THE STEADY STATE KINETIC CONSTANTS OF THE Mg-ACTIVATED MYOFIBRILLAR ATPase

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Received 10 March 1972

1. Introduction

Although the kinetic constants (K_m, V etc.) of myosin and actomyosin have been studied in extenso [1–9], there are wide discrepancies between workers, even over the order of the Michaelis constant (K_m). Quoted values of K_m for myosin vary between 10^{-4} M (Ca-activated) to 5×10^{-7} M (Mg-activated), and those for actomyosin by at least 20-fold [8, 9]. Two groups of workers [10, 11] have recently reported nearly identical K_m's of about 5×10^{-6} M for the myosin and myofibrillar ATP-ases (Mg-activated), which agree quite well with values quoted for the actin–heavy meromyosin system [12, 13].

This paper shows that the discrepancies probably arise from the fact that the myofibrillar enzyme, and possibly myosin also, show two quite distinct K_m's, one of the order of 10^{-4} M and the other of 10^{-6} M, with associated maximal velocity values (V) of 6.6 and 1.0 (in relative terms), respectively. The K_m's increase as the level of free Mg^{2+} is raised, but the values of V are unaffected, showing that Mg^{2+} is an inhibitor of the type where the inhibitor–substrate complex (*EIS*) has a lowered affinity for S, but breaks down at the same rate as the substrate complex (*ES*) [14].

2. Experimental procedure

Myofibrils were prepared from the back muscles of rabbits, and their ATPase activity at 35° was measured by the methods of Bendall [15]. The medium was: 40 mM imidazole, 78 mM KCl, pH 7.2, I = 0.12 to 0.13. It included 100 units per ml of purified creatine kinase (CK: EC 2.7.3.2) and 2 or 4 mM PC, to remove ADP as it was formed, since the latter otherwise inhibits the reaction. (The other product, P_i, does not inhibit even at concentrations of $40 \times S$). The amount of CK represents a large excess, adequate even at Mg^{2+} levels of less than 6 μM to maintain the ATP level above 90% of the initial. The ATP level was assayed in all the experimental samples by the spectrofluorimetric method of Scopes [16]. Note that the free Ca^{2+} level was maintained at 10^{-5} to 10^{-4} M, to ensure that the fibrillar ATPase was fully active [17].

The splitting of ATP was estimated by assaying aliquots for P_i and creatine, at 10, 20, 40, 100 and 180 sec intervals, by the enzymatic methods of Scopes [16]. These two assays generally agreed with one another within ± 5%.

The concentrations of $MgATP^{2-}$, $CaATP^{2-}$, Mg^{2+} and Ca^{2+} were calculated by Dr. R.H. Abbott (A.R.C. Muscle Contraction Unit, Oxford), using a modified version of the computer programme of Perrin and Sayce [18]. The $\log_{10}$ values of the main affinity constants employed were as follows: $MgATP^{2-}$ 4.9, $CaATP^{2-}$ 4.5, $KATP^{3-}$ 1.0, $NaATP^{3-}$ 1.0. These represent the most consistent values to be found in [19] for I = about 0.1. A small correction has to be applied for the chelating effect of PC. The $KATP^{2-}$ and $NaATP^{2-}$ constants are considerably lower than those expected from the recent thermodynamic constants of about 2.34 given by Mohan and Rechnitz [20], which reduce to about 1.45 at $I = 0.12$ (Debye-Hückel correction). The latter, however, yield values of the various chelates and free metals which are inconsistent with the detailed results to be described.

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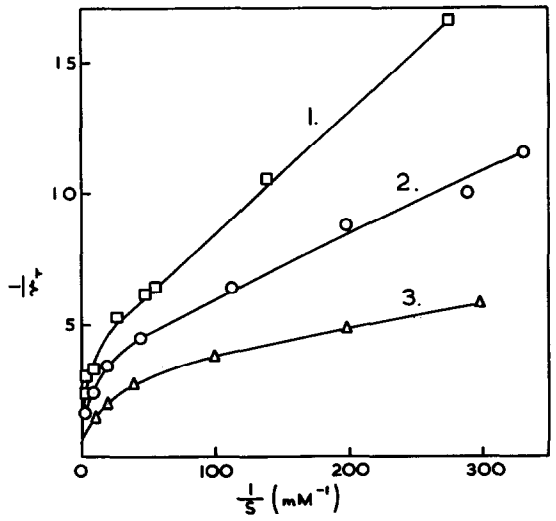

