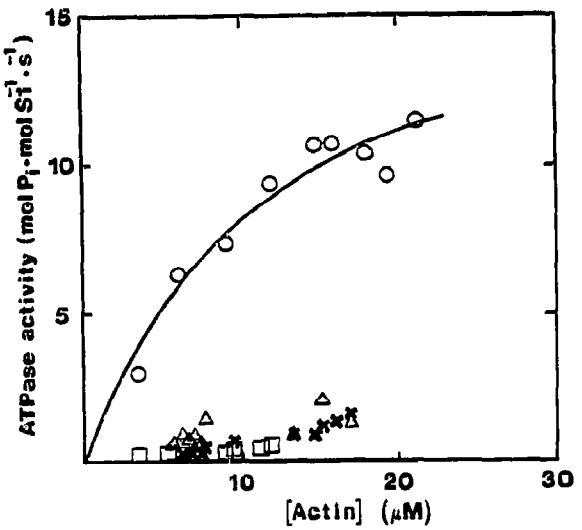

Fig. 3. The ATPase activity generated by acto-S1 complexes. The assay was performed as described in Section 2 incubating 25 μg/ml S1 with either wild-type or mutant actin filaments at 25°C. Wild-type actin is denoted by (○), D3Δ,D4Δ actin by (△), D3K,D4K actin by (□), and D3A,D4A actin by (×). The ATPase activity of S1 alone was 0.1 mol P_i/mol S1 × s.

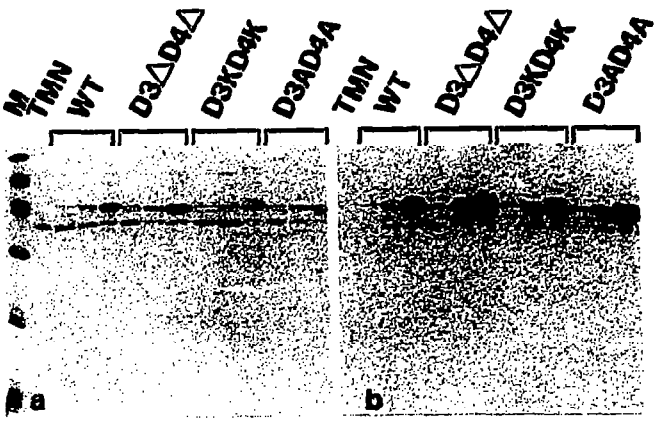

Fig. 4. The actin–tropomysin interaction as probed by a co-sedimentation assay and subsequent SDS-PAGE analysis. Tropomyosin and each of the actins were mixed at three different ratios and centrifuged. The resulting supernatants and pellets after SDS-PAGE are shown in panels (a) and (b), respectively. Lanes with wild-type or mutant actins are labeled as in Fig. 1, and the tropomyosin/actin ratio in the incubation mixtures analysed in each lane in the triplets indicated was from left to right: 1:1.8, 1:7, 1:28. TMN is a control sample treated as the others except that actin was not added, and M shows molecular weight markers (from top to bottom 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa and 14.4 kDa). Note that the amount of tropomyosin decreases in the supernatants while it increases in the corresponding pellets as the amount of added actin is increased.

shifts in the position of β-actin towards the alkaline side of the pH gradient when analyzed by IEF (Fig. 1b), and the mutations also shifted the elution position from the hydroxyapatite column relative to that of endogenous yeast actin [11].

