THE EFFECT OF TREATMENT WITH 5,5'-DITHIOBIS-(2-NITROBENZOIC ACID) ON THE INITIAL RAPID PROTON LIBERATION DURING HYDROLYSIS OF ADENOSINE TRIPHOSPHATE BY MYOSIN

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SUMMARY. The initial rate of proton liberation during MgATP hydrolysis by myosin was followed in a stopped flow spectrophotometer: before and after treatment with 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB) with and without removal of the corresponding light chain. At pH 8, 20°, and in the presence of $MgCl_2$, the biphasic pattern of the initial rate of proton liberation for native myosin became monophasic following treatment with DTNB, removal of the corresponding light chain, and regeneration of the steady state ATPase activity. The rate constant characterizing the single exponential term increased with MgATP concentration attaining a maximum value of 100 s^{-1} at 300 µM MgATP with an apparent 2° rate constant of 7×10^5 $M^{-1}s^{-1}$. Both the biphasic and monophasic pattern of initial proton liberation observed for myosin and subfragment 1 respectively (Pemrick, S.M. and F.G. Walz, 1972. J. Biol. Chem. 247: 2959) can be explained by differences in the relative amounts of the DTNB light chain.

Several laboratories have shown that an adequate description of the initial rapid proton liberation (proton burst) observed during the presteady state interaction of myosin with MgATP requires two exponential terms (1) (2), whereas, a single exponential term (similar to the faster of the two rate constants for myosin) is sufficient to describe this process for the isolated catalytic region of the myosin molecule, subfragment 1 (S1)[3] (3)(2). Recent experiments by Koretz et al (3) describe a monophasic pattern of proton liberation for myosin, heavy meromyosin (HMM), and S1, which contradict their earlier

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[3] Abbreviations: adenosine-triphosphatase-ATPase; 5,5'-dithiobis-(2-nitrobenzoic acid)-DTNB; subfragment 1-S1; heavy meromyosin-HMM; ethylenediaminetetraacetic acid-EDTA; dithiothreitol-DTT.

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findings (1). The present report shows that when the presteady state pattern of proton liberation is observed under a variety of conditions two exponential terms are required to fit the data for native myosin. In addition this report gives an experimental explanation for the biphasic behavior of myosin. Myosin treated with DTNB releases approximately 80 percent of a single light chain component (plus minor amounts of two other light chain fractions (4), and upon regeneration of the thiol groups, the EDTA-K^{+}/Ca^{2+}-steady state ATPase activity is recovered (4)(5)(6). However, the presteady state pattern of proton liberation for DTNB treated myosin is monophasic. Myosin contains approximately 2 moles and S1 0.38 moles of the DTNB light chain (4)(7). It is, therefore, suggested that differences between myosin and S1 in the presteady state pattern of proton liberation are due to differences in the relative amounts of the DTNB light chain.

MATERIALS AND METHODS

Rabbit muscle myosin was prepared as described previously (2) and was not subjected to either chromatography on DEAE sephadex or ammonium sulfate precipitation. Myosin (15 mg/ml) was reacted with DTNB according to the procedure of Gazith *et al* (5) as described by Weeds and Lowey (4). The DTNB treated myosin was either: A) separated from the light chain fraction (dilution-precipitation), solubilized in 1 M KCl, and dialyzed against 0.5 M Tris-HCl, 5 mM DTT (pH 7.6) for several days; B) dialyzed directly against the above solution (reassociated myosin).

Protein concentration was determined by a modified Lowry (8) or microbiuret (9) procedure.

The steady state ATPase activity was assayed by means of the pH stat (at pH 6.9) (2) and the Tausky-Shorr (at pH 7.5) (10) method. The assay medium contained 0.5 M KCl, 2.5 mM K_2ATP and either 15 mM $CaCl_2$ (for the Ca^{2+}-ATPase) or 1 mM K_2EDTA (for the EDTA-K^{+}-ATPase). Prior to assaying the EDTA-K^{+}-ATPase, the samples were incubated (1 hr., 4°) in 10 mM K_2EDTA, 0.5 M KCl.

