

Rapid exchange of actin-bound nucleotide in perfused rat heart

Michael Bárány¹ and Pieter P. de Tombe²

¹Department of Biochemistry and Molecular Genetics and ²Department of Physiology and Biophysics, University of Illinois College of Medicine, Chicago, Illinois 60612

Submitted 8 September 2003; accepted in final form 3 December 2003

Bárány, Michael, and Pieter P. de Tombe. Rapid exchange of actin-bound nucleotide in perfused rat heart. *Am J Physiol Heart Circ Physiol* 286: H1394–H1401, 2004; 10.1152/ajpheart.00866.2003.—In the rat heart the actin-bound nucleotide contained both ATP and ADP. The ratio of bound ATP to bound ADP depended on the functional state of the heart; it was higher in hearts stopped reversibly in diastole (low Ca^{2+} , high Mg^{2+} , or high K^{+}), than in stimulated (inotropic agents or pacing) hearts. Immunoblotting and gel electrophoresis showed the existence of G-actin (30% of total actin) in the cytoplasm of the heart. Pure actin was isolated from rat hearts: in G-actin the bound nucleotide readily exchanged with ATP or ADP, and in F-actin the bound nucleotide did not exchange with ATP or ADP. The free and bound nucleotides were separated in the intact heart by extraction with 75% methanol at -15°C . In rat hearts perfused with ^{32}P -labeled orthophosphate the actin-bound nucleotide rapidly exchanged with the cytoplasmic ATP. The full exchange of the bound ATP was immediate, whereas the full exchange of the bound ADP was slower. The full exchange of the bound ATP was independent of the heartbeat frequency, whereas the full exchange of the bound ADP was frequency dependent. The data suggest that the transformation of actin monomer-ATP to actin polymer-ADP is a part of the normal contraction-relaxation cycle of the rat heart.

actin nucleotide; nucleotide exchange

THE CONTRACTILE MECHANISM of the heart is a complex process involving the interactions of myosin, actin, and ATP and the binding of Ca^{2+} to troponin C, followed by a sequence of reactions between troponin C and troponin I and between troponin T and tropomyosin. Structurally the contraction takes place between the thick filaments (containing myosin) and the thin filaments (containing actin, the troponin complex, and tropomyosin). It is generally believed that the major role of actin in contraction is the activation of myosin ATPase, thus causing force development, shortening, and the conversion of chemical energy into mechanical energy (chemomechanical transduction).

Actin is a unique protein that exists in two forms, globular (G) monomer and filamentous (F) polymer. G-actin contains bound ATP, which, on polymerization, is transformed into F-actin-bound-ADP and P_i (31). It is generally accepted that actin polymerization is the basic mechanism for motility in nonmuscle cells (10, 27). On the other hand, no convincing evidence has been obtained for actin polymerization in skeletal muscle (24).

Mehta and Gunst (25) and Jones et al. (17) were the first to report the presence of G-actin in smooth muscle with the methods of DNase inhibition and phalloidin staining, respectively. We examined the G-actin/F-actin distribution in smooth

muscle by labeling the muscle with ^{32}P and separating the free nucleotides from the actin-bound nucleotides by an alcohol-water extraction technique (3). Briefly, ^{32}P labeling results in the synthesis of $[\gamma, \beta\text{-}^{32}\text{P}]\text{ATP}$ in the cytosol, which exchanges with G-actin bound ATP. Polymerization of G-actin into F-actin results in F-actin-bound $[\beta\text{-}^{32}\text{P}]\text{ADP}$. Hence, ^{32}P labeling of actin allows for the determination of steady-state G-actin monomer to F-actin polymer distribution. Using this method we showed exchange of the actin-bound nucleotide with cytoplasmic ATP in smooth muscle (3), thus demonstrating the presence of G-actin in smooth muscle. Subsequently, several laboratories reported actin polymerization in smooth muscle related to contractile activity: pressure-induced actin polymerization in cerebral arteries was suggested as a mechanism underlying myogenic behavior (9). It was also suggested that profilin plays a role in the regulation of actin polymerization in response to contractile stimulation of carotid arteries (33). Inhibitors of actin polymerization attenuated force in pressurized mesenteric arteries (30) or inhibited contractile differentiation of rat portal veins in organ culture (35). Thus these results suggest that polymerization of G-actin monomer into filamentous F-actin may play an important role in contractile activation in smooth muscle independent of activation of myosin ATPase activity in response to myosin light chain phosphorylation.

In contrast to the extensive work on actin polymerization in smooth muscle, virtually no information exists about this reaction in cardiac muscle. Nevertheless, it is generally assumed that the actin filament in striated muscle is a relatively stable cellular structure that does not undergo dynamic alterations within the time frame of one or several cardiac cycles. In the experiments reported here, we used our recently developed technique in smooth muscle (3) to determine the turnover rate of G-actin into F-actin in the heart. We used a perfused rat heart preparation to answer the following questions: First, what is the distribution between G-actin monomer and F-actin polymer in the heart? Second, what is the rate of G-actin polymerization to F-actin in the heart? Finally, does the contractile state of the heart affect this distribution or turnover rate?

We found that a significant amount of the actin-bound nucleotide was in the ATP form, suggesting the presence of a sizable G-actin monomer concentration in the heart. Furthermore, the transformation of G-actin into F-actin was too fast to be measured with our technique at 37°C and could only be assessed at 25°C . Finally, we found a reduction in G-actin content in contracting hearts vs. diastolic-arrested hearts. Our results suggest that a large fraction of actin in the heart is in the form of G-actin monomer and the rate of G-actin

Address for reprint requests and other correspondence: M. Bárány, Dept. of Biochemistry and Molecular Genetics, M/C 536, Univ. of Illinois at Chicago, 1819 W. Polk St., Chicago, IL 60612 (E-mail: mbarany@uic.edu).

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polymerization is regulated in conjunction with contractile state. Preliminary results of this work were published in abstract form (4).