The polymerizing behaviour of the three mutants in the presence of 2 mM $MgCl_2$ or 0.1 M KCl was indistinguishable from that of the wild-type actin except for the D3Δ,D4Δ mutant which showed a slightly reduced steady-state level of relative viscosity, Fig. 2. The critical concentration of actin polymerization (A_{cc}) for the wild type β-actin under these two salt conditions was determined at 0.02 mg/ml in the presence of Mg^{2+} ions (A_{cc}(Mg)) and 0.08 mg/ml in the presence of K^+ ions (A_{cc}(K)). The corresponding values for D3Δ,D4Δ actin were 0.06 mg/ml and 0.12 mg/ml respectively, while those of D3K,D4K actin and D3A,D4A actin did not differ significantly from the wild-type values as illustrated by the viscosity curves in Fig. 2. Thus positions 3 and 4 appear to be of little importance for the formation and stability of actin filaments, though the increased A_{cc} values obtained with D3Δ,D4Δ actin indicate a slightly reduced polymerizability due to this mutation. The reason for an interference with the polymerization in this case is unclear, especially when considering the wild-type behaviour of the other two terminal mutants.

Table I

The average sliding velocity and fraction of motile filaments of the different actins as recorded by the myosin 'motor' assay

Actin	Sliding Velocity (μm/s)	Fraction of motile filaments
Wild type	2.7, s = 0.4 (n = 34)	51% (n = 164)
D3Δ,D4Δ actin	1.7, s = 0.4 (n = 13)	17% (n = 393)
D3K,D4K actin	0	0% (n = 316)
D3A,D4A actin	1.5, s = 0.4 (n = 13)	6% (n = 326)

The standard deviation and the numbers of filaments traced for each actin are indicated by s and n, respectively.

Myosin subfragment 1 (S1) decoration recently showed that D3K,D4K actin filaments have almost completely lost their ability to bind S1 while the D3Δ,D4Δ and D3A,D4A mutant actins bound S1 although with a somewhat distorted arrowhead pattern [11]. However, it became obvious that also these latter mutations dramatically influenced the chemo-mechanical transduction process by reducing the hydrolysis of ATP to a similar level as that seen with D3K,D4K actin which in turn had an activity close to the ATPase activity seen with S1 alone (Fig. 3). These results demonstrated a pronounced influence of residues 3 and 4 in actin on the actomyosin interaction under non-rigor conditions (in the presence of ATP). The 'motor assay' supported these observations showing that the introduction of the D3Δ,D4Δ and D3A,D4A mutations decreased the average wild-type velocity by 37% and 44%, respectively. In addition the number of moving filaments was reduced (Table I). In the case of the D3K,D4K actin filaments, no motility was observed, and in one experiment the filaments did not adhere to the myosin-coated glass surface.

The amino-terminal mutants were also screened for their tropomyosin binding properties in a sedimentation assay analyzing the supernatants and pellets by SDS-PAGE (Fig. 4). In the absence of filamentous actin, tropomyosin remained in the supernatant but with increasing concentrations of actin filaments, increasing amounts of tropomyosin were observed in the pelleted material indicating the formation of actin–tropomyosin complexes. All three mutants showed a behaviour indistinguishable from that of the wild type demonstrating that within the limitations of the assay these mutations did not influence the tropomyosin binding properties of actin. This result agrees with the proposed location of the tropomyosin binding site in actin subdomains 3 and 4 [25].

4. DISCUSSION

Of the regions in the actin molecule that have been suggested to interact with myosin, the amino-terminal sequences have attracted the largest attention. Despite the crosslinking of the myosin heavy chain to residues 1–12 in actin [12], studies employing antibodies which discriminate between the amino- and carboxy-terminal parts of the actin sequence 1–28 have located the myosin association to be close to the carboxy-terminal end of this segment [19,20,24]. However, recent studies of the acto–S1 interaction, again with antibodies directed against residues 1–7 in actin, led to the conclusion that this part of actin contributes to the formation of the actomyosin complex in the presence of ATP [23,27]. Hence residues 1–28 in actin may form at least two different myosin contacts as has also been suggested in a more recent crosslinking study [22].

