FLUORESCENCE CHANGES ASSOCIATED WITH THE BINDING OF RIBOSE-5-TRIPHOSPHATE TO MYOSIN SUBFRAGMENT 1

Evidence for a second triphosphate binding site

John F. ECCLESTON

Department of Biochemistry and Biophysics, University of Pennsylvania, School of Medicine, Philadelphia, PA 19104, USA

Received 11 February 1980

1. Introduction

Recent discussions on the interaction of myosin subfragment 1 with ATP have been based on the protein having a single nucleotide binding site at which the ATPase reaction occurs (reviewed [1,2]). However, reports have appeared which have been interpreted as showing the presence of a second nucleotide-binding site on subfragment 1. This evidence is mainly based on deviations from Michaelis-Menten kinetics of the steady-state rate of hydrolysis of ATP by myosin and its proteolytic fragments [3–6].

This report describes the kinetics of the fluorescent changes of subfragment 1 on interaction with ribose-5-triphosphate (RTP). It is difficult to reconcile these results with a single binding site on subfragment 1. RTP has been shown [7] to be slowly hydrolysed by myosin although no quantitative data are given. It is a potent competitive inhibitor of RNA polymerase and is suggested to be bound at the active site in the 2-*endo* conformation of the pentose moiety [8] but no structural studies have been made yet to confirm this hypothesis.

2. Materials and methods

Subfragment 1 from rabbit skeletal muscle was prepared and characterized as in [9]. RTP was prepared by the condensation of ribose-5-phosphate with pyrophosphate [10] and characterized by chromatographic and electrophoretic methods [9]. In addition, the ^{31}P NMR spectrum was identical to that of ATP. Concentrations of RTP were determined by the orcinol method [11].

Steady-state rates of hydrolysis of ATP by subfragment 1 were determined by a linked-enzyme assay [12]. RTP hydrolysis was followed by assaying for P_i formation [13].

3. Results

When subfragment 1 is mixed with RTP in a stopped-flow spectrofluorimeter, there is a rapid exponential increase in the intrinsic fluorescence of the subfragment 1. At low concentrations of RTP, the observed rate constant of this process is proportional to the concentration of RTP but at high concentrations the rate approaches a plateau value (fig.1). This is consistent with a two-step process comparable to that occuring with ATP and subfragment 1 although at a slower rate [1,14,15]. The second order binding

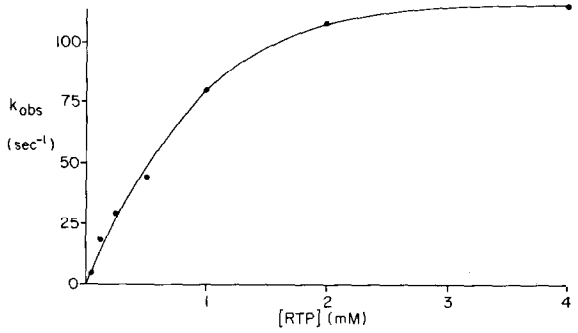

Fig.1. Dependence on RTP concentration of the rate of increase of protein fluorescence on mixing 10 μM subfragment 1 with RTP in a stopped-flow fluorimeter at 25°C. The solutions also contained 0.1 M KCl, 5 mM $MgCl_2$ and 50 mM Tris adjusted to pH 8.0 with HCl.

rate constant obtained from the linear part of fig.1 is $1.2 \times 10^5\ M^{-1} . s^{-1}$.

If subfragment 1 is mixed with greater than stoichiometric amounts of ATP at 25°C, the higher fluorescent state is maintained after the initial binding since this is the steady-state intermediate, until all of the ATP has been hydrolysed when it decays to the low fluorescent state. With RTP, however, the high fluorescent state is not maintained at all. It decays to a low fluorescent state biphasically in a manner that varies with the initial concentration of RTP.

Fig.2 shows the dependence of the rate of decay of the high fluorescent form on the RTP concentration. At 63 μM RTP, a small fast decay process is followed by a larger amplitude slow process. As the concentration of RTP is increased, the amplitude of the fast process increases and that of the slow process decreases until a 1 mM RTP, no slow process can be observed. The rates of both processes were determined from traces at more appropriate time scans than shown here. These traces are shown to allow a direct comparison of the processes at different RTP concentrations. The fraction of the fluorescence decay which occured rapidly increased hyperbolically with an app. K_d 300 μM. The rate constant for the faster process is relatively independent of RTP concentration, being 0.71 (±0.22 SD) s^{-1} over a 60-fold range of RTP concentration. The rate of the slow process (from the 63 μM RTP trace) is ~0.02 s^{-1}.