Electrophoresis on 10 percent polyacrylamide gels in 2 percent SDS and

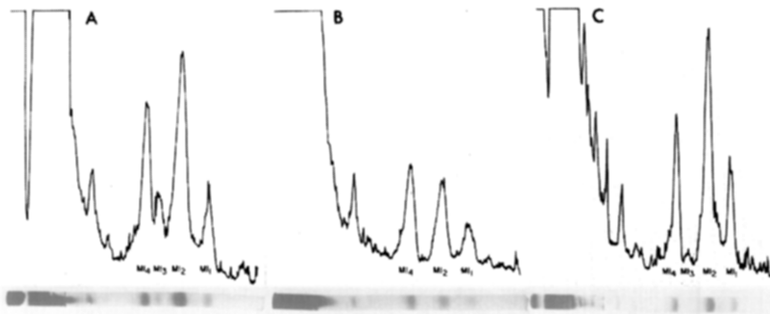


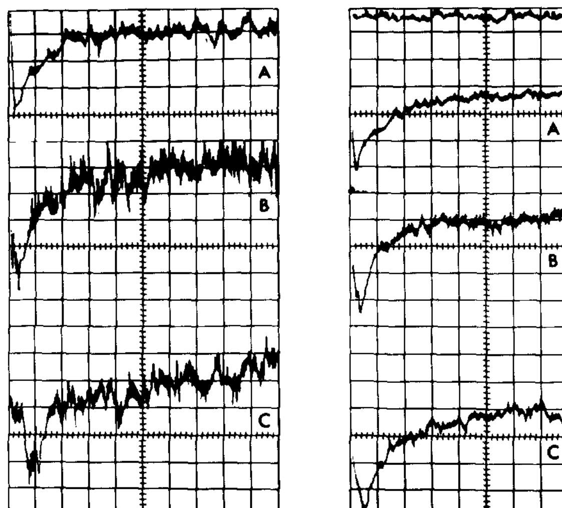
Figure 1. Electrophoretic patterns and densitometric scans for native and treated myosin: A, native myosin; B, DTNB treated myosin with removal of the released light chain fraction; C, DTNB treated myosin followed by reassociation of the DTNB light chain. 140 μg samples in 8 M urea, 1% SDS and 2% β-mercaptoethanol were applied to 10% gels. Conditions: 8mA/tube (5 hours) in 10 mM sodium phosphate buffer (pH 7.6), 1% SDS (11); stained in 1% fast green and scanned at 600 nm.

8 M urea followed the procedure of Weber and Osborn (11) as described for myosin (4). The gels were stained (1 hr.) in 1 percent fast green-7 percent acetic acid (12), destained, and scanned at 600 nm (Gilford recording spectrophotometer with a linear transport attachment).

A Durrum-Gibson stopped flow apparatus was used for the rapid kinetic studies. The "time constant" was always set at less than 1/10 of the horizontal setting of the oscilloscope. Determination of the initial rate of proton liberation and attainment of the steady state rate were described previously (2).

RESULTS

The Ca^{2+} and EDTA-K^+-ATPase activities are essentially unaltered for DTNB treated myosin following sulfhydryl regeneration, when the light chain fraction has been removed (Table 1). The Ca^{2+}- and EDTA-K^+-ATPase values are in the range reported by Weeds and Lowey (4). However, if the DTNB light chain is allowed to reassociate, only 20 percent of the original activity remains, which does not reflect a change in the conformation of the myosin molecule without reassociation of the light chain. The reassociated myosin was diluted 10-fold, the precipitate removed, and the supernatant concentrated and



Figures 2 (left) and 3 (right). Oscilloscope traces of photomultiplier signals due to the change in transmission after miximg myosin and MgATP. Final concentrations were: 3.7 μM myosin, 10 mM $MgCl_2$, 0.4 M KCl, 50 μM o-cresol sulfonephthalein and 60 μM MgATP (left) or 30 μM Mg-ATP (right); pH 8, 20°, 572 nm. Vertical scale, 20 mV/division at at full scale setting of 0.8 V. Horizontal scale for both Figs.: A, 50; B, 20; C, 10 msec./division.

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analyzed for protein (A_{280}, microbiuret, and Lowry methods). The results were negative.