As described earlier [11] deletion of the aspartic acid residues at positions 3 and 4 in the actin amino terminus or their replacements with alanines caused recognizable changes in the S1-decoration pattern of these mutated actin filaments. Filaments containing the deletion mutant bound S1 with irregular spacings, and filaments with alanine at these positions formed complexes with a more blunt angle between the S1 and the filament. Here these mutations are shown to strongly impair both the acto-S1 ATPase activity and translocational activities in the motor assay. This strongly supports the contention that the amino terminus of actin is an important element in the interaction between actin and myosin during chemo-mechanical transduction. This view is further strengthened by the results obtained with the D3K,D4K mutant actin, which clearly demonstrate that a complete charge shift in the amino terminus abolishes the actomyosin interaction. It is noteworthy that this mutant actin polymerizes with the characteristics of wild-type actin suggesting that this part of the molecule is non-influential in the formation and stability of the actin filament.

Sutoh et al. [37] in a recent study of amino-terminal actin mutants expressed in *Dictyostelium discoideum* reached similar conclusions with respect to the importance of the amino terminus for the actomyosin interaction as those presented here. In contrast, actin genes carrying mutations which partly or fully neutralized the negative charge at the amino terminus did not visibly alter the phenotype of the yeast *S. cerevisiae* [38]. These findings are difficult to reconcile, but may be related to the fact that *S. cerevisiae* like other eukaryotes, has at least two different myosins at its disposal, myosin I ('minimyosin') and myosin II (classical musle myosin) encoded by the *MYO2* and *MYO1* genes respectively [39,40]. The 'minimyosin' is essential for yeast viability [39] while the myosin II is non-essential although cells lacking this protein express an altered budding pattern and a distorted cell wall deposition [40,41]. The unaltered phenotype of yeast cells with the mutated endogenous actin mentioned above therefore seems to suggest that the mutated actin can be utilized by the 'minimyosin' more efficiently than the wild-type actin can in the absence of myosin II. A more plausible explanation, perhaps, is that there are other components in vivo that allow myosin II to function also with the actins containing the mutations in the amino terminus.

Previously we reported on the construction of a yeast strain lacking endogenous actin but which was rescued by the presence of the plasmid expressing β-actin [42]. Preliminary experiments with this strain using plasmid shuffling [43] to replace the wild-type β-actin with the amino-terminal mutants D3Δ,D4Δ and D3A,D4A actin demonstrated that these actins are viable while cells expressing only D3K,D4K actin could not be isolated suggesting that this mutant action is non-viable (PA and RK, to be published elsewhere). This again indicates that the D3K,D4K mutation more severely disturbs actin function.

Acknowledgements: We thank Mrs. H. Vretman and Mr. T. Hult for technical assistance. This work was supported by the Swedish Natural Science Research Council (UL and RK), and by the Swedish Cancer Foundation (UL).

REFERENCES

[1] Kabsch, W., Mannherlz, H.G., Suck, D., Pai, E.F. and Holmes, K.C. (1990) Nature 347, 37–44.
[2] Winkelmann, D.A., Baker, T.S. and Rayment, I. (1991) J. Cell Biol. 114, 701–713.
[3] Higashi-Fujime, S. (1991) Int. Rev. Cytol. 125, 95–138.
[4] Wakabayashi, K., Ueno, Y., Amemiaya, Y. and Tanaka, H. (1988) in: Molecular Mechanism of Muscle Contraction (Sugi, H. and Pollack, G.H., eds.) pp. 353–367, Plenum Press, New York.
[5] Nagano, H. and Yanagida, T. (1985) J. Mol. Biol. 177, 769–785.
[6] Harada, Y., Noguchi, A., Kishino, A. and Yanagida, T. (1987) Nature 326, 805–808.
[7] Toyoshima, Y.Y., Kron, S.J., McNally, E.M., Niebling, K.R., Toyoshima, C. and Spudich, J.A. (1987) Nature 328, 536–539.
[8] Curmi, P.M.G., Stone, D.B., Schneider, D.K., Spudich, J. and Mendelson, R.A. (1988) J. Mol. Biol. 203, 781–798.
[9] Sellers, J.R. and Homsher, E. (1991) Curr. Biol. 1, 347–349.

