THE ATPase ACTIVITIES OF RAT CARDIAC MYOSIN ISOENZYMES

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1. Introduction

Rat ventricular myosin contains three isoenzymes which can be separated by polyacrylamide gel electrophoresis in the presence of pyrophosphate buffers [1]. Two of these isoenzymes, V_1 and V_3 (the fastest and slowest migrating components, respectively), contain homodimers of two chemically distinct heavy chains, while the intermediate component, V_2, contains 1 mol of each of these two heavy chains [2]. The light chains in these isoenzymes have identical electrophoretic mobilities suggesting that the main structural differences reside in the heavy chains [1]. The distribution of these isoenzymes varies with the age and thyroid status of the rat. V_3 predominates in rats made hypothyroid by hypophysectomy or thyroidectomy [1,2], while replacement therapy with physiological doses of the hormone leads to a shift in the distribution of the isoenzymes towards V_1, which appears to be due to stimulation of specific mRNA synthesis [3]. In 3–4-week-old rats, only V_1 is present [1]. Thus it is possible to prepare myosin isoenzymes of both V_1 and V_3 forms from suitable animals without the need to separate the mixture. Staining the gels for enzymatic activity suggests that the Ca^{2+}-activated ATPase of V_1 is substantially higher than that of V_3. However, this activity is not physiologically meaningful, nor is it possible to measure other ATPase activities on gels. Here we report studies on the ATPase activities of these two isoenzymes, measured under a number of different conditions to show that these phenotypes differ in their kinetic properties. V_1 has a higher ATPase activity than V_3 except for the activity measured in the absence of divalent cations. Marked differences are observed in the physiologically important actin-activated ATPase.

2. Methods and materials

V_1 myosin was prepared from ventricles isolated from 4-week-old rats. For the preparation of V_3 myosin, rats were thyroidectomised at 4.5 months and treated with the antithyroid agent, propyl thiouracil at a dosage of 0.02% (w/v) in their drinking water for 11–12 weeks. The hearts were stored in a buffer solution containing 50% glycerol at −20°C. Individual myosin preparations were made from 2–5 hearts, giving 1–2 g tissue. Myofibrils were prepared as in [4]. The hearts were chopped finely with scissors and blended in buffer containing 0.1 M NaCl, 10 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium azide and 50 μM phenyl methane sulphonyl fluoride. Four successive washings in this buffer were carried out; in the 2nd washing 0.5% Triton X-100 was included to improve the separation of the myofibrils, and this detergent was washed out in the final 2 washings, each with 10 vol. buffer. To extract the myosin the myofibrils were resuspended in a solution containing 0.6 M NaCl, 0.05 M Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP and 2 mM sodium pyrophosphate and centrifuged at 55 000 rev./min (300 000 × *g*) for 2.5 h. The supernatant was dialysed overnight against 30 mM NaCl, 10 mM imidazole (pH 7.0), 1 mM sodium azide and 50 μM phenylmethane sulphonyl fluoride to precipitate the myosin, which was redissolved in 0.6 M NaCl, 10 mM sodium phosphate (pH 7.0) and clarified by centrifugation at 40 000 × *g* for 1 h. Protein concentrations were measured by A_{280}, with suitable correction for the presence of residual ATP, and an $E^{1\%}_{280} = 5.60$ used. Although this value was determined for skeletal myosin, and it may not strictly apply to rat cardiac myosins, the amino acid compositions of V_1 and V_3 myosins are remarkably similar [2], so that for these

comparisons there is no reason to believe that the two isoenzymes will have significantly different extinction coefficients. In one preparation the myosin concentration was determined by Folin's method using bovine serum albumin as a standard. The concentration measured in this way was 8% above that estimated by A_{280}.