Polyacrylamide gel electrophoresis of myosin in the presence of SDS and 8 M urea gives the well established 4 band pattern (Fig. 1) (13)(14)(15) designated ML_1, ML_2, ML_3, and ML_4 in order of decreasing mobility (14); ML_1 and ML_4 represent the alkali light chains (4). It is assumed that ML_2 and ML_3 represent the phosphorylated and unphosphorylated forms of the DTNB light chain (15), however, no attempt was made to quantitate the amount of bound phosphate in the present myosin preparation before or after treatment with DTNB

Protein analysis of the supernatant obtained upon precipitation of DTNB treated myosin indicated 70-100 percent of the calculated DTNB light chain had been removed. However, the electrophoretic pattern of this treated myosin indicates that ML_3 and 60 percent of ML_2 are absent. In addition minor amounts of the alkali light chains are removed (Fig. 1B). For reassociated myosin, the relative amounts of the 4 light chain fractions are similar to those of native

TABLE 1. STEADY STATE ATPase ACTIVITIES

		Ca^{2+} [a]	EDTA-K^{+} [a]
Myosin	Preparation A	.46	.81
	" B	.66	.90
DTNB-treated-myosin	Preparation A	.66[b]	2.54[b]
	" B	.53	.76
Reassociated myosin[c]	Preparation A	.085	.18

[a]All data expressed as µmoles Pi/min/mg and obtained by the Taussky-Shorr method (10), unless otherwise indicated.
[b]Data expressed as µmoles Pi/min/mg and obtained by the pH stat method.
[c]Myosin which has been reacted with DTNB followed by reassociation of the DTNB light chain.

myosin; however, only a minor amount of the DTNB light chain persists as MI_3 (Fig. 1C).

The initial rate of proton liberation for myosin is compared in Figs. 2 and 3 at 2 MgATP concentrations, and at several sweep speeds. At 60 µM MgATP (Figs. 2A and B) the oscilloscope trace appears flat (steady state) after approximately 80-100 msec. A semilog plot of these data would suggest that they are best described by two exponential terms. However, at a faster sweep speed (Fig. 2C) the biphasic pattern of the data is more apparent. The oscilloscope trace is flat after 10 msec., but this does not indicate attainment of the steady state rate, since the above traces at slower sweep speeds indicate a steady state rate only after 80-100 msec. Therefore, 2 exponential terms are required to fit the data for native myosin. This observation is confirmed at the lower MgATP concentration (Figs. 3A, B, and C).

The kinetics of the initial burst of proton liberation are simplified when the process is monophasic (Figs. 4A, B and C). For myosin treated with DTNB and the corresponding light chain removed, at 200 µM MgATP, and at 3 sweep speeds, a steady state rate of proton liberation is apparent after 60 msec. A semilog plot of the change in transmission as a function of time (Fig. 5) for the data in Fig. 4C is linear. Therefore, a single exponential term is sufficient to describe the approach to a steady state rate of proton liberation.

The rate constant, k, describing the exponential term for the DTNB treated

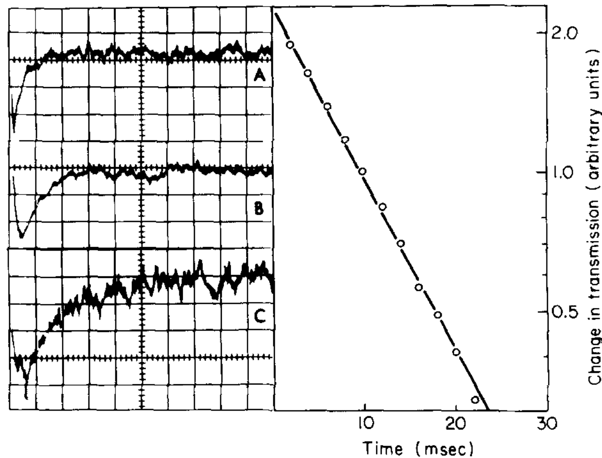

Figure 4. (left). Oscilloscope traces of the change in transmission after mixing DTNB treated myosin and MgATP. Final concentrations: 3.7 μM DTNB treated myosin, 200 μM MgATP, 10 mM $MgCl_2$, 0.4 M KCl, 50 μM o-cresol sulfonephthalein, 0.5 mM DTT; pH 8, 20°, 572 nm. Vertical scale: 50 mV/division at a full scale of 0.8 V. Horizontal scale: A, 50; B, 20; C, 10 msec./division.

Figure 5. (right). Semilog plot of the change in transmission with time observed in trace 4C.

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myosin increases with the MgATP concentration (Fig. 6). attaining a maximum value of 100 s^{-1} at 300 μM MgATP. There is some indication that the concentration dependence below 100 μM MgATP may not be linear. Assuming a linear relationship, the apparent 2° rate constant is $7 \times 10^5\ M^{-1}s^{-1}$.

If the DTNB light chain is allowed to reassociate, the presteady state pattern of proton liberation appears to be biphasic. However, until it is possible to recover at least 75 percent of the steady state ATPase activity (see Table 1), these last results remain tentative.

