CONFORMATIONAL DIFFERENCES IN MYOSIN. EVIDENCE FOR CHANGE IN RATE LIMITING STEP OF THE MAGNESIUM STIMULATED ITPase OF MYOSIN

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1. Introduction

Studies on enzyme conformation under equilibrium and steady state conditions give information about that intermediate which, preceding the rate limiting step of the reaction cycle, accumulates and hence represents the long lived species. However direct kinetic observations on a number of additional short lived forms of the enzyme in the pre-steady state are needed to identify the rate limiting step [1]. In the case of myosin it has been shown that chemical modification of either the substrate [2] or the enzyme itself [3] may result in another step becoming the rate limiting one rather than accelerating or slowing down all the elementary steps involved proportionally. We wish to report here changes in the rate limiting step induced by variation of ionic strength and temperature. This will be shown for the Mg^{2+}-stimulated ITPase of myosin where the predominant species in the steady state is an enzyme product complex whose conformation depends on the environmental conditions.

2. Methods

Myosin was prepared from white rabbit skeletal muscle according to Trayer and Perry [4] with 5 mM EDTA present in all solvents during the precipitation cycles subsequent to extraction [5]. The molecular weight of myosin bearing 2 active sites was taken to be 470 000 daltons [6, 7]. Alkylation of myosin was performed on 0.14–0.16 mg protein/ml for 5 min under various conditions as indicated in the text with 10–100 molar excess of *N*-ethylmaleimide (NEM) over active sites. Usually NEM was added 1 min after addition of the nucleotide and the reaction terminated 5 min later by addition of at least a 100-fold excess of dithiothreitol over NEM, unreacted reagents were removed by a washing procedure as described in detail elsewhere [5]. In the cases where the alkylation reaction was performed in the presence of 0.8 M KCl, the samples of 2–4 ml were dialysed after addition of dithiothreitol twice against 500 ml of 20 mM Tris–maleate pH 7.1 containing 10 mM EDTA prior to enzymic analyses. Subsequent to alkylation the K^+-stimulated ATPase activity of the dissolved myosin was measured in 10 mM EDTA, 1 M KCl and 25 mM Tris–HCl pH 7.6 or just in the dialysis solvent at pH 7.1 [8]. This ATPase activity of myosin was found to decrease with increasing amounts of NEM used during alkylation and the initial slopes of % loss in activity per μM NEM were taken as inactivation rates [5]. Steady state ITPase measurements were carried out on 1–2 mg of protein/ml in the presence of 5 mM $MgCl_2$, 2.5 mM ITP and 50 mM Tris–HCl or Tris–maleate for the pH ranges above and below 7.4 respectively. All buffers were prepared at the temperatures used for the enzyme reaction. Liberated phosphate was determined according to Fiske and Subbarow [9]. Protein concentrations were determined by the biuret method standardized on nitrogen estimations [10]. Distilled and deionized water was used throughout.

3. Results

The specific activities of the Mg^{2+}-stimulated ITPase given in table 1 were measured under condi-

Table 1

In activation rates of K^+-stimulated ATPase of myosin due to previous alkylation in the presence of 2.5 mM nucleotide, 5 mM 5 mM $MgCl_2$ and 25 mM Tris–HCL pH 7.6. The corresponding steady state activities of Mg^{2+}-stimulated ITPase were measured under the same conditions as the alkylation was performed.

Nucleotide	KCL (M)	Temperature (°C)	Steady state rate (μmole *P*/min/mg protein)	Life time of hydrolytic cycle per active site (sec)	Inactivation rate (% per μM NEM)	± σ	No. of experiments
ITP	0.8	25	0.0109	23	0.62	0.28	8
IDP	0.8	25	–	–	2.12	0.70	8
ITP	0.8	0	0.000366	703	7.40	–	3
IDP	0.8	0	–	–	7.32	–	3
ITP	0	25	0.0523	5	7.29	1.28	5
IDP	0	25	–	–	7.42	1.08	8
ITP	0	0	0.00175	146	7.89	–	3
IDP	0	0	–	–	7.04	–	3

tions under which alkylation was performed. The corresponding life times of a hydrolytic cycle per active site were calculated from these activities under the assumption that the 2 active sites of each myosin molecule [7] function independently of each other [11–13]. The alkylation reaction was carried out in the presence of a 5000-fold molar excess of ITP or IDP over active sites and 5 mM $MgCl_2$ at high and low ionic strength as well as at 25° and 0°C. The low inactivation rate found only at high ionic strength and 25°C indicates that under these experimental conditions myosin exists mainly in a form whose fast reacting thiol groups connected with the K^+-stimulated ATPase are rather protected from alkylation. The fast inactivation rate of about 7 occurring under all other conditions studied so far implies that usually these thiol groups are more exposed in myosin so that they react readily with NEM. Such differences in reactivity of a definite class of thiol groups have been reported to reflect different conformational states that a protein may adopt at least in the region of these groups [14].