Fig. 1. Lineweaver-Burk plot ($1/v_r$ against $1/S$) at three Mg^{2+} concentrations, for the myofibrillar ATPase at pH 7.2, 35°, I = 0.12–0.13. Medium: 40 mM imidazole, 78 mM KCl, 2–4 mM PC + 100 units CK/ml, and 0.9 mg myofibrillar protein/ml. Curve 1: 3.9 mM Mg^{2+}; curve 2: 0.04 mM Mg^{2+}; curve 3: 0.0047 mM Mg^{2+}. Lines through points are calculated from constants in table 1.

3. Results and discussion

Assessment of the true initial velocity of ATP-splitting is made difficult by the fact that the progress curves tend to fall off fairly rapidly with time, no doubt because of rapid super-contraction of the myofibrils as soon as ATP is added [14]. To overcome this, the relative velocity (v_r) was used instead and was estimated by plotting the amount split at 10, 20, 40, 100 and 180 sec intervals against that split at the same times in a medium (control) containing 3.8 mM ATP, 4 mM $MgCl_2$, 0.4 mM $CaCl_2$ and 4 mM PC (+ CK). The relevant concentrations of reactive species were: $MgATP^{2-}$ 3.35 mM, Mg^{2+} 0.65 mM, $CaATP^{2-}$ 0.3 mM and Ca^{2+} 0.098 mM. Plots of this type were always found to be linear, except for occasional small blank errors, showing that interference from supercontraction occurs to the same extent regardless of ATP concentration, even down to less than 5 μM ATP. v_r is given by the slopes of the curves (relative to a mean absolute v in the control of 2.4 μmole/min/mg protein, for the first 10 sec of the reaction). Note that the substrate (S) is assumed to be $MgATP^{2-}$, since none of the other forms of ATP ($CaATP^{2-}$, $KATP^{3-}$, ATP^{4-} etc.) bear any consistent relation to v_r.

The Lineweaver-Burk plots in fig. 1, of $1/v_r$ against $1/S$ at three Mg^{2+} concentrations, are all markedly non-linear and hyperbolic, clearly showing that no single K_m value can account for the results. However, the three plots all straighten out and become nearly linear as $1/S$ increases (at 80 mM^{-1} at 0.04 mM Mg^{2+}, for instance), suggesting that the enzyme possesses two active centres, one with a high and the other with low K_m, associated respectively with high and low V values. Furthermore the slopes (K_m/V) increase markedly with increasing Mg^{2+}. Kinetic analysis of this type of plot is somewhat complex, but it can be carried out approximately by assuming that when the plots become linear at high values of $1/S$, any interference from the higher of the two postulated K_m's is minimal. The linear portions can then be extrapolated back to the ordinate to give an approximate value of $1/V$ corresponding to the lower K_m (for clarity, $1/V_2$ and K_2, respectively). By the method of least squares, the four available points at each of the two higher Mg^{2+} concentrations give intercepts of 4.08 and 3.96 on the ordinate (= $1/V_2$), showing that V_2 is about 0.25 in relative terms, irrespective of Mg^{2+} concentration (V_2 in absolute terms = about 0.6 μmole/min/mg protein). The curve for 0.0047 mM Mg^{2+} has to be treated as a special case, because of significant interference from the higher K_m, even at very low S.

Knowing V_2, the apparent K_2 values (K_2 (app)) can be evaluated, and are 5.34 and 11.60 μM, respectively, at 0.04 and 3.9 mM Mg^{2+}. K_2 (app) at 0.0047 mM Mg^{2+} is approx. 2.2 μM, after correction for interference from the higher K_m (app).