2.1. *ATPase activities*

ATPase activities were measured in a Radiometer pH-stat at pH 8.0, 25°C. The enzymic activity in the absence of divalent cations (the K^+/EDTA ATPase) was measured in 0.6 M KCl, 1 mM EDTA and 5 mM ATP; Ca^{2+}-ATPase in 0.4 M KCl, 10 mM $CaCl_2$ and 5 mM ATP; actin-activated ATPase activities in 2.5 mM ATP, 3.75 mM $MgCl_2$ and KCl at 21 mM, 71 mM and 121 mM, with actin varied from 2–95 μM. The assays were set up at the lowest [KCl] and after a few minutes 4 M KCl was added to give the next [KCl] and the enzymatic activity measured again. In this way activities at different ionic strength values were measured for the same actomyosin sample. V_{max} and K_m values for the actin activated ATPase were obtained from Eadie-Hofstee plots as in [5]. Myofibrillar ATPase activities were measured under similar conditions using 0.1 ml of myofibril suspension and no additional KCl. The protein concentration in the myofibrillar samples was estimated by A_{280} in the presence of 1% SDS to solubilise the proteins. An $E^{1\%}_{280} = 7.0$ was used, based on the known extinction coefficients of the myofibrillar proteins in skeletal muscle and the relative proportions of individual components. To convert this protein concentration to a myosin concentration, it was assumed that 54% by weight of the myofibrillar protein is myosin. Alkali inactivation of the ATPase activity [6] was measured as follows: myosin was mixed with an equal volume of 0.5 M KCl, 0.05 M glycine, 1 mM EDTA (pH 9.5) giving a final pH 9.0 and incubated at 20°C for different times. The pH was reduced to 8 at the end of the incubation and ATPase activities measured in the absence of divalent cations as above.

3. Results and discussion

Myosin isolated from 4-week-old rats gave a single band on polyacrylamide gel electrophoresis in pyrophosphate buffers corresponding to the V_1 isoenzyme reported earlier [1]. Myosin isolated from hearts of hypothyroid rats also gave a single band under these conditions but the mobility was somewhat slower, corresponding to the V_3 isoenzyme. We will term these two myosins V_1 and V_3, respectively. The Ca^{2+}-ATPase activities of the two isoenzymes differ by a factor of >3 as shown in table 1, in good agreement with earlier measurements made on gels [1]. There was a large variation between the two preparations of V_3, which may reflect partial inactivation of the ATPase or possibly some chemical modification of preparation 1 may have occurred resulting in activation of this particular ATPase. It is well known that thiol modification can produce markedly activated Ca^{2+}-ATPase values [7]. Since the two V_3 preparations did not show significant variation in the ATPase measured in the absence of divalent cations (the K^+/EDTA ATPase), it appears more likely that spurious activation of preparation 1 may account for the differences in the Ca^{2+}-ATPase.

Table 1
ATPase activities (μmol P_i . mg myosin^{-1} . min^{-1}) of V_1 and V_3 cardiac myosin isoenzymes

ATPase	V_1		V_3	
	1	2	1	2
Ca^{2+}	0.822	0.700	0.278	0.155
K^+/EDTA	0.182	0.205	0.196	0.190
K^+/EDTA after 20 min at pH 9.0	0.182	0.205	0.140	0.152
K^+/EDTA after 60 min at pH 9.0	0.161	0.202	0.107	0.104
Myofibrillar	0.163	0.165	0.126	0.135

Conditions for these various assays are given in section 2

There appears to be no significant difference between V_1 and V_3 myosins in their ATPase activities in the absence of divalent cations as shown in table 1. This is in good agreement with earlier experiments on cardiac myosins from normal and thyrotoxic rabbits [8]. Although the number and purity of the isoenzymes had not been established in [8], evidence in [9] suggests that different myosins are synthesized in these different thyroid states. Whilst the myosins cannot be distinguished by the K^+/EDTA ATPase activities directly, they show different susceptibility to inactivation at pH 9.0 as indicated in table 1. Thus after 1 h at pH 9.0, V_1 shows a 2–12% loss of activity compared with 45% for V_3. In this respect V_1, which has a much higher Ca^{2+}-ATPase resembles fast-

twitch muscle myosin, while V_3 behaves more like slow-twitch muscle myosin.

The important ATPase from the physiological standpoint is the actin-activated ATPase. This has been measured in two ways. Table 1 shows the activity found in isolated myofibrils, where V_1 gave values ~27% higher than V_3. This assay has the disadvantage that the preparations are not homogeneous and it is also difficult to obtain an accurate estimate of the myosin concentration. We have also measured the actin activation of the myosin ATPase directly, using increasing concentrations of actin to obtain a maximum velocity. Whilst this method has been used extensively for myosin subfragments [10], it has been little used with myosin itself. One problem in interpreting the results concerns the validity of using a Michaelis-Menten model for the interaction of two filamentous interacting systems. Nevertheless it seems preferable to analyse the effects of different concentrations of actin rather than choosing some arbitrary ratio of myosin and actin, since it is not possible to reproduce the conditions in muscle itself. Fig.1 shows comparative data for V_1 and V_3 at two different ionic strength values. In all cases the Eadie-Hofstee plots are biphasic with a change in slope at 15–20 μM actin. Similar biphasic plots have been consistently observed with rabbit skeletal muscle myosin and other myosins (B. P., Wagner, A. W., unpublished). The results extrapolate to a V_{max} corresponding to a very low K_m of ~1 μM actin, while above the breakpoint, there appears to be a second and higher V_{max} with correspondingly higher K_m. A detailed discussion of the possible reasons for this biphasic behaviour of the Eadie-Hofstee plots will be presented elsewhere (B. P., Wagner, A. W., in preparation). Here we will consider only the comparison between the two cardiac myosin isoenzymes.

