Tropomyosin-Troponin-Induced Changes in the Partitioning of Free Energy Release of Actomyosin-Catalyzed ATP Hydrolysis as Measured by ATP-Phosphate Exchange

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ATPase activity and ATP-P_i exchange of unregulated (without tropomyosin-troponin) and regulated (with tropomyosin-troponin) acto-HMM were measured in media containing 0.2 mg/ml actin, HMM, and (when present) tropomyosin-troponin, 2 mM $MgCl_2$, 10 mM KCl, 2 mM NaN_3, 10 mM P_i (pH 7.0), 3 mM ATP. The following mean values for ATPase activity and for the rate of incorporation of P_i into ATP (each per mg HMM and per min) were obtained: unregulated acto-HMM 0.33 µmol P_i and 0.33 nmol P_i, regulated acto-HMM 0.54 µmol P_i and 1.06 nmol P_i. The ratio of P_i incorporation rate to ATPase activity was 1.01×10^{-3} for unregulated and 2.02×10^{-3} for regulated acto-HMM. From these ratios and from the overall free energy change of ATP hydrolysis it was calculated that under the prevailing experimental conditions in unregulated acto-HMM 62% and in regulated acto-HMM 66% of the free energy change of ATP hydrolysis occurs after the release of phosphate from actomyosin. It is probably this part of the free energy change that is used by the muscle for the performance of work.

Introduction

The basic mechanism underlying muscle contraction is the cyclical interaction between actin and myosin driven by the hydrolysis of ATP. This interaction, in the ordered muscle structure, gives rise to the "crossbridge cycles" (for a recent summary see [1]). *In vitro,* this interaction manifests itself as "actomyosin ATPase". For the understanding of muscle contraction it is necessary to correlate distinct reaction intermediates of actomyosin ATPase with specific states of the crossbridge cycle and to explain in which step the chemomechanical energy transformation takes place.

The starting point of the present investigation was the growing evidence that interaction in the presence of ATP of pure actin and myosin (which exhibits all basic features of actomyosin ATPase) can be further improved by the addition of the regulatory protein complex tropomyosin-troponin. This complex not only adds calcium sensitivity to pure reconstituted actomyosin but increases, under certain circumstances, the activation of myosin ATPase by actin (Shigekawa and Tonomura [2], Lehman and Szent-Györgyi [3], Bremel *et al.* [4], Dancker [5]; for summary see [6–8]). This "potentiation" [4, 6] is mainly performed by the tropomyosin part of the complex (Katz [9], Eaton *et al.* [10], see also [2–4]) which is able to transform the actin filament into a "high affinity state" [5, 7, 8] which has a higher affinity to myosin than pure actin has.

In this study the problem of actin-myosin interaction is approached by exploiting the fact that certain steps of actomyosin-catalyzed ATP hydrolysis are reversible. Wolcott and Boyer [11], Paulsen [12], Cardon and Boyer [13] observed incorporation of radioactively labeled P_i into ATP (ATP-P_i exchange) during myosin or actomyosin ATPase activity. This exchange was markedly enhanced by actin and, in actomyosin, by conditions which favor actin-myosin interaction [12]. An ATP-P_i exchange has also been observed in glycerinated fibers of insect flight muscle (Mannherz [14], Ulbrich and Rüegg [15, 16] or of vertebrate striated muscle (Gillis and Maréchal [17]). This ATP-P_i exchange was also enhanced under conditions of high actin-myosin interaction so that Ulbrich and Rüegg [15, 16], Reiermann *et al.* [18], Paulsen [12] interpreted this

Abbreviations and terms used: Acto-HMM: mixture of HMM and actin without tropomyosin-troponin (unregulated) or mixture of HMM and actin with tropomyosin-troponin (regulated). HMM, heavy meromyosin; PEI, polyethylene imine; P_i, inorganic orthophosphate; R, gas constant; T, temperature in kelvin; TCA, trichloroacetic acid.

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ATP-P_i exchange as being due to an "energy rich" actomyosin-ADP complex.

Thus, the ATP-P_i exchange seems to reflect the "strength" of actin-myosin interaction. Therefore, when the regulatory proteins strengthen actin-myosin interaction they should stimulate ATP-P_i exchange. In this paper I show that this is in fact the case. I will further show that from the ATP-P_i exchange the partitioning of the free energy change of ATP hydrolysis catalyzed by actomyosin can be deduced.