MATERIALS AND METHODS

Heart perfusion. Sprague-Dawley rats weighing 300–400 g were used, and the hearts were dissected under Nembutal anesthesia (15 mg Nembutal/100 g rat injected intraperitoneally). The protocol was approved by the Animal Care Committee of the University of Illinois at Chicago. The heart was placed into ice-cooled physiological salt solution (PSS) containing (in mM) 131.6 NaCl, 5.0 KCl, 1.2 MgSO₄, 1.5 CaCl₂, 14.9 NaHCO₃, and 10.0 glucose. The aorta was connected to the cannula of a home-made Langendorff perfusion apparatus, and the heart was perfused retrogradely with a pressure of 76 mmHg.

The spontaneously beating heart was equilibrated with PSS under 95% O₂-5% CO₂, at a temperature of 25°C or 37°C until constant flow rate and beating rate were obtained (10–15 min). The normal PSS was then exchanged for PSS containing 1.0 mCi carrier-free [³²P]orthophosphate, and the heart was perfused with aid of a Control Company model 3386 pump (Friendswood, TX) for 5 min. Subsequently, excess ³²P from the extracellular space of the heart was washed out with normal PSS for 3.5 min. The same type of ³²P perfusion was performed with hearts paced with a World Precision Instruments A320 stimulator (Sarasota, FL) or with the heart exposed to positive or negative inotropic agents. Under these conditions, the electrical stimulation or chemical treatment was continued during the wash period. Two hearts were used for each experiment, which was completed within 1 h (stock ³²P solution was divided into 2 parts; each part was used for the perfusion of 1 heart). At the end of the experiment, the ventricle was isolated on an ice-cooled watch glass and cut in the middle. On a random basis, one half ventricle was placed into a blender and kept in ice and the other half ventricle was dropped into 100 ml of 75% methanol and kept in ice.

For perchloric acid (PCA) extraction, the ventricles were blended with 1.5% PCA at 0°C for 1 min. The suspension was centrifuged briefly, and the supernatant was poured into a small volume of 10 N KOH solution to neutralize PCA. The perchlorate precipitate was centrifuged off, and the specific activity (SA) of phosphocreatine (PCr) was determined from an aliquot of the supernatant (20); the remainder of the supernatant was concentrated by SpeedVac and used to determine the SA of ATP and ADP by Dowex-1 chromatography.

For the 75% methanol extraction (100 ml), the ventricles were transferred to a -15°C freezer and extracted for 2 days. The ventricles were reextracted twice, each with 100 ml of fresh 75% methanol at -15°C for 2 h. All of the free nucleotides were removed from the hearts by this procedure because reextraction of the residue did not release any material that absorbed at 260 nm. It was demonstrated previously that repeated alcoholic extraction of smooth muscle removes all the free nucleotides but not the actin-bound nucleotides (3). The methanol-treated ventricles were extracted with 1.5% PCA at 0°C for 1 h. After centrifugation, the supernatant was neutralized and centrifuged and the supernatant was concentrated for Dowex-1 chromatography. In most experiments, 75% methanol extraction at -15°C was used to analyze the actin-bound nucleotides; similar results were obtained when the extraction was carried out with 65% or 70% methanol at -15°C or with 65–75% methanol at 0°C.

Dowex-1 chromatography. The procedure was described previously (3). Figure 1 illustrates the elution pattern of compounds that absorbed at 260 nm and their radioactivity in the neutralized PCA extract. NAD was eluted in the early part of the chromatogram, and it was radioactive. P_i, ADP, and ATP were also radioactive. The elution pattern of the ³²P-labeled actin-bound nucleotides is shown in Figs. 3 and 4.

Determination of exchange of actin-bound nucleotides in perfused heart. The percent exchange of the actin-bound ATP and ADP was calculated as

$$\% \text{ actin-ATP exchange} = (\text{SA of actin-ATP} / \text{SA of cytosolic ATP}) \times 100 \quad (1)$$

$$\% \text{ actin-ADP exchange} = (\text{SA of actin-ADP} / \text{SA of } \beta\text{-P of cytosolic ATP}) \times 100 \quad (2)$$

where β -P is the middle phosphate of ATP and

$$\text{SA of } \beta\text{-P of cytosolic ATP} = \text{SA of cytosolic } [\gamma, \beta\text{-}^{32}\text{P}]\text{ATP} - \text{SA of } [^{32}\text{P}]\text{PCr} \quad (3)$$

SA is expressed in counts per minute per micromole of nucleotide or counts per minute per micromole of PCr.

Preparation of rat heart actin. Fresh hearts were washed with cold PSS to remove blood, and the ventricles were minced with scissors. The mince was extracted with 3 vols of 0.35 M KCl and 0.15 M KP_i, pH 6.5, at 0°C for 20 min; after centrifugation the residue was washed with 5 vols of 0.4% NaHCO₃ at 0°C for 10 min, followed by a 50-min wash with 10 vols of distilled water. Five treatments were performed with cold acetone, 5, 3, and 1 vol, each for 10 min. The dried powder was extracted with 30 vols of 0.5 mM ATP at 0°C for 1 h. Actin in the supernatant was polymerized by 0.1 M NaCl and 2 mM MgCl₂ at 37°C for 1 h and then left at 0°C overnight. NaCl was added to the F-actin solution to 0.6 M, and it was stirred at 0°C for 30 min and then centrifuged at 30,000 rpm at 5°C for 3 h. The pellet was washed with distilled water to remove the salts and dissolved by homogenization in 0.5 mM ATP to yield a nonviscous G-actin (4–5 mg/ml).