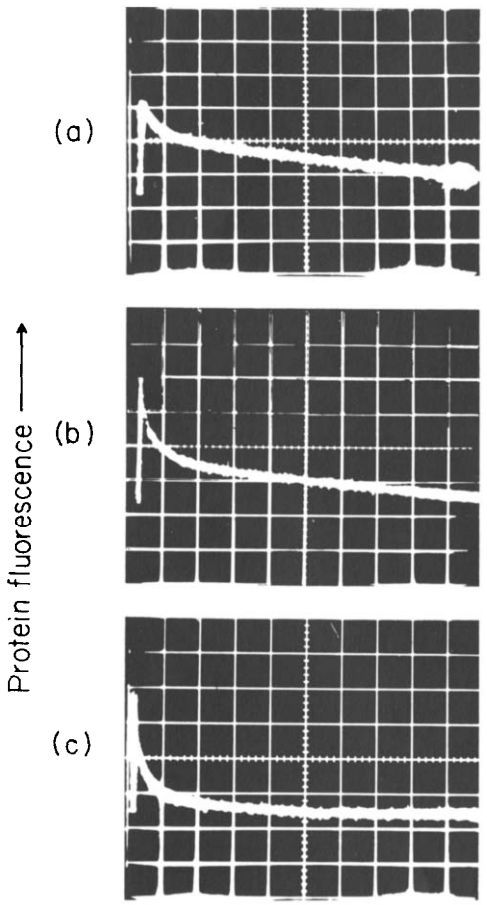

Fig.2. Stopped-flow spectrophotometric record of protein fluorescence during the interaction of subfragment 1 with RTP. One syringe contained 10 μM subfragment 1 and the other contained: (a) 63 μM; (b) 250 μM; (c) 1 mM RTP (final reaction concentrations). Solvent conditions were as in fig.1. Each major division is 5 s.

The rate of hydrolysis of RTP by subfragment 1 was determined by measuring the rate of P_i production in a solution containing 2 μM subfragment 1 and 1 mM RTP in 50 mM Tris–HCl, 0.1 M KCl, 5 mM $MgCl_2$ (pH 8.0 at 20°C). A control containing 2 μM subfragment 1 and 1 mM ATP was also measured. The steady-state rate of hydrolysis of RTP was found to be 0.016 s^{-1} compared to 0.038 s^{-1} for ATP.

The effect of RTP on the hydrolysis of ATP by subfragment 1 was measured using a linked-assay system. RTP is not a substrate for pyruvate kinase, and does not interfere with the linked assay. ATP was varied over 3–300 μM. Competitive inhibition by RTP was observed with K_i values of 330 μM and 210 μM at 0.29 mM and 1.16 mM RTP, respectively.

4. Discussion

The major finding of this work is that the relative amplitude of the 2 first-order fluorescence decay processes after the initial binding of RTP to subfragment 1 is controlled by the concentration of RTP. It is difficult to explain this result on the basis of a single binding site on subfragment 1. Before discussing a 2-site model, single RTP site models are first considered.

RTP exists as an equilibrium mixture of α and β anomers which may bind to subfragment 1 to give 2 different intermediates which then follow 2 different pathways:

$$M + \alpha RTP \xrightarrow{k_1} M.\alpha RTP \xrightarrow{\text{slow}}$$

$$M + \beta RTP \xrightarrow{k_1} M.\beta RTP \xrightarrow{\text{fast}}$$

In this scheme the initial 2 step process inferred from fig.1 (and which may well involve RTP cleavage) is considered as a single second-order process with a rate constant (k_1) of $1.2 \times 10^5\ M^{-1} . s^{-1}$.

This scheme could explain the biphasic decay process but does not explain the change in the relative amplitude of the 2 processes. Similary inter-conversion of the α- and β-forms of RTP on or off the enzyme cannot explain the results shown in fig.2. Two different populations of subfragment 1 could also give rise to a biphasic fluorescence decay, however, the changing relative amplitude is again incompatible with this explanation.

The results are compatible with two binding sites for RTP on subfragment 1. At low RTP concentrations it is envisaged that only a single site is occupied, and this situation is characterized by a slow fluorescence decay of the high fluorescent species. At higher RTP concentrations a second site is occupied with a K_d of 300 μM and this second site acts as an effector molecule on RTP at the first site, accelerating the rate of the fluorescence decay. However, it appears that the affinity for the second site is relatively low. Moreover the biphasic nature of the fluorescence decay indicates the process is not simple, otherwise a gradual increase in the rate constant of the decay would be expected rather than a biphasic process with changing relative amplitude.

The finding that RTP acts only as a competitive inhibitor of ATP hydrolysis by subfragment 1 at first sight argues against 2 binding sites. However, RTP binding away from the active site will not necessarily interfere with the ATPase activity. More surprising is the high K_i value of RTP as a competitive inhibitor; it might be expected that the measured K_i could equal its catalytic center activity divided by k_1 or 0.08 μM. (The fact that the data points in fig.1 extrapolate through the origin argues that RTP once bound does not readily dissociate from the site at which the fluorescence change in induced.) The possibility also exists that the initial fluorescence change is caused by RTP binding away from the ATPase active site and that RTP binding with K_d 300 μM at the active site is responsible for the effect in the protein fluorescence decay.

The role of the proposed second site for RTP binding in muscle is uncertain. RTP is not present in muscle, certainly not at concentrations used here where these effects are obtained. The second site could however be a binding site for some other nucleotide, possible ATP, although the relatively constant level of ATP in muscle argues against a regulation process.

If the proposed second site does indeed bind ATP, it is necessary to determine this in a direct method, and to explain why no evidence for this has so far been convincing. Weak binding is difficult to detect by many physical methods and also there is no a priori reason why the binding should perturb some property of subfragment 1 such as fluorescence or absorption. However, it would be expected to alter the kinetic processes by analogy with the results described above with RTP. A second site might explain the discrepencies between measured and calculated values for the K_m of the hydrolysis of ATP by subfragment 1.

Acknowledgements

I would like to thank Drs D. R. Trentham, M. R. Webb and D. Dennis for helpful discussions and Ms P. Hinney for the synthesis of RTP. This work was supported by grants from the National Institutes of Health (AM 23030) and the Whitehall Foundation.

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