Carrying the assumptions one step further, it might be expected that the V value (V_1) associated with the higher of the two K_m's (K_1) would also be independent of Mg^{2+} concentration. We can write the Michaelis equation [14] for the two sites, for any particular Mg^{2+} concentration, as:

$$v_1 + v_2 = v_{obs} = \frac{V_1}{1 + \frac{K_1\,(app)}{S}} + \frac{V_2}{1 + \frac{K_2\,(app)}{S}} \qquad (1)$$

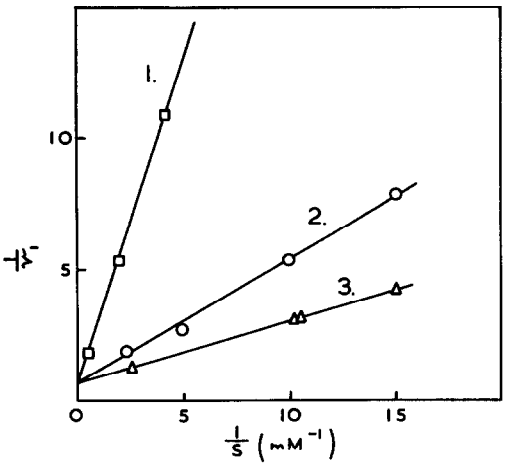

Fig. 2. Plot of $1/v_1$, as calculated in text, against $1/S$ at three Mg^{2+} concentrations. Conditions as in fig. 1.

Table 1
Kinetic constants of the myofibrillar ATPase.

	Site 1	Site 2
K_m (μM)	172.0	1.54
K'_m (μM)	418.0	11.83
K_i (μM)	8.89	8.83
V_{rel}	1.65	0.25
V_{abs} (μmole/min/mg protein)	3.96	0.60
V_{max} (μmole/min/mg protein)	4.56	

We can assess the second term on the right (= v_2) from the above constants, and thus v_1 (= $v_{obs} - v_2$) can be found for moderate to high values of S (> 50 μM). $1/v_1$ can then be plotted against $1/S$ (fig. 2) to give reliable estimates of the slopes (K_1(app)/V_1) at varying Mg^{2+} concentration. It is seen that the three linear plots in fig. 2 cut the ordinate at about the same point, and thus again yield a common value of the maximal velocity (V_1 in this case).

The above form of plot is not accurate enough to assess V_1 reliably, and it is preferable to re-arrange the relevant terms in equation 1) in the form:

$$1 = \frac{v_1}{V_1} + \frac{K_1\,(\text{app})}{V_1} \times \frac{v_1}{S} = \frac{v_1}{V_1} + \text{slope} \times \frac{v_1}{S} \quad (2)$$

v_1 can then be plotted against the term on the extreme right (modified Scatchard plot) to yield a value of V_1 on the v_1-axis and, theoretically, of 1.0 on the abscissa. This form is independent of Mg^{2+} concentration and so the results at the three Mg^{2+} concentrations can be combined to yield a common plot which, by the method of least squares, gives intercepts of 0.996 on the v_1/S axis, and of 1.654 on the v_1-axis (for N = 12, r = 0.996). V_1 in absolute terms = 3.96 μmole/min/mg protein, and thus $V = V_1 + V_2$ = 4.56 μmole/min/mg protein, which is considerably higher than any value of v observed in practice. The results of this plot and the earlier one for V_2 and K_2 are summarised in table 1. The values for K_1 (app) are 3.95, 0.79, and 0.255 mM, in descending order of Mg^{2+} concentration.

It is obvious from figs. 1 and 2 that Mg^{2+} is an inhibitor of the reaction, and it is also apparent that the *EIS* complex breaks down at the same rate as *ES*, but has a lower affinity for the substrate (a higher K_m (app)). This is confirmed by a further series of experiments, carried out at varying Mg^{2+} concentration (= m) and a constant S of 1.92 mM (see fig. 3), where the plot of $1/v_r$ against m is seen to be markedly hyperbolic. The general equation for this type of inhibition [14] is:

$$v_r = \frac{V}{1 + \frac{K_m}{S} \times \frac{1 + am}{1 + bm}} \quad (3)$$

where $a = 1/K_i$ and K_i is the dissociation constant of *EI*; $b = K_m/K_iK'_m$, where K'_m is the Michaelis constant of the *EIS* complex.

In the present case, two sites are present and so two equations of the type of equation 3 are required, one for V_1, K_1 and the other for V_2, K_2. Further, there is no evidence, a priori, that K_i and K_m/K'_m are identical for both sites.