The dried powder previously extracted with 0.5 mM ATP was further extracted with 30 vols of a solution containing 0.25 M KI, 0.02 M Tris-HCl, pH 7.4, and 1 mM ATP (modified from Ref. 2) at 0°C for 1 h. The supernatant was dialyzed against a solution of 0.1 M NaCl and 2 mM MgCl₂, with frequent changes, at 4°C for 20 h. The workup of the F-actin solution was the same as described in the previous paragraph. On addition of salts to 0.1 M NaCl and 2 mM MgCl₂, this actin solution became very viscous. This actin migrated as a single band on acrylamide gels (see Figs. 8 and 9).

Exchange of actin-bound nucleotides in vitro. [$\gamma, \beta\text{-}^{32}\text{P}$]ATP and [$\beta\text{-}^{32}\text{P}$]ADP were prepared by perfusing rat hearts with 2 mCi ³²P_i at 37°C for 30 min and isolating the nucleotides by Dowex-1 chromatography. The SA of [$\gamma, \beta\text{-}^{32}\text{P}$]ATP was in the range of 8×10^7 cpm/ μ mol and that of [$\beta\text{-}^{32}\text{P}$]ADP was 4×10^7 cpm/ μ mol. The labeled nucleotides were diluted with the corresponding unlabeled nucleotides to have a SA of ~100,000 cpm/ μ mol ATP and 50,000 cpm/ μ mol ADP in the exchange reaction mixture [conditions: G- or F-actin, 2.5–3 mg/ml; molar ratio of ³²P-labeled free nucleotide to

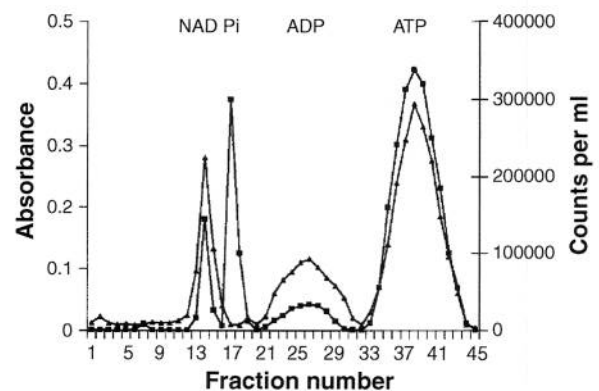


Fig. 1. Dowex-1 chromatography of perchloric acid extract of perfused rat heart. Perfusion was carried out at 25°C for 5 min with physiological salt solution (PSS) containing ³²P_i and reduced Ca²⁺ concentration (0.2 mM). The heart was in diastole during the entire perfusion and washing. Δ , Absorbance; \blacksquare , counts per milliliter.

actin-bound nucleotide, 15–20; 18 mM NaCl, 2 mM Tris·HCl, 2 mM MgCl₂ (in case of F-actin), pH 7.1; 25°C].

Before the experiment started, free nucleotides were removed from the actin solutions by treatment with Dowex-1 X8, 200–400 mesh, 25 mg/ml (modified from Ref. 5) and the reaction was initiated by the addition of actin. Three-milliliter aliquots were removed from the reaction mixture at 30-s and 2-, 4-, 8-, and 16-min intervals and quickly mixed with 80 mg of Dowex-1 to stop the exchange reaction. Three-milliliter aliquots containing no actin were also treated with Dowex-1 to obtain blank values. The resin was removed by brief centrifugation at 400 g, and the upper two-thirds of the supernatant was taken for analyses. PCA was added to the supernatant to release the actin-bound nucleotides, and after centrifugation absorbance at 260 and 280 nm and radioactivity were measured in the supernatant. The actin-containing precipitate was dissolved in 1 N NaOH, and the protein content was determined. From these data the number of moles of bound nucleotide per 42,000 g of actin was calculated; the values ranged from 0.74 to 1.29.

In calculating the SA of the actin-bound nucleotide, the small blank absorbance and radioactivity values were subtracted from the values measured for the bound nucleotides. Next, the SA of the actin-bound nucleotide was compared with the SA of [γ , β -³²P]ATP or [β -³²P]ADP in the medium; this term is called percent exchange

$$\% \text{ exchange} = (\text{SA of } [\gamma, \beta\text{-}^{32}\text{P}]\text{ATP in actin-bound nucleotide} / \text{SA of } [\gamma, \beta\text{-}^{32}\text{P}]\text{ATP in medium}) \times 100 \quad (4)$$

or

$$\% \text{ exchange} = (\text{SA of } [\beta\text{-}^{32}\text{P}]\text{ADP in actin-bound nucleotide} / \text{SA of } [\beta\text{-}^{32}\text{P}]\text{ADP in medium}) \times 100 \quad (5)$$

Preparation of myofibrils from ³²P-labeled rat hearts. Ventricles from ³²P-perfused hearts were chopped with scissors. A part of the mince was treated with PCA for determination of the SA of PCr and ATP. The rest of the mince was blended at 0°C with a solution containing 0.01 M iodoacetamide, 0.04 M NaCl, and 0.01 M Tris·HCl, pH 7.4, for 15 s (iodoacetamide in this solution inhibits the enzymic hydrolysis of ATP). The suspension was centrifuged at 600 g for 25 min at 4°C, and the residue was blended with 0.04 M NaCl and 0.01 M Tris·HCl for 1 s and then centrifuged as before. This step was repeated. The final pellet was suspended in the NaCl-Tris·HCl solution and clarified at 100 g to remove connective tissue. The myofibrils in the supernatant were analyzed for bound nucleotide content, composition of the nucleotides, and their radioactivity.

Preparation of mitochondria from ³²P-labeled rat hearts. Ventricles from ³²P-perfused hearts were blended at 0°C with a solution containing 0.1 M NaCl and 0.05 M Tris·HCl, pH 7.4, in two bursts, each lasting 30 s. The homogenate was centrifuged at 600 g, and the mitochondria were isolated from the supernatant by centrifugation at 10,000 g. The pellet was repeatedly homogenized at 0°C in NaCl-Tris, with the aid of a glass Potter-Elvehjem homogenizer, and centrifuged at 10,000 g. Mitochondria were also prepared from ³²P-labeled rat hearts by sucrose-sorbitol homogenization (13). No difference was found in the ³²P content of mitochondria prepared by the two methods.