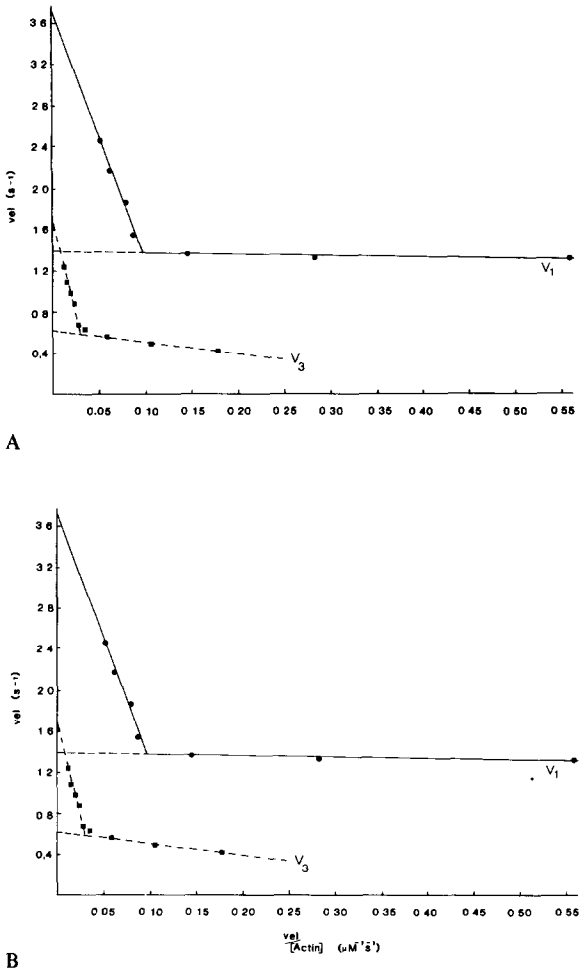

Fig.1. Eadie-Hofstee plot of the actin activated ATPase of V_1 and V_3 myosins: (A) in 21 mM KCl; (B) in 71 mM KCl. Assay conditions were as in section 2. (●——●) V_1 myosin; (■–.–■) V_3 myosin.

Table 2
Values for V_{max} and K_m for actin-activated ATPase activities

KCl	V_1		V_3	
	V_{max} (s^{-1})	K_m (μM actin)	V_{max} (s^{-1})	K_m (μM actin)
21 mM	1.4	<0.2	0.62	1.1
	3.7	24	1.7	34
71 mM	0.61	<0.2	0.19	0.6
	1.4	25	0.98	64
121 mM	0.31	<0.5	–	–
	0.81	34	0.51	56

Assays were done under the conditions in section 2 and extrapolated values for V_{max} and K_m obtained from Eadie-Hofstee plots shown in fig.1

Table 2 summarises the values for V_{max} and K_m for V_1 and V_3, showing that V_1 has both a higher V_{max} and lower K_m than V_3. The second order rate constant (V_{max}/K_m) is thus considerably greater for V_1, and this is true at all salt concentrations for both low and high K_m data. (It was not possible to obtain results at very low actin concentrations at 121 mM KCl because of the very low activities observed.) The second preparation of these myosins gave almost identical results at 21 mM KCl to those in table 2 for

both V_1 and V_3, but the data points at higher salt concentrations were more scattered, though in every case the maximum activity of V_1 was higher than V_3. Thus the V_1 isoenzyme has an actin-activated ATPase which is greater than that of V_3 under all conditions measured.

These experiments, taken with earlier data on electrophoretic and peptide mapping studies [1,2], confirm that different isoenzymes of myosin are produced in rat hearts during development and in response to varied thyroid status. The differences in the actin-activated ATPase activities of the two isoenzymes suggest that cardiac contractility can be modified by the control of gene expression in response to thyroid hormone.

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