DISCUSSION

The present results indicate that the rate constant characterizing the slower burst reaction for native myosin is due to the DTNB light chain. This observation does not contradict recent hypotheses (2)(16) of interaction between the two S1 moieties of the myosin molecule characterized by the 2 rate constants for the initial proton liberation. Since a monophasic pattern of initial proton liberation is observed without complete removal of the DTNB light chain, and a

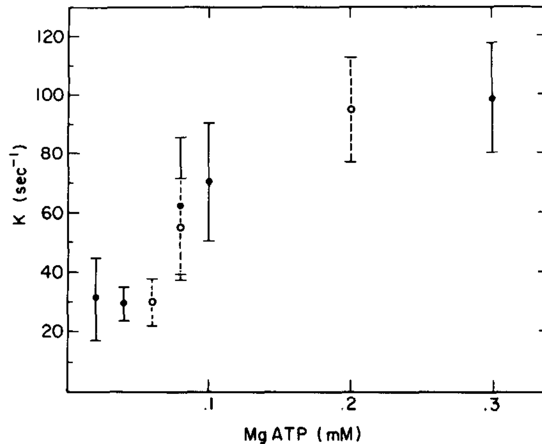

Figure 6. DTNB treated myosin. The effect of MgATP on the time constant. Data are included from two preparations, and indicate for each MgATP concentration the mean and S.D.; 3.7 μM DTNB treated myosin, 0.4 M KCl, 0.5 mM DTT, 50 μM o-cresolsulfonephthalein, and 10 mM $MgCl_2$ final concentrations; pH 8, 20°, 572 nm.

small fraction of the S1 molecules may retain the DTNB light chain (4)(7), the data suggest either interaction between sites or a heterogeneous population of myosin molecules. In the latter case, a threshold level of a particular population is necessary before a biphasic pattern of proton liberation is observed.

The value of k at low MgATP concentrations measures the apparent rate of substrate binding, k_{+1} (1)(17)(18). Although the value of k_{+1} for DTNB treated myosin is similar to that obtained for native myosin from rate measurements of phosphate liberation (17), it is lower by a factor of 2 from the value obtained for myosin and S1 from rate measurements of proton liberation. Since the steady state ATPase activities are similar for myosin, native and DTNB treated, and S1, the presteady state reaction scheme may differ for S1 and DTNB treated myosin, and in the latter case there is an increase in the rate of an intermediate step. It is not known whether or not pretreatment of native myosin with a phosphatase enzyme would result in a monophasic pattern of initial proton liberation.

REFERENCES

1. Finlayson, B. and Taylor, E.W. (1969) Biochemistry 8, 802-810.
2. Pemrick, S.M. and Walz, F.G. (1972) J. Biol. Chem. 247; 2959-2961.
3. Koretz, J.F., Hunt, T. and Taylor, E.W. (1972) Cold Spring Harbor Symp. on Quant. Biol. 37, 179-184.

4. Weeds, A.G. and Lowey, S. (1971) J. Mol. Biol. 61, 701-725.
5. Gazith, J., Himmelfarb, S. and Harrington, W.F. (1970) J. Biol. Chem. 245, 15-22.
6. Weeds, A.G. (1969) Nature 223, 1362-1364.
7. Taylor, E.W. (1972) Ann. Rev. Biochem. 41, 577-616.
8. Geiger, P.J. and Bessman, S.P. (1972) Anal. Biochem. 49, 467-473.
9. Itzhaki, R.F. and Gill, D.M. (1964) Anal Biochem 9, 401-410.
10. Taussky, H.M. and Shorr, E. (1952) J. Biol. Chem. 202, 675-685.
11. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
12. Gorovsky, M.A., Carlson, K. and Rosenbaum, J.L. (1970) Anal. Biochem. 35, 359-370.
13. Perrie, W.T., Perry, S.V. and Stone, D. (1969) Biochem. J. 113, 28P-29P.
14. Perrie, W.T. and Perry, S.V. (1970) Biochem. J. 119, 31-38.
15. Perrie, W.T., Smillie, L.B. and Perry, S.V. (1973) Biochem. J. 135, 151-164
16. Walz, F.G. (1973) J. theor. Biol. 41, 357-373.
17. Lymn, R.W. and Taylor, E.W. (1970) Biochemistry 9, 2975-2983.
18. Lymn, R.W. and Taylor, E.W. (1971) Biochemistry 10, 4617-4624.