Determination of free nucleotide content of rat hearts. With repeated 75% methanol extraction, 5.82 ± 0.65 μmol of adenine nucleotides were obtained per gram of heart (n = 14). This value included the NAD and NADP content of the heart, 0.77 μmol/g (22). The ATP content of the methanol extract was ~4.0 μmol/g. With ³¹P-NMR, 5.4 μmol of ATP in 73% intracellular water of the rat heart (3.94 μmol ATP/g heart) was reported (12).

Determination of bound nucleotide content of rat hearts. Weighed pieces of ventricles (230–270 mg) were extracted with 30 ml of 0.04 M NaCl and 0.01 M Tris·HCl, pH 7.4, with stirring at 4°C for 16–18 h. After centrifugation at 600 g, the residue was extracted twice with

the NaCl-Tris solution (no free nucleotide remained in the last supernatant). The final residue was treated with PCA to liberate the actin-bound nucleotides. A value of 1.03 ± 0.12 μmol nucleotide/g wet heart weight was found (n = 10). With 75% methanol extraction and subsequent PCA treatment, 1.06 ± 0.08 μmol bound nucleotide/g heart was found (n = 21). As shown under *Determination of free nucleotide content of rat hearts*, rat hearts contain 4 μmol free nucleotides/g, thus the actin-bound nucleotides comprise ~20% of the total nucleotides.

Quantifying the actin content of rat hearts by gel electrophoresis gave 38.8 mg actin/g heart. With a molecular mass of 42 kDa for heart actin (7), 0.92 μmol of actin monomer was found per gram of heart. This compares well with the 1.03 and 1.06 μmol actin/g heart measured by the bound nucleotide method.

Determination of myosin content of rat hearts. This procedure is based on the observations (6, 32) that a muscle washed free of ATP and subsequently extracted with 0.6 M KI solution yields actomyosin containing monomeric actin. On dilution to 0.024 M KI, the myosin component of the actomyosin precipitates while the actin component of the actomyosin remains in the supernatant. We found 77.3 ± 12.1 mg myosin/g wet heart weight (n = 6). Because the heart contains ~42 mg actin/g, there is a 2.6-fold molar excess of actin monomer over the myosin heavy chain (molecular mass 200 kDa) in the rat heart.

Determination of rat heart wet weight. Weighed ventricles were extracted with 100 vols of 0.1 N NaOH solution at 25°C for 24 h (21). After centrifugation to remove connective tissue, 184.9 ± 6.5 mg protein/g heart remained in the supernatant (n = 10). This value is somewhat higher than the 160 mg protein reported (18). In our experiments, the heart residue left over after 75% methanol or 1.5% PCA extraction was extracted with 0.1 N NaOH and the amount of protein solubilized was converted into grams of wet heart weight with the factor of 184.9.

Miscellaneous. The band densities of proteins on gels were determined by digital analysis with a Kodak Digital Science DC 120 Zoom digital camera and Kodak Digital Science 1D 2.0.2 software (Eastman Kodak). The protein content of the heart preparations was determined by the modified biuret method (1) after dissolution in 0.1 N NaOH. Radioactivity was measured by liquid scintillation counting. ³²P_i was obtained from ICN. Data are expressed as means ± SE.

RESULTS

Exchange of actin-bound nucleotide in vitro. The exchange of the bound nucleotide of rat heart G- and F-actin with [γ , β -³²P]ATP was investigated (Fig. 2). The percent exchange (Eq. 4) was 92–95% for G-actin and virtually zero for F-actin. Under the conditions of Fig. 2, 20-fold molar excess of [γ , β -³²P]ATP was used over the molar concentration of the actin-bound nucleotide; thus the maximal theoretical percent exchange was 95%. The exchange was immediate; that is, at the earliest experimental time (30 s), maximal exchange occurred. The same exchange pattern was found at 37°C as at 25°C; i.e., there was no exchange for F-actin, whereas there was full exchange for G-actin.

When [γ , β -³²P]ATP was substituted with [β -³²P]ADP, no exchange was found with F-actin-ADP at either 25°C or 37°C (calculated with Eq. 5) but considerable exchange was found with G-actin-ATP. From these data one can conclude that the F-actin-ADP is not exchangeable with either ATP or ADP in the medium. These data also show that the exchangeability of heart actin is the same as that of skeletal muscle actin (23, 24).

Distribution of actin-bound nucleotides in perfused hearts. Two bound nucleotides, ADP and ATP, and three radioactive peaks, P_i, ADP, and ATP, were found by Dowex-1 chroma-

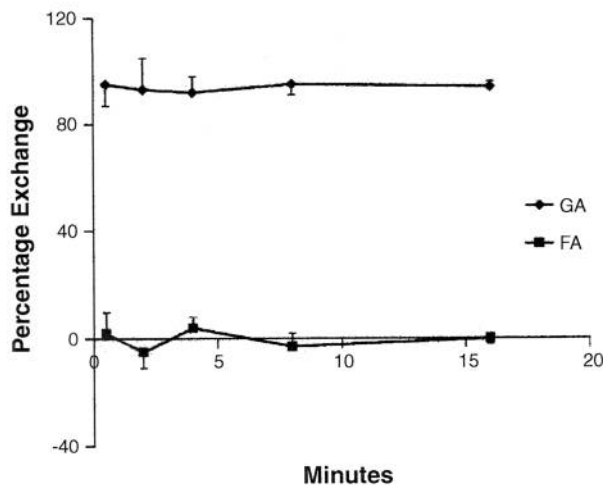


Fig. 2. Exchange of the bound nucleotide in rat heart G-actin (GA) and F-actin (FA) with $[\gamma, \beta\text{-}^{32}\text{P}]\text{ATP}$ in the medium as a function of time at 25°C. Each data point represents the average of 3 experiments. For details see MATERIALS AND METHODS.

tography (Figs. 3 and 4). The absorbance of the actin-bound ATP was higher than that of the actin-bound ADP. A comparison of Fig. 3 with Fig. 4 revealed that the ratio of the actin-bound ATP to the actin-bound ADP depended on the functional state of the heart. Thus, from the areas of the ATP and ADP peaks, a contribution of 54% for ATP and 46% for ADP was determined in the heart stimulated with isoproterenol (Fig. 3), whereas in the heart stopped in diastole with excess Mg^{2+} the contribution was as much as 77% for ATP and only 23% for ADP (Fig. 4).