Experimental procedures

The preparation of actin, myosin and tropomyosin-troponin (as unseparated complex) has been described elsewhere [5]. HMM was prepared by digestion with α-chymotrypsin [19]. Incorporation of P_i into ATP and ATPase activity (*cf.* [5]) were measured in parallel assays which were identical to one another except that in the ATPase assays P_i was substituted by histidine. That ATPase activity was the same in both media was checked in the following way: Addition of ATP to acto-HMM causes a decrease of light scattering intensity. When the ATP is hydrolyzed by the ATPase activity of HMM, light scattering intensity rises again, so that in this way ATPase activity can be determined without phosphate measurement. Since such light scattering measurements revealed no difference of the ATPase activity in both media, it is justified to take the ATPase activity in the P_i-deficient assay as identical to that of the incorporation assay. ATPase activity was measured immediately before and repeated immediately after the incorporation experiments.

The concentrations in the incubation mixtures were: HMM 0.2 mg/ml, actin 0.2 mg/ml, tropomyosin-troponin (when present) 0.2 mg/ml, $MgCl_2$ 2 mM, KCl 10 mM, NaN_3 2 mM, ATP 3 mM, P_i (pH 7.0) 10 mM. We adjusted the specific radioactivity of P_i in such a way that we added so much $^{32}P_i$ (from Amersham-Buchler, Braunschweig, W-Germany) to a stock solution of 0.1 M P_i that in the final assays $^{32}P_i$ gave rise to $4-8 \times 10^5$ cpm per μmol of P_i.

In each experiment the time course of incorporation was measured in the following way: For each time intervall to be measured (mostly 3, 6, 9 and 12 min) 3 ml assays were prepared, the reaction was started by the addition of ATP and after the desired time 0.5 ml 10% TCA was added. After removing the precipitated protein by centrifugation each assay was diluted with water to about 25 ml and applied to a column (10×1 cm) of Dowex 1×8 in the chloride form. P_i and ADP were eluted with 0.01 M HCl, 0.05 M KCl [20]. When the optical absorption at 260 nm had dropped below 0.01 extinction units HCl was raised to 0.06 M in order to elute the ATP. We frequently checked by thin layer chromatography on PEI cellulose plates that the ATP fraction was free of ADP (the PEI cellulose plates were washed in 0.1 M HCl, rinsed with distilled water, then the probes were applied and the plates were developed with 0.1 M HCl).

The ATP eluted from the columns (about 50 ml per column) was concentrated by freeze-drying, redissolved in 2 ml H_2O, the ATP concentration was determined by optical absorption at 260 nm and the radioactivity was counted by standard liquid scintillation techniques. From the amount of radioactivity (which, together with the specific activity of the added P_i gives the amount of phosphate incorporated into ATP) and from the measured ATP concentration we could calculate the "specific activity" of ATP (that is the fraction of ATP that has become radioactive) by dividing the amount of radioactive phosphate by the amount of ATP that contains this phosphate.

Before calculating the specific activity of ATP, however, a correction had to be made, because control experiments without proteins had shown that we always eluted, together with ATP, an amount of radioactivity which could not be ATP but was probably due to various polyphosphates. This activity corresponded to about $1-1.5$ nmol P_i. Therefore this amount was subtracted and from the remaining radioactivity the ratio P_i/ATP (the specific activity of ATP) was calculated. When after 1 minute incubation time the radioactivity which was found in the ATP fraction corresponded to less than 1.5 nmol P_i, only 1 nmol P_i was subtracted, otherwise 1.5 nmol were subtracted.

Calculation of the incorporation rate (rate of ATP-P_i exchange)

Since ATP was hydrolyzed during the course of the experiments, the actual ATP concentrations varied. It was therefore desirable to calculate the incorporation rate at the beginning of the experiment (when nearly all the ATP was still present). In order

to do this, one has to know the initial velocity of the increase of the specific activity of ATP. For all experiments the specific activity of ATP was plotted against time (as in Fig. 1) in order to extrapolate back to the beginning of the experiment. Fortunately these curves were linear with time as long as no more than about 30% of the ATP were hydrolyzed. It is therefore assumed that the slope of these curves represents the initial incorporation rate. Then, the incorporation rate could be calculated from diagrams like that of Fig. 1 by the following formula: incorporation rate (μmol $P_i \times$ mg $HMM^{-1} \times min^{-1}$) = $\frac{\Delta a \times A}{\Delta t \times HMM}$ with a, amount of radioactive P_i divided by the amount of ATP that contains this phosphate; t, time in minutes; A, initial amount of ATP per ml ($= 3\ \mu mol \times ml^{-1}$); HMM, amount of HMM per ml ($mg \times ml^{-1}$).