The calculation of the constants in equation (3) can be simplified as follows: knowing the variation of K_1 (app) and K_2 (app) with Mg^{2+} concentration

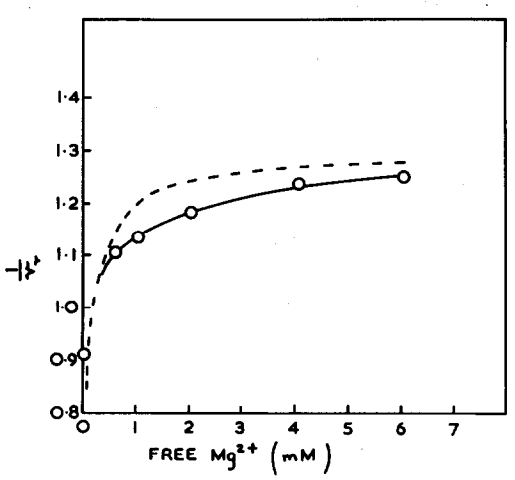

Fig. 3. Plot of $1/v_r$ against free Mg^{2+} concentration with S held constant at 1.92 ± 0.03 (SEM). Conditions as in fig. 1. Full line through points equals curve of best fit; broken line calculated from constants in table 1.

(see earlier) we can write a general equation:

$$K_m\,(\text{app}) = \frac{K_m\,(1 + am)}{1 + bm} \qquad (4a)$$

and thus:

$$K_m\,(\text{app}) - K_m + bmK_m\,(\text{app}) - amK_m = 0 \qquad (4b)$$

Equation 4b can be solved as a simultaneous equation in the three unknowns K_m, a and b, for which three values of m and K_m (app) are available in each of the two cases (i.e. site 1 with $K_m = K_1$ etc and site 2 with $K_m = K_2$ etc). The calculated values of K_1, K_2, K_1', K_2' and K_i are given in table 1. The only value in common for the two postulated sites is $K_i = 8.89$ μM, which is about 80 × the dissociation constant of the Mg^{2+}–myosin complex [21]. This might be expected if the main modifying effect of actin on the myosin ATPase is indeed to lower its affinity for Mg^{2+}, as Lymn and Taylor have suggested [8].

The proof of the validity of the overall equation derived from the above constants is provided by the closeness of fit of the experimental values of $1/v_r$ to the lines calculated at the three different Mg^{2+} concentrations in fig. 1, and also by the reasonably good fit of the values in fig. 3, at varying Mg^{2+} concentration and constant S = 1.92 mM. The agreement is still quite good, even when the 'free' ATP level (ATP^{4-} + $KATP^{3-}$ + $HATP^{3-}$) is increased above 2 mM, conditions which are unavoidable when it is required to keep S (= $MgATP^{2-}$) high and Mg^{2+} low. For instance, at 'free' ATP = 4.6, S = 4, Mg^{2+} = 0.03 mM, the observed value of v_r is 1.42 compared with the calculated value of 1.65.

It is concluded that two active sites are present at the ATPase centre, possibly associated with the two 'heads' of the myosin molecule [22]. Binding of ATP at one site (low K_m and V) interferes, either by steric hindrance or a charge effect, with binding at the second site, so that the affinity is reduced and K_m rises by more than 100-fold, with a concomitant increase in V by 5.6-fold. This suggests that the two enzymic heads of myosin lie closely apposed to one another in space. These considerations have, of course, to be seen in the light of recent studies of the transient kinetics of the system [11, 12], which seem to show that the rate-determining step in ATP-splitting is the release of products from the enzyme, itself modified by actin.

The results help to explain the wide discrepancies in the literature over the exact value of K_m (e.g. [1–13]), particularly as K_m (app) is so highly dependent on both Mg^{2+} concentration and on the actual range of ATP concentrations employed in any one set of experiments. The ATP concentration has to be varied by more than 300-fold to reveal the true nature of the splitting reaction and of the inhibitory effect of free Mg^{2+} upon it.

Acknowledgements

The author wishes to thank Dr. R.K. Scopes for analysing many of the samples and for his constant interest and advice, and Dr. R.H. Abbott for calculations of chelate and free metal concentrations. He also acknowledges with gratitude the expert technical assistance of Mr. C.C. Ketteridge.

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