In Fig. 5 the percentage distributions of ATP and ADP were compared between hearts stopped reversibly in diastole by low Ca^{2+} , high Mg^{2+} , or high K^{+} and hearts stimulated by isoproterenol, high Ca^{2+} , or electrical pacing. In the resting heart the distribution was ATP $75 \pm 6\%$ and ADP $25 \pm 4\%$, whereas in the stimulated heart the distribution was ATP $53 \pm 7\%$ and ADP $47 \pm 8\%$.

Exchange of actin-bound nucleotides in ^{32}P -perfused hearts. The maximal radioactivity in the ATP peaks matched the maximal absorbance in both stimulated and resting hearts (Figs. 3 and 4), indicating a high level of ^{32}P incorporation into the actin-bound ATP. On the other hand, the radioactivities of

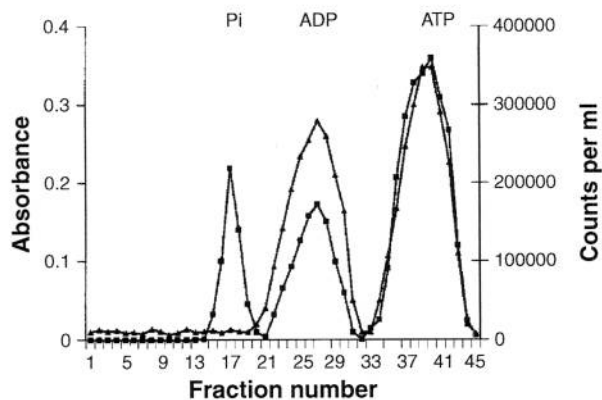


Fig. 3. Dowex-1 chromatography of actin-bound nucleotide from rat heart perfused with isoproterenol. Perfusion was carried out at 25°C for 5 min with PSS containing $^{32}\text{P}_i$ and 10^{-5} M isoproterenol. For symbols see Fig. 1.

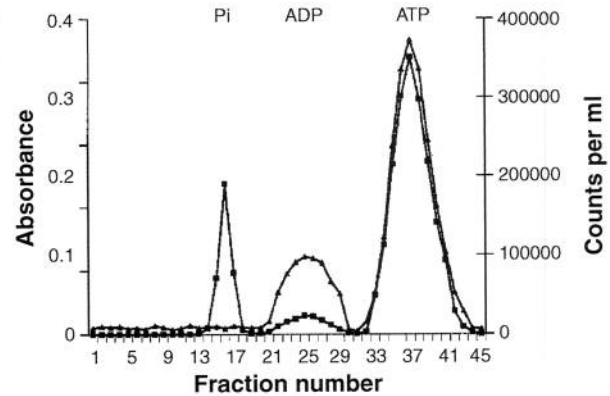


Fig. 4. Dowex-1 chromatography of actin-bound nucleotide from rat heart perfused with Mg^{2+} . Perfusion was carried out at 25°C for 5 min with PSS containing $^{32}\text{P}_i$ and 10 mM Mg^{2+} (and the normal 1.5 mM Ca^{2+}). The heart was in diastole during the entire perfusion and washing. For symbols see Fig. 1.

the ADP peaks remained below the maxima of the corresponding absorbance peaks, suggesting that the ^{32}P incorporation into the actin-bound ADP was not complete. This reveals a difference in the labeling pattern between bound ATP and bound ADP.

To quantify the incorporation of $^{32}\text{P}_i$ into the actin-bound nucleotides, we measured the SA of their ^{32}P label and compared it with the SA of the ^{32}P label in the cytoplasmic nucleotides (Eqs. 1 and 2). The exchange was so rapid at 37°C that its kinetics could not be measured. Therefore, we lowered the perfusion temperature from 37°C to 25°C.

In spontaneously beating hearts, the complete exchange of actin-bound ATP was immediate whereas the full exchange of the bound ADP took over 20 min (Fig. 6). The exchange of the bound ATP occurred at the lowest stimulation frequency, whereas the full exchange of the bound ADP required a stimulation frequency of $\sim 200 \text{ min}^{-1}$ (Fig. 7). From the

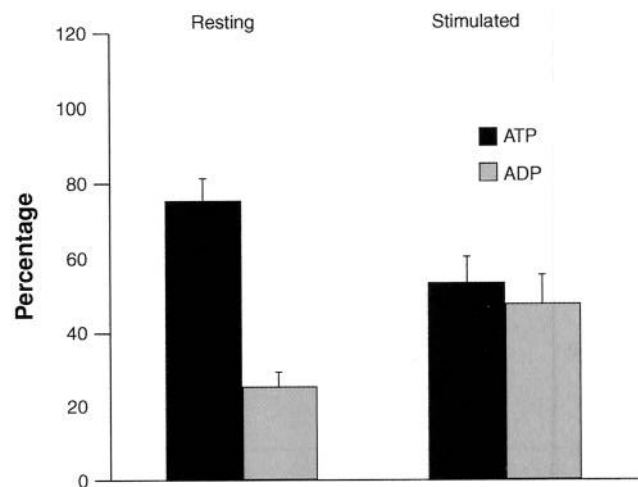


Fig. 5. Distribution of actin-bound ATP and ADP in resting and stimulated rat heart. "Resting" refers to hearts stopped in diastole by PSS containing 0.2 mM Ca^{2+} , 10 mM Mg^{2+} , or 50 mM K^{+} . "Stimulated" refers to hearts stimulated by PSS containing 10^{-5} M isoproterenol, 5 mM Ca^{2+} or paced at 300 min^{-1} in normal PSS. ^{32}P perfusion was carried out at 25°C for 5 min under resting or stimulated conditions. The same conditions also prevailed during the washing period; $n = 9$ for resting or stimulated.