Protein concentrations were determined photometrically at 280 nm wavelength using extinction coefficients of 1 for actin, 0.64 for HMM and 0.38 for tropomyosin-troponin (all in $cm^2 \times mg$).

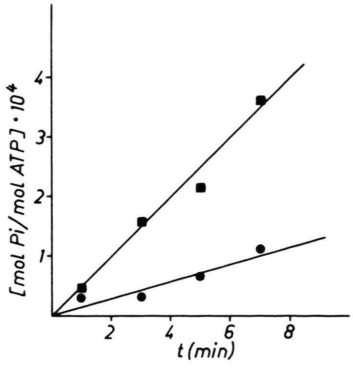

Fig. 1. Time dependence of incorporation of radioactive phosphate (P_i) into ATP. In all probes radioactivity equivalent to 1.5 nmol P_i was subtracted before the quotient P_i/ATP was calculated. The incorporation assays (3 ml each) contained 0.2 mg/ml actin, 0.2 mg/ml HMM and (in regulated acto-HMM) 0.2 mg/ml tropomyosin-troponin, 2 mM $MgCl_2$, 10 mM KCl, 2 mM NaN_3, 10 mM $^{32}P_i$ (pH 7.0). ATP was initially 3 mM. T: 22 °C; ● unregulated acto-HMM (without tropomyosin-troponin); ■ regulated acto-HMM (with tropomyosin-troponin).

Results

From Fig. 1 it can be seen that the regulated acto-HMM incorporated P_i much faster into ATP than unregulated acto-HMM did. Fig. 2B shows that this difference in not due to some unknown exchanging activity in tropomyosin-troponin. Neither actin alone nor actin plus tropomyosin-troponin without HMM exhibited a time-dependent incorporation of P_i into ATP. Measurable incorporation as well as the difference between unregulated and regulated actin were only obvious when actin could interact with HMM (note also the difference in incorporation between HMM alone and acto-HMM in Fig. 2A). The difference between regulated and unregulated acto-HMM is further not due to a stimulation of synthesis of enzyme-bound ATP from ADP and P_i [21, 22] because this synthesis should be ready after about 1 min [13, 21]. Therefore, all radioactive ATP which appeared after 1 min must be free ATP.

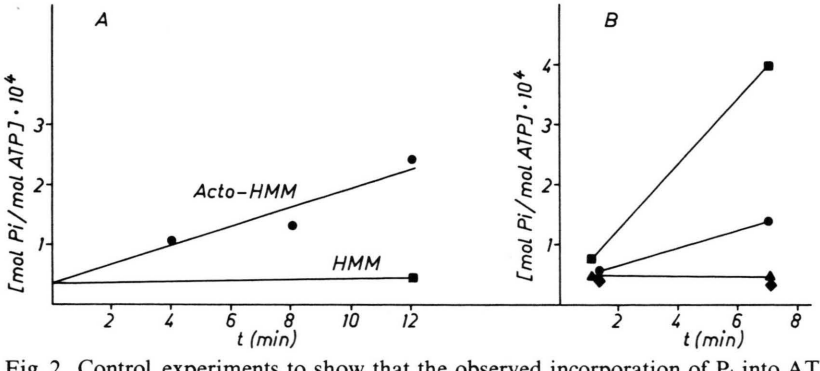

Fig. 2. Control experiments to show that the observed incorporation of P_i into ATP was due to actin-myosin interaction and not due to contaminations of unidentified enzymes. – A: Unregulated Acto-HMM (without tropomyosin-troponin) or HMM alone, other conditions as in Fig. 1. As background 1 nmol P_i was subtracted. – B: As A, with the following proteins: ▲ actin alone (without HMM); ◆ actin plus tropomyosin-troponin, without HMM; ● unregulated acto-HMM (without tropomyosin-troponin); ■ regulated acto-HMM (with tropomyosin-troponin); all concentrations as in Fig. 1.

Table 1. Rate of incorporation of P_i into ATP and ATPase activity of unregulated and regulated acto-HMM.