[10] Schutt, C.E. and Lindberg, U. (1992) Proc. Natl. Acad. Sci. USA 89, 319–323.
[11] Aspenström, P. and Karlsson, R. (1991) Eur. J. Biochem. 200, 35–41.
[12] Sutoh, K. (1982) Biochemistry 21, 4800–4804.
[13] Sutoh, K. and Hatano, S. (1986) Biochemistry 25, 435–440.
[14] Sutoh, K. and Mabuchi, I. (1984) Biochemistry 23, 6757–6761.
[15] Sutoh, K. and Mabuchi, I. (1986) Biochemistry 25, 6186–6192.
[16] Doi, Y., Higashida, M. and Kido, S. (1987) Eur. J. Biochem. 164, 89–94.
[17] Mimura, N. and Asano, A. (1987) J. Biol. Chem. 262, 4717–4723.
[18] Trayler, I.P., Trayer, H.R. and Levine, B.A. (1987) Eur. J. Biochem. 164, 259–266.
[19] Mejean, C., Boyer, M., Labbe, P.J., Marlier, L., Benyamin, Y. and Roustan, C. (1987) Biochem. J. 244, 571–577.
[20] Miller, L., Kainoski, M., Yunossi, Z., Bulinski, J.C. and Reisler, E. (1987) Biochemistry 26, 6064–6070.
[21] Bertrand, R., Chaussepied, P. and Kassab, R. (1988) Biochemistry 27, 5728–5736.
[22] Yamamoto, K. (1989) Biochemistry 28, 5573–5577.
[23] DasGupta, G. and Reisler, E. (1989) J. Mol. Biol. 207, 833–836.
[24] Labbe, J.P., Mejean, C., Benyamin, Y. and Roustan, C. (1990) Biochem. J. 271, 407–413.
[25] Milligan, R.A., Whittaker, M. and Safer, D. (1990) Nature 348, 217–221.
[26] Van Eyk, J.E. and Hodges, R.S. (1991) Biochemistry 30, 11676–11682.
[27] DasGupta, G. and Reisler, E. (1992) Biochemistry 31, 1836-1841.
[28] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[29] Karlsson, R. (1988) Gene 68, 249–257.
[30] Weeds, A.G. and Taylor, R.S. (1975) Nature 257, 54–56.
[31] Smillie, L.B. (1982) Methods Enzymol. 85, 234–241.
[32] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[33] Laemmli, U.K. (1970) Nature 227, 680–685.
[34] Matsudaira, P. and Burgess, A. (1978) Anal. Biochem. 87, 386–396.
[35] Seals, J.R., McDonald, J.M., Bruns, D. and Jarett, L. (1978) Anal. Biochem. 90, 785–795.
[36] Kron, S.J. and Spudich, J.A. (1986) Proc. Natl. Acad. Sci. USA 83, 6272–6276.
[37] Sutoh, K., Ando, M., Sutoh, K. and Toyoshima, Y.Y. (1991) Proc. Natl. Acad. Sci. USA 88, 7711–7714.
[38] Johannes, F.J. and Gallwitz, G. (1991) EMBO J. 10, 3951–3958.
[39] Johnston, G.C., Prendergast, J.A. and Singer, R.A. (1991) J. Cell. Biol. 113, 539–551.
[40] Watts, F.Z., Shiels, G. and Orr, E. (1987) EMBO J. 6, 3499–3505.
[41] Rodriguez, J.R. and Paterson, B.M. (1990) Cell Motility and the Cytoskeleton 17, 301–308.
[42] Karlsson, R., Aspenström, P. and Byström, A. (1991) Mol. Cell. Biol. 11, 213–217.
[43] Boeke, J.D., Trueheart, J. and Matsculis, G. (1987) Methods Enzymol. 154, 164–175.