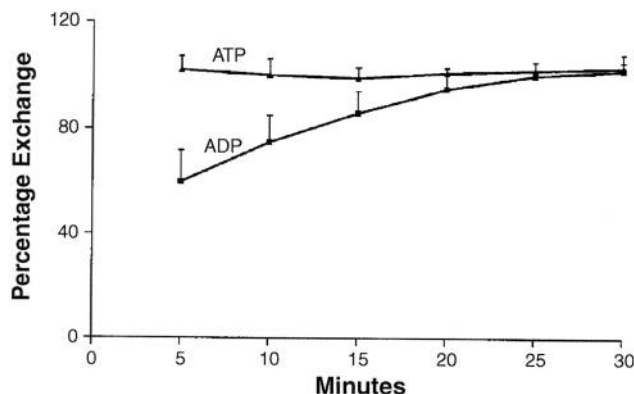


Fig. 6. Exchange of actin-bound ATP and ADP in rat heart as a function of time. The heart was beating spontaneously at 25°C while perfused with ^{32}P -labeled PSS for the time indicated on the x-axis. The washing time for removal of ^{32}P from the extracellular space was 3.5 min in all cases. Each data point represents the average of 3 experiments.

profiles of Figs. 6 and 7 one can conclude that the exchange of the actin ATP is a faster reaction than the exchange of the actin ADP.

Mitochondria. The heart is rich in mitochondria (19). We have found on average 13 mg mitochondrial protein/g rat heart, containing a total of 0.04 μmol of bound ATP and bound ADP. When isolated from ^{32}P -labeled heart, the mitochondrial ATP and ADP were fully exchanged. Because the actin-bound nucleotide amounts to 1 $\mu\text{mol/g}$ heart, the small quantity of mitochondrial ATP and ADP has only a negligible effect on the conclusions drawn in the present work.

Myofibrils. Myofibrils from hearts perfused with $^{32}\text{P}_i$ contained 0.6–0.75 μmol bound ^{32}P -ADP/100 mg protein. Full exchange of the myofibril-bound ADP was reached in spontaneously beating hearts in 10 min.

Analysis of PCA extract. ^{31}P -NMR studies on hearts of live animals and on perfused hearts indicated that “ P_i is virtually undetectable” (Ref. 14 and references therein) because of the rapid incorporation of P_i into ADP formed during cellular metabolism. Therefore, the ADP found in the PCA extract of a well-oxygenated, perfused rat heart is essentially the actin-bound ADP; this was shown to be the case in smooth muscle

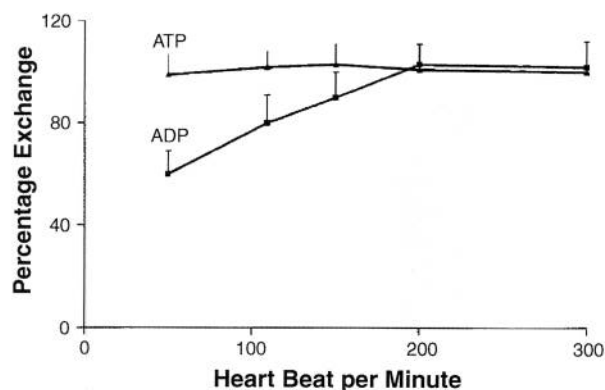


Fig. 7. Exchange of actin-bound ATP and ADP in rat heart as a function of pacing frequency. For frequencies from 100 to 300 min^{-1} the heart was electrically stimulated at 25°C; for the frequency of 50 min^{-1} the heart was spontaneously beating at 20°C. Stimulation was on both during the 5-min ^{32}P perfusion and the 3.5-min washing period. Each data point represents the average of 3 experiments.

(3). The current study with rat hearts also confirmed that the PCA extract contained actin-bound ADP. For instance, the percent exchange of the actin-ADP in hearts stimulated at a frequency of 300 min^{-1} was $101 \pm 5\%$ when analyzed by the methanol-water extraction method and $95 \pm 7\%$ when measured by the PCA extraction method.

Evidence for existence of G-actin in rat heart muscle. When ventricles were homogenized in 0.15 M NaCl and 0.01 M Tris-HCl, pH 7.4, and centrifuged at 600 g, the supernatant contained on average 52 mg cytosolic proteins/g heart. Gel electrophoresis of the supernatant showed a strong band in the 42-kDa region (Fig. 8), with mobility identical to that of rat heart actin. Immunoblotting of the supernatant gel showed that the monoclonal antibody specific to sarcomeric actin stained a single band with mobility identical to that of rat heart actin (Fig. 8).

The staining intensity of the 42-kDa band was determined in four different experiments, and it was found to be $24.4 \pm 3.0\%$ of the total staining intensity of all the bands. This means that 12.7 mg of actin was present in the cytosol per gram of heart ventricle, or 30% of the total actin (42 mg).