Exp.	Unregulated			Regulated		
	Incorp.	ATPase	Inc. / ATPase	Incorp.	ATPase	Inc. / ATPase
1	0.42	0.30	1.40×10^{-3}			
2	0.47	0.27	1.74×10^{-3}			
3				0.97	0.45	2.16×10^{-3}
4				2.00	0.47	4.26×10^{-3}
5	0.24	0.42	5.71×10^{-4}			
6	0.22	0.23	9.57×10^{-4}			
7	0.28	0.35	8.00×10^{-4}	0.78	0.62	1.26×10^{-3}
8	0.52	0.47	1.11×10^{-3}	1.44	0.71	2.03×10^{-3}
9	0.34	0.39	8.72×10^{-4}	0.80	0.54	1.48×10^{-3}
10	0.25	0.29	8.62×10^{-4}	0.68	0.52	1.31×10^{-3}
11	0.21	0.26	8.08×10^{-4}	0.75	0.45	1.67×10^{-3}
	0.33 ± 0.04	0.33 ± 0.03	$(1.01\pm0.12)\times10^{-3}$	1.06 ± 0.18	0.54 ± 0.04	$(2.02\pm0.39)\times10^{-3}$

The mean values are given with their respective standard errors of the mean. Incorporation is expressed in nmol $P_i\times$ mg $HMM^{-1}\times min^{-1}$; ATPase activity in μmol $P_i\times$ mg $HMM^{-1}\times min^{-1}$.

Table I summarizes the results of all experiments. It gives the rate of incorporation, the ATPase activity and the ratio of the rate of incorporation to ATPase activity. Although there is some scatter in the data, the rate of incorporation and, most important, the ratio of incorporation rate to ATPase activity is significantly higher in regulated than in unregulated acto-HMM. This does not only follow from the mean values averaged over all experiments but follows much more convincingly when unregulated and regulated acto-HMM were compared in the same experiments (expts. 7 – 11). The ratio of incorporation rate to ATPase activity is about 1:1000 in unregulated and about 1:500 in regulated acto-HMM. Ratios very close to this latter value have been deduced by other authors for "natural actomyosin" (which contains the regulatory proteins) [11, 12], and in glycerinated muscle fibers the ratio might be even lower [16, 17].

Discussion

ATP-P_i exchange and ATPase activity

Rüegg and his collaborators [12, 15, 16, 18, 23] have frequently stressed the proportionality between ATPase activity (experimentally modified by variation of calcium concentration, by addition of troponin I, by variation of oscillating frequency of insect flight muscle) and ATP-P_i exchange and have thus clearly shown that the exchange between P_i and ATP depends on actin-myosin interaction. Therefore, a stimulation of actomyosin ATPase by troponin-tropomyosin should stimulate ATP-P_i exchange simply on this basis. The main result of this paper, however, is not that tropomyosin-troponin in fact stimulated ATP-P_i exchange but that these proteins stimulated ATP-P_i exchange more than they stimulated ATPase activity. Hence the regulatory proteins alter the partitioning of free energy change of ATP hydrolysis catalyzed by actomyosin.

Thermodynamic interpretation of the ATP-P_i exchange

This interpretation is based on the assumption that the ATP-P_i exchange reflects the reversibility of steps of the ATPase reaction of actomyosin.

For total ATP hydrolysis the ratio of the reverse rate (V_r) to the forward rate (V_f) is

$$\frac{V_r}{V_f} = \frac{[ADP]\,[P_i]}{[ATP]\,K_{ATP}} \qquad (1)$$

(see Appendix for proof).

With the following values for the concentrations of reactants and products (which, in the present experiments, prevail after the hydrolysis of 10% of the initial ATP) and for K_{ATP} (= overall equilibrium constant of ATP hydrolysis): ATP = 2.7×10^{-3} M, ADP = 3×10^{-4} M, $P_i = 1.03\times10^{-2}$ M, $K = 10^5$ M ([24, 25]), the ratio V_r/V_f is 1.14×10^{-8} which is several

orders of magnitude lower than the ratio found in the present experiments. This shows that ATP-P_i exchange cannot comprise the reversibility of total ATP hydrolysis but only of a part of it. In other words, P_i leaves the reaction before ATP hydrolysis is complete (as it has already been deduced from kinetic evidence for myosin ATPase [26]). The following reaction sequence which is a condensed version of common ideas about actomyosin ATPase (*cf.* [25] for review) is consistent with this observation:

$$\begin{aligned} &AM + ATP \overset{K_1}{\rightleftarrows} M \cdot ADP \cdot P + A \overset{K_2}{\rightleftarrows} AM \cdot ADP \\ &\quad + P_i \overset{K_3}{\rightleftarrows} AM + ADP + P_i \end{aligned} \tag{2}$$

with A and M being actin and myosin, respectively, K_1, K_2, and K_3 are the equilibrium constants of the three steps with $K_1 K_2 K_3 = K_{ATP}$ (overall equilibrium constant of ATP hydrolysis).

In terms of reaction chain (2) ATP-P_i exchange reflects the reversibility of the following partial reaction:

$$AM + ATP \overset{K_1 K_2}{\rightleftarrows} AM \cdot ADP + P_i \tag{3}$$

with $K_1 K_2$ being the equilibrium constant of this partial reaction. V_f of this reaction can be deduced from the ATPase activity (which, in fact, is measured as the appearance of P_i). ATPase activity is a net rate and equals $V_f - V_r$, so that we have (in the case of regulated actin, see Table I)

$$\frac{V_f - V_r}{V_r} = 500 \quad \text{or} \quad \frac{V_f}{V_r} = 500 + 1 .$$

This relation shows that exchange rate divided by ATPase activity is sufficiently close to V_r/V_f.

From Eqn. (1) it can be seen that the ratio V_r/V_f immediately gives the free energy change associated with the respective reaction. For the hydrolysis of ATP to ADP and P_i the free energy change is

$$\Delta G = R\,T \ln \frac{[ADP]\,[P_i]}{[ATP]\,K_{ATP}} . \tag{4}$$

This relation is true for any concentration of reactants and products and is of course also true when the respective concentrations result from a steady state (in fact, "thermodynamics at this simple level applies more simply to steady-state systems than to closed systems" [27] p. 269).

Combination of Eqns (1) and (4) gives

$$\Delta G = R\,T \ln \frac{V_r}{V_f} . \tag{5}$$

This relation can be applied to any chemical reaction and is of course also true when a particular reaction is part of a larger reaction. Eqn. (5) does further not depend on the reaction path nor on the concentration of any reaction intermediates. This can easily be illustrated for ATP hydrolysis catalyzed by the reaction chain (2). For each particular step of this reaction chain the rate (V) equals the steady state concentration times the respective rate constant. Multiplication of all rates gives the following identity:

$$\frac{[M \cdot ADP \cdot P]\,[A]\,k_{-1}[AM \cdot ADP]\,[P_i]\,k_{-2}[AM]\,[ADP]\,k_{-3}}{[AM]\,[ATP]\,k_1[M \cdot ADP \cdot P]\,[A]\,k_2[AM \cdot ADP]\,k_3} \equiv \frac{V_{-1} V_{-2} V_{-3}}{V_1 V_2 V_3} .$$

On the left-hand side all reaction intermediates can be cancelled so that we have, together with

$$\frac{k_{-1} k_{-2} k_{-3}}{k_1 k_2 k_3} = \frac{1}{K_{ATP}} \text{ and with Eqn. (4)}$$

$$\frac{[ADP]\,[P_i]}{[ATP]\,K_{ATP}} = \frac{V_{-1} V_{-2} V_{-3}}{V_1 V_2 V_3} = \exp\,(\Delta G/R\,T) .$$

This expression is analogous to Eqn. (17) of T. L. Hill [28]. We see that the relation between rates on the one hand and the ratio of product and reactant concentrations or of free energy change on the other hand contains no reaction intermediates.

We are now in a position to calculate the partitioning of the free energy change of reaction chain (2). For the partial reaction (3) (comprising steps 1 and 2 of reaction (2)) we obtain by applying Eqn. (5): $\Delta G_{12} = R\,T \ln \alpha$ with α: rate of ATP-P_i exchange divided by ATPase activity. α was taken as 1.01×10^{-3} for unregulated acto-HMM and as 2.02×10^{-3} for regulated acto-HMM (see Table I). The free energy change of step 3 of reaction (2), that is of the total of events following after the release of phosphate, is $\Delta G_3 = \Delta G_t - \Delta G_{12} \cdot \Delta G_t$ is more difficult to obtain because the actual concentrations of ATP, ADP and P_i which have to be inserted into Eqn. (4) are uncertain, since our experiments are made under ATP-hydrolyzing conditions. A reasonable but somewhat arbitrary choice is to select a moment where only 10% of the initial ATP have been hydrolyzed because at this moment we still observe the initial incorporation rate. Then we have

Table II. Free energy change of actomyosin-catalyzed ATP hydrolysis $AM + ATP \overset{1}{\rightleftarrows} M.ADP.P \overset{2}{\rightleftarrows} AM.ADP + P_i \overset{3}{\rightleftarrows} AM + ADP + P_i$.