Although in the presence of both substrate and product the inactivation slopes are low at high ionic strength and 25°C, they are significantly different from one another. During turnover of ITP the essential thiol groups are better protected than in the presence of the product IDP. The average of the ratio of the slopes in the presence of IDP to those with ITP from 8 experiments in which the alkylation with the 2 different nucleotides was always done in parallel, is 4.0 (ranging from 2–10). In the case of IDP experiments were also performed in the additional presence of 10 mM phosphate without having an effect on the slopes. Evidence that under these conditions both IDP and phosphate in conjunction with a divalent cation bind independently to myosin will be published later [15]. That the lower slope in the presence of ITP is connected with its turnover is shown in fig. 1.

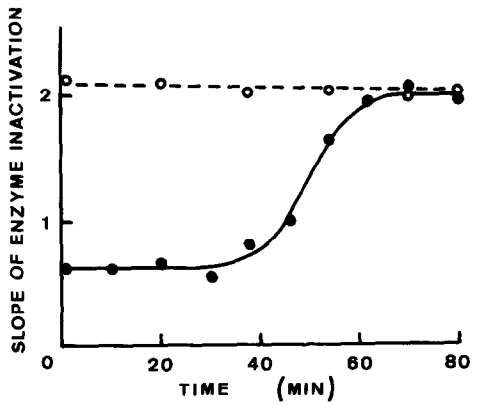

Fig. 1. Time course of change in inactivation rate of K-ATPase due to previous alkylation. Alkylation was performed after incubation of myosin with Mg-ITP for different periods of time. At zero time 0.36 μM myosin was mixed with 70 μM ITP in 5 mM $MgCl_2$, 25 mM Tris–HCl pH 7.6, 0.8 M KCl at 25°C. (●——●——●) ITP; (○——○——○) control samples with IDP.

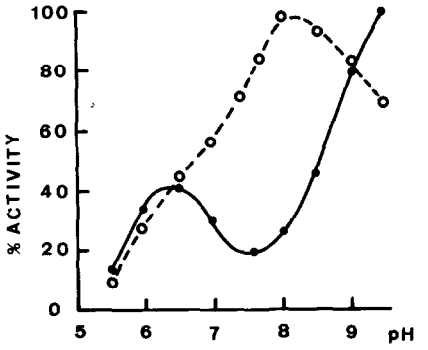

Fig. 2. Steady state rates of Mg-ITPase of myosin as a function of pH in the presence of 0.8 M KCl as indicated in Methods. (●——●——●) 25°C; (○——○——○) 0°C.

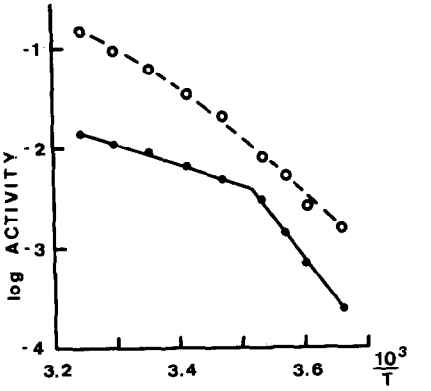

Fig. 4. Arrhenius plot of steady state rates of Mg-ITPase of myosin at pH 7.6 as indicated in Methods. (●——●——●) in 0.8 M KCl; (○——○——○) no added KCl.

In this experiment the alkylation reaction was performed after different times of incubation of myosin with an initial 90-fold molar excess of substrate over active sites. As the ITP becomes depleted with time the slope increases and approaches that of the controls mixed with IDP. The midpoint of the changing slope at 49 min would indicate an approximate life time of the hydrolytic cycle of 33 sec, a value in agreement with that deduced from steady state measurements with high substrate concentrations.

The Mg-ITPase steady state rate examined as a function of pH conssitently exhibits a minimum near pH 7.5 only at 25°C and KCl concentrations above 0.4 M (figs. 2 and 3). This minimum which is the well known characteristic of the Ca^{2+}-stimulated ATPase of myosin [16, 17] is absent under the other conditions. Although the logarithm of the activities at low ionic strength is almost a linear function when plotted against reciprocal temperature, the curve does not show the distinct change in slope found at high ionic strength (fig. 4).