Viscometric studies were used to establish the form of actin, G or F, in the supernatant. The viscosity was expressed as a logarithm of the relative viscosity, which is proportional to the protein concentration in actomyosin systems (28). The viscosity of the actin in the supernatant was about one-eighth of the viscosity of the pure rat cardiac F-actin, indicating that the actin in the supernatant was in the G form. The fact that actin

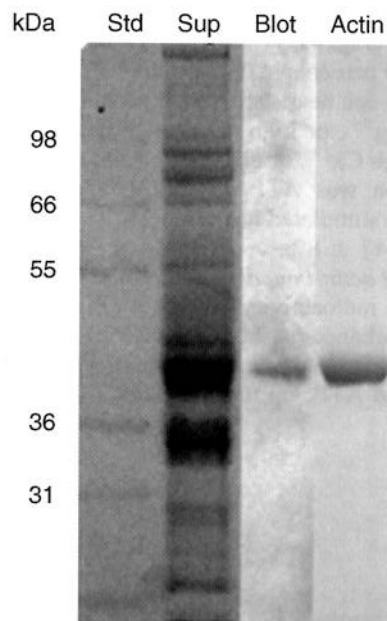


Fig. 8. Gel electrophoresis and immunoblotting of rat heart cytosolic proteins. The ventricles were homogenized in 0.15 M NaCl and 0.01 M Tris-HCl, pH 7.4, the suspension was centrifuged at 600 g, and the supernatant was electrophoresed on 10% Bis-Tris gel (Invitrogen). The staining pattern of 50 μg of supernatant proteins is shown in the lane labeled Sup. A duplicate gel not stained was used for Western blotting. The C4 monoclonal antibody to sarcomeric actin (a gift from Dr. James Lessard) was used to probe the blot. A peroxidase-labeled goat antibody to mouse IgG and the ECL Western blotting system of Amersham Biosciences were used to visualize the antibody reacted band, shown in the lane labeled Blot. Ten microliters of molecular weight standard (Std) from Invitrogen and five micrograms of purified actin were used as markers.

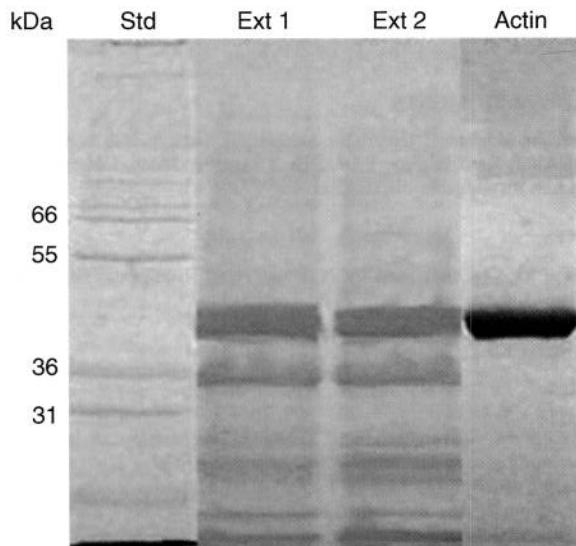


Fig. 9. Electrophoresis of water extracts of 75% methanol-extracted heart residue. The methanolic residue was extracted twice, each for 1 h, with 10 vols of distilled water at 0°C (Ext 1 and Ext 2). The 10% Bis-Tris gel was loaded with 30 μ g of protein from the first or second extract, 15 μ l of molecular weight standard (Std) from Invitrogen, and 10 μ g of purified actin.

in the supernatant in 0.15 M NaCl remained in the G form suggests that there is an inhibitor present in rat heart cytosol that prevents the polymerization of actin.

On the basis of nucleotide analysis (Figs. 3–5), it was postulated that the 75% methanol-extracted residue of the rat heart contains G-actin. Gel electrophoresis provided direct evidence for the existence of G-actin in this residue (Fig. 9). In both the first and second distilled water extracts of the methanolic residue, the strongest-stained band migrated with a mobility identical to that of pure rat heart actin. The staining intensity of the actin band in the water extracts was ~55% of the total band intensities. Along with the proteins, nucleotides were also extracted from the methanolic residue, which were identified predominantly as ATP on the Dowex-1 column. Furthermore, the water extracts possessed low viscosity. All these characteristics suggest that the water extracts contained G-actin.

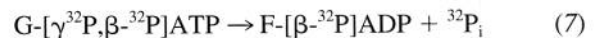
DISCUSSION

Our results provide a new insight into the characteristics of the cardiac thin filaments, namely, that the actin component is in a dynamic state and undergoes a fast reorganization. This concept differs greatly from the currently accepted view that actin is in a steady state with “a long lifetime” and the renewal of the actin filament is controlled by the rate of actin protein synthesis. The dynamic state of actin is now well established in smooth muscle (3, 9, 17, 25, 30, 33, 35). Importantly, agents that blocked actin polymerization also inhibited smooth muscle contraction without any significant effect on intracellular Ca^{2+} concentration, membrane potential, or myosin light chain phosphorylation (9, 25, 30, 33). This indicates that actin has an independent control on smooth muscle contractility and this may underlie a potential regulatory pathway for contractile function. No such studies are known in cardiac muscle.

The following observations support cardiac actin dynamics.

1) The actin-bound nucleotide in the rat heart exists in two

forms, ATP and ADP. The ratio of bound ATP to bound ADP depended on the functional state of the heart; it was higher in hearts stopped in diastole than in stimulated hearts (Fig. 5). It is conventional to associate ATP-actin with G-actin and ADP-actin with F-actin; accordingly, the nucleotide data indicate that rat heart actin occurs both in the G and F forms. 2) Both actin-bound ATP and ADP were labeled in hearts perfused with PSS containing $^{32}\text{P}_i$. The labeling of the bound nucleotides was so fast that with our technique it could not be followed at 37°C, only at 25°C. The labeling of the bound ATP was immediate, whereas the labeling of the bound ADP lagged behind (Figs. 6 and 7). To explain these data, one must recall that ATP bound to rat heart G-actin readily exchanges with free ATP in the medium but ADP bound to rat heart F-actin does not exchange with free nucleotides (Fig. 2). The labeling of actin-bound ADP in the perfused heart can be described by the following reactions



That is, the double-labeled ATP in the cytoplasm rapidly exchanges with the ATP bound to G-actin (Eq. 6). Subsequently, the double-labeled G-actin polymerizes while its $[\gamma, \beta^{32}\text{P}]\text{ATP}$ is hydrolyzed to F-actin- $[\beta^{32}\text{P}]\text{ADP}$ and ^{32}P phosphate (Eq. 7). That is, the F-actin-ADP does not exchange directly, but the ^{32}P ADP label is introduced into F-actin through the polymerization of G-actin- $[\gamma, \beta^{32}\text{P}]\text{ATP}$.