Acto-HMM	ΔG_t [kJ · mol^{-1}]	ΔG_{12} [kJ · mol^{-1}]	ΔG_3 [kJ · mol^{-1}]	$\Delta G_3/\Delta G_t$
unregulated	− 44.8	− 16.9	− 27.9	0.62
regulated	− 44.8	− 15.2	− 29.6	0.66

Conditions: ATP: 2.7×10^{-3} M; ADP: 3×10^{-4} M; P_i: 1.03×10^{-2} M, HMM, actin, tropomyosin-troponin (when present): all 0.2 mg/ml. *T*: 22 °C ΔG_t: overall free energy change of ATP hydrolysis; ΔG_{12}: free energy change of step 1 plus step 2, ΔG_3: free energy change of step 3.

the following values for Eqn. (4): 2.7×10^{-3} M ATP, 3×10^{-4} M ADP, 1.03×10^{-2} M P_i. The results of these calculations are given in Table II. From the high ATP-P_i exchange we must conclude that after the release of P_i about two third of the total free energy change of ATP hydrolysis are still in the actomyosin system. Since a similar situation prevails in the ordered actin-myosin system of muscle fibers [14 − 16], one has to assume that in muscle work is performed after the release of phosphate. This is substantiated by the observation of this paper that in regulated actomyosin (which is certainly nearer to the physiological situation than unregulated actomyosin) the free energy change of just this step is increased.

The equilibrium constants of the actomyosin ATPase scheme (1)

Sequence (2) is a minimum sequence for interpreting the ATP-P_i exchange with three equilibrium constants K_1, K_2, and K_3. The observation that about two third of the free energy change of ATP hydrolysis are associated with step 3 does not necessarily imply that K_3 must be large, it simply implies that the relevant steady state concentrations are far from equilibrium. For step 3 of reaction sequence (2) the following expression corresponds to Eqn. (4)

$$\Delta G_3 = R\,T \ln \frac{[AM]\,[ADP]}{[AM \cdot ADP]\,K_3}.$$

With $\Delta G_3 = -29.6$ kJ mol^{-1} and $[ADP] = 3 \times 10^{-4}$ M one obtains

$$\frac{[AM]}{[AM \cdot ADP]} \sim 0.02\,K_3.$$

This relation must be satisfied by any choice of rate constants for reaction sequence (2).

Concluding remarks

The high binding constant for ATP-myosin binding [11, 13, 21, 22] suggests that in myosin ATPase (in the absence of actin) the main free energy drop occurs during the binding of ATP to myosin prior to the hydrolysis of myosin-bound ATP. It is obvious that the same cannot be true for the physiological actomyosin system in muscle because the whole energy of ATP hydrolysis would be dissipated before work can be done by reattached crossbridges [8]. Therefore the binding of ATP to myosin has to be weaker in the presence of actin (*cf.* [29]) in order to keep free energy in the system mainly by keeping actin and myosin dissociated. In fact, displacement of bound ATP by actin from myosin has been observed [30]. However, as shown in this paper, the free energy stored in the dissociated actin-myosin mixture is not immediately released during recombination of actin and product-bearing myosin, but a considerable part, and an even larger one in regulated actomyosin, is still in the recombined actomyosin complex after the release of phosphate. One therefore has to conclude that the force in muscle which drives the working part of the crossbridge cycle is related to the chemical potential difference between the states AM · ADP and AM + ADP. Since the regulatory proteins increase just this potential difference they increase the part of the free energy change of ATP hydrolysis which can be converted to mechanical energy. The regulatory proteins therefore increase the efficiency of the actomyosin system which can be at the best, under the conditions of this paper, about 60%.