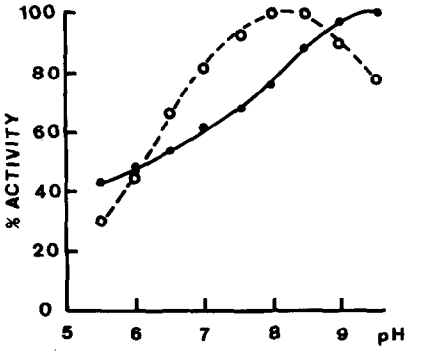

Fig. 3. Steady state rates of Mg-ITPase of myosin as a function of pH at low ionic strength as indicated in Methods. (●——●——●) 25°C; (○——○——○) 0°C.

4. Discussion

In the hydrolytic cycle of myosin when ATP or ITP in conjunction with a divalent cation is the substrate, the steady state rate is determined by the product dissociation step [18, 19]. The use of various techniques has established that the conformation of the long lived myosin product complex has a different conformation to that resulting from binding of myosin with the products whether the experiments are performed at high or at low ionic strength [13, 20–23]. The simplest kinetic scheme on the basis of these results together with those from fast kinetic studies can be written as in eq. 1. The species designated M_3ADPP occurring subsequent to the hydrolytic step represents that conformation of the protein whose essential thiol groups are protected [5] and which corresponds to the high fluorescent state [13]. The distinction

$$M_1 + ATP \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} M_2\,ATP \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} M_3ADPP \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}}$$

$$M_4ADPP \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} M_5ADP + P \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} M_1 + ADP + P \quad (1)$$

between the forms M_4ADPP and M_4ADPP and M_5ADP is based on their respective dissociation rates at low substrate concentrations, i.e. slightly below that of active sites [18, 19]. However in the millimolar range of ATP the dissociation rate of its product ADP approaches that of the phosphate [18]. Hence in the presence of high substrate concentration the transient state M_5ADP can be neglected at least under steady state conditions. With ITP as substrate the essential thiol groups are more protected than in the presence of IDP only at high ionic strength and 25°C. This difference implies that only under these latter conditions in the presence of ITP the protein adopts a conformation similar to that found in the presence of ATP at 25°C at high and low ionic strength [23], at least in those regions of the molecule where these thiol groups are located. Hence during the turnover of ITP the long lived species differs in its conformation from that induced by simple binding of the product IDP. However, that this species is a myosin–product complex must be inferred from the fact that the hydrolysis of ITP exhibits a phosphate burst under these conditions [24]. The rate of phosphate production was found to increase with substrate concentration. At the highest concentration of ITP studied so far, i.e. only about 50 times that of active sites, this rate is about 14 sec^{-1} per site but has not yet reached its maximum. On the other hand we found the steady state turnover at high substrate concentrations of about 3000 times over that of sites to be 0.086 sec^{-1} under comparable conditions to those used by Lymn and Taylor [24]. In other words the rate limiting step must be subsequent to the hydrolytic one. It is thus concluded that the predominant species during hydrolysis of ITP corresponds to the hydrolytically induced M_3ADPP state and that also in the case of ITP turnover k_{+3} is the rate limiting step at high ionic strength and above 20°C. At 0°C and high ionic strength no difference in the reactivity of the essential thiol groups is found. Since under these conditions the dissociation of the products is also the rate determining factor [18] the predominant species during turnover is the enzyme–product complex M_4IDPP. Therefore the lowering of temperature causes a shift from one rate limiting step to another whereby the life time of the hydrolytically induced M_3IDPP state is shortened in relation to that of the next state M_4IDPP. This change may be reflected in the rather sharp and large change in the slope of the Arrhenius plot of the steady state ITPase at high ionic strength which occurs around 12°C.

In addition to the change in rate limiting step a difference in the conformation of myosin affecting the essential thiol groups and thus the active sites also occurs in the presence of IDP between 0° and 25°C. This transition may be the cause of the shift in the rate limiting process which is at present under investigation. Temperature dependent transitions of this type are reported to cause sharp changes in the slope of Arrhenius plots [1]. No such change in the conformation of myosin as reflected by thiol group reactivity either in the presence of ITP or IDP between 0° and 25°C is evident at low ionic strength. Neither is there a marked change in slope of the Arrhenius plot. Since it has been reported that the product dissociation is rate limiting during the turnover of ITP at low ionic strength also [18], the results reported here indicate that k_{+4} is the rate limiting step over the entire temperature range. In conclusion both high ionic strength and a temperature above 20°C are needed for the step k_{+3} to be rate limiting during hydrolysis of ITP in the presence of magnesium. Furthermore it is this rate k_{+3} which has a minimum at pH 7.5 whereas in all other cases the rate of k_{+4} continuously rises with increasing pH.

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