Evidence was presented for the existence of G-actin protein per se in the cytoplasm of the heart by immunoblotting and gel electrophoresis (Fig. 8) and viscometry. As a matter of fact, as much as 30% of the total rat heart actin was found to be in the G form in the cytoplasm. As described under MATERIALS AND METHODS, there is a 2.6-fold molar excess of actin monomer over the myosin heavy chain in the rat heart and, therefore, a significant part of the actin could be in the G form. It should be noted that our value of the 30% actin content of heart cyto-

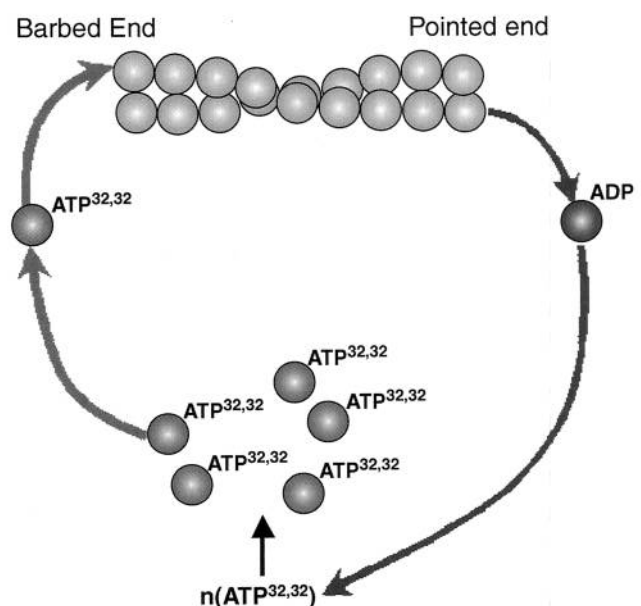


Fig. 10. A scheme for the exchange of actin-bound ATP and ADP in beating rat heart. For explanation see the text.

plasm is considerably higher than the 13% value reported by Seidel et al. (29). This difference is likely due to differences in the methods used: we separated the proteins by gel electrophoresis and scanned the intensity of the protein bands, whereas Seidel et al. measured DNase activity in the cytoplasm and compared it with that of pure G-actin. We believe that the analytical gel electrophoresis is more accurate than the comparative enzymic method.

Based on the treadmilling process (26, 34), Fig. 10 depicts a scheme of how these reactions may take place in the perfused heart. The double-labeled ^{32}P -ATP in the cytoplasm exchanges with the bound ATP of free G-actin. Individual G-actin- ^{32}P -ATP molecules are attaching to the barbed end of the F-actin double helix in the thin filament while hydrolyzing the bound double-labeled ATP to single-labeled bound ADP. (The transformation of bound ATP to bound ADP is not shown in Fig. 10. Furthermore, Fig. 10 shows the attachment of the G- ^{32}P -ATP only to one of the twin strings of F-actin; in reality, the attachment to both strings takes place on a random basis.) To keep the filament length constant (under physiological conditions), a nonlabeled ADP-actin monomer must leave the actin string at the pointed end. The G-actin-ADP in the cytoplasm exchanges its nucleotide to be transformed to G-actin- ^{32}P -ATP; this ends the cycle.

There are reports in the literature that treadmilling is not involved in nucleotide exchange of F-actin (8, 11). These experiments were carried out in solution and lasted for several hours, unlike the nucleotide exchange in the beating heart, which takes place in the thin filament compartment of the heart and is completed in minutes. Furthermore, the bound nucleotide of rat heart F-actin did not exchange with free nucleotides (Fig. 2). In analyses of polymerization dynamics (15, 16), diffusion exchange was preferred over treadmilling. We believe, however, that a random diffusion process would allow for less control over the obligatory sequence of actin polymerization in the structure of thin filaments than that provided by treadmilling.

The cycling rate of the G-actin-ATP to F-actin-ADP transformation was very fast at a physiological temperature (37°C). Moreover, the dynamic rate of actin monomer incorporation into the thin filament appears to be regulated in conjunction with the contractile state. Site-directed polymerization of actin in cardiac thin filaments, in response to stimulation, generates a movement (treadmilling) of the actin helix from the Z line toward the center of the sarcomere. The ongoing replacement of actin monomers may facilitate cross bridge formation by increasing the chance for a myosin head to be in optimal juxtaposition to actin and thereby enhance myofilament force development. One may speculate, therefore, that enhanced actin treadmilling would lead to enhanced contractility. It should be noted that the estimated rate of actin incorporation into the thin filament at 25°C amounted to ~1 cycle per 3 s. This implies that this mechanism operates over the time course of several beats. However, we were not able to determine the rate at 37°C. We cannot, therefore, exclude a role for this process during the cardiac cycle at physiological temperatures.

In summary, we found a significant amount of G-actin monomer in the heart that is rapidly transformed into F-actin in the cardiac sarcomere. Furthermore, the rate of actin polymerization increased in conjunction with the contractile state. Our results suggest that the actin monomer-ATP to actin polymer-

ADP transformation is of physiological significance in the heart.

ACKNOWLEDGMENTS

We thank Mariano E. Rivera for enthusiastic and expert assistance and Drs. Katalin Ajtai, Kate Bárány, Primal De Lanerolle, James Lessard, and Bao-Shiang Lee for valuable discussions.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute P01-HL-62426 Project 4 (to P. P. de Tombe).

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