Appendix

The thermodynamic discussion of this paper is based on the concept that for any chemical reaction

$A \rightleftarrows C$ the following relations hold:

$$\Delta G = R\,T \ln \frac{V_r}{V_f} \tag{1}$$

where

$$\frac{V_r}{V_f} = \frac{[C]}{[A]\,K} \tag{2}$$

with K = equilibrium constant.

The validity of relation (2) is demonstrated in the following paragraphs.

1.) Direct interconversion of A and C.

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} C \quad \text{with } V_f = [A]\,k_1 \quad \text{and} \quad V_r = [C]\,k_{-1}.$$

Hence: $\dfrac{V_r}{V_f} = \dfrac{[C]\,k_{-1}}{[A]\,k_1}$ or $\dfrac{V_r}{V_f} = \dfrac{[C]}{[A]\,K}$.

That the ratio of products to reactants determines the ratio V_r/V_f remains also valid when second order reaction are considered, *e. g.* $D + A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} C + F$ since such a reaction can be easily transformed into a pseudo-first order reaction by incorporating one reaction partner into the rate constants:

$$A \underset{[F]\,k_{-1}}{\overset{[D]\,k_1}{\rightleftarrows}} C$$

so that the formal treatment remains unaltered. For the sake of simplicity only first order reactions are therefore considered in this appendix.

2.) Relation (2) remains of course also true when an intermediate B lies between A and C.

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} B \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} C.$$

Here V_f or V_r is the velocity with which A appears as C or C appears as A (mostly measured as the appearence of radioactive A in C or radioactive C in A). Then we have $V_f = [B_a]\,k_2$ where B_a is that part of B which stems from A (which would be labelled if A is radioactively labelled). From simple steady-state considerations it follows for the concentration of B

$$[B] = \frac{[A]\,k_1 + [C]\,k_{-2}}{k_{-1} + k_2}.$$

Then it follows for $[B_a]$ (that part of B which contains the label of A)

$$[B_a] = \frac{[A]\,k_1}{k_{-1} + k_2}.$$

Hence $V_f = \dfrac{[A]\,k_1\,k_2}{k_{-1} + k_2}$.

An analogous reasoning gives for $[B_c]$ (that part of B which stems from C and which would contain the label of C)

$$[B_c] = \frac{[C]\,k_{-2}}{k_{-1} + k_2} \quad \text{and for } V_r$$

$$V_r = [B_c]\,k_{-1} = \frac{[C]\,k_{-2}\,k_{-1}}{k_{-1} + k_2}.$$

Then

$$\frac{V_r}{V_f} = \frac{[C]\,k_{-2}\,k_{-1}}{[A]\,k_1\,k_2} = \frac{[C]}{[A]\,K}.$$

3.) When the reaction is catalyzed by an enzyme the situation is just the same:

$$A + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EB \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} C + E.$$

$V_f = [EB_a]\,k_2$ and $V_r = [EB_c]\,k_{-1}$ where EB_a and EB_c are the parts of EB stemming from A and C, respectively.

Then

$$[EB] = \frac{[E]\,([A]\,k_1 + [C]\,k_{-2})}{k_{-1} + k_2}$$

$$[EB_a] = \frac{[E]\,[A]\,k_1}{k_{-1} + k_2}$$

$$[EB_c] = \frac{[E]\,[C]\,k_{-2}}{k_{-1} + k_2}.$$

In this case V_r and V_f are still proportional to [C] and [A], respectively, but the proportionality factor $\left(\dfrac{[E]\,k_1\,k_2}{k_{-1} + k_2}\right.$ in the case of $\left.V_f\right)$ is no longer a constant (as in the uncatalyzed case), since [E] depends on the concentration of both A and C, but nonetheless we have

$$\frac{V_r}{V_f} = \frac{[E]\,[C]\,k_{-2}\,k_{-1}}{[E]\,[A]\,k_1\,k_2} = \frac{[C]}{[A]\,K}.$$

It can be seen that in all cases the same expression results which contains only the concentration of reactants, products and the overall equilibrium constant but no reaction intermediates. As long as the only flux is that between A and C, relation (2) is valid as it stands. Only when the net flux from A to C drives another flux against its corresponding po-

tential (as in active transport or in weight-lifting muscle, but this is not the situation of actomyosin ATPase activity in solution), the potential associated with the driven flux has to be included into K, which is then no longer the equilibrium constant of the uncoupled reaction.

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