The Role of Magnesium in the Relaxation of Myofibrils*

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ABSTRACT: Mg was necessary for relaxation of intact myofibrils even under conditions where it did not displace bound calcium. After most of the bound calcium had been removed by treatment with ethylene glycol bis(2′-aminoethyl ether)-*N*,*N*′-tetraacetic acid and magnesium so that the myofibrils contained only 0.5 μmole of calcium/g of protein (a residual amount of bound calcium which could not be removed under any circumstances), myofibrils in the presence of magnesium remained fully relaxed until sufficient calcium had been added to raise the amount of bound calcium above 1.4 μmoles of calcium/g of protein. If, however, magnesium was removed from the medium, syneresis was 85% complete with only 0.6 μmole of bound calcium/g of protein and remained constant on increasing the amount of bound calcium. It appeared that syneresis was independent of bound calcium when the magnesium concentration (contaminating magnesium was present) was very low. Syneresis was incomplete because of the low magnesium. With 0.6 μmole of bound calcium/g of protein the rate of adenosine triphosphate hydrolysis in the absence of added magnesium was twice that in its presence. Adenosine triphosphatase activity, however, increased with increasing bound calcium but this activity probably was not related to contraction but represented calcium-activated hydrolysis without energy transfer. Relaxation as well as syneresis and adenosine triphosphatase activity required magnesium as magnesium adenosine triphosphate. The activation of the adenosine triphosphatase activity with low magnesium adenosine triphosphate was determined only by the concentration of magnesium adenosine triphosphate and was not influenced by the concentration of free magnesium ion, but the inhibition of adenosine triphosphatase activity at higher concentrations of total magnesium depended upon both the concentrations of magnesium adenosine triphosphate and the free magnesium ion. While most of the bound calcium could be removed and 40% of the residual calcium was exchangeable, the myofibrils contained 4 μmoles/g of protein unexchangeable magnesium. Since this amount of unexchangeable magnesium equaled the content of monomeric actin in myofibrils, it appeared likely that myofibrillar actin contains magnesium as the tightly bound divalent cation rather than calcium.

The regulation of actomyosin contraction by Ca is a process of considerable complexity. It involves the participation of a protein complex of two or more additional proteins (Ebashi and Ebashi, 1964; Ebashi and Kodama, 1965; Hartshorne and Mueller, 1968) and requires the presence of ATP and Mg in concentrations of about 0.1 mM (Weber and Winicur, 1961; Maruyama and Watanabe, 1962; Weber and Herz, 1962, 1963; Endo, 1964; Levy and Ryan, 1965; Kaminer, 1968). Without any one of these factors, contraction occurs in the absence of Ca and relaxation cannot take place.

The experiments described in this paper were designed to clarify the role of Mg in relaxation. Earlier experiments showed that Mg displaces Ca from myofibrils (Weber and Herz, 1963). Without added Mg in the medium a considerable amount of Ca (about 2 μmoles/g of protein) remains bound even after lowering the Ca^{2+} concentration in the medium to 0.01 μM by chelation of Ca with EGTA (Weber and Herz, 1963). However, these experiments do not prove that Mg causes relaxation by displacing Ca from the regulatory site, because the total number of myofibrillar sites which bind Ca is about two times the number of binding sites which regulate contraction (Weber and Herz, 1963). Therefore Mg may displace Ca from sites not related to the regulation of contraction. In order to decide whether Mg causes relaxation by displacing Ca or by another mechanism, we attempted to remove all Ca from the myofibrils prior to the withdrawal of Mg. Since the Mg requirement for relaxation persisted even after removal of bound Ca, Mg is not just needed for the displacement of Ca. A brief summary of some of these experiments has been published previously (Weber, 1966).

Experimental Section

Removal of bound Ca from intact, KCl-washed myofibrils (Weber, 1959) was achieved by treatment with 1 mM EGTA and 1 mM Mg in the presence of 0.1 M KCl and 10 mM imidazole (pH 7.0) at 0°, followed by two to five washes with 0.1 M KCl. Even though 1 mM Mg was present during the EGTA treatment not all of the Ca could be removed but 0.45–0.5 μmole/g of protein residual Ca always remained bound (Figure 1).

Myofibrils low in bound Mg were obtained by three washes with a solution containing 1 mM EDTA, 10 mM CaEDTA, 0.1 M KCl, and 10 mM imidazole (pH 7.3) at 0°, followed by one wash with 0.1 M KCl which diluted the CaEDTA to about 0.8 mM. This treatment reduced the total Mg content in the preparation from 5.4 to 4.0 μmoles per g of protein.

Determination of Ca and Mg. Except for some of the measurements described in Table I, Ca or Mg present in myofibrils was determined after extraction in 2% trichloroacetic acid by atomic absorption with a Perkin-Elmer spectrophotometer, Model 303 (Willis, 1960). A comparison with the Ca measured after ashing of the myofibrils showed that all of the bound Ca could be extracted by trichloroacetic acid. In

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TABLE I: Ca and Mg Bound to Myofibrils following Different Pretreatment.

Prepn	Pretreatment	μmoles/g of Protein Ca	Mg	Ca + Mg
1	0.1 M KCl	3.6	4.4	8
1	1.0 mM Mg	2.1	5.6	7.7
1	1.0 mM Mg + 1.0 mM EGTA[a]	1.0	6.2	7.2
2	1.0 mM Mg + 1.0 mM EGTA[a]	1.2	7.0	8.2
3	1.0 mM Mg + 1.0 mM EGTA[a]	1.1	6.8	7.9
4	0.1 M KCl	3.5	5.4	8.9
4	10 mM Ca-EDTA–1 mM EDTA		4.0	
5	1.0 mM Mg + 1.0 mM EGTA[b]	0.5		

Conditions for the pretreatment are given in the Experimental Section: [a] followed by five washes with 0.1 M KCl–10 mM imidazole (pH 7.0); [b] followed by only two washes.

some of the experiments in Table I, after extraction with perchloric acid (about 0.3 N) and neutralization, Ca was titrated as described previously (Weber *et al.*, 1966), and total divalent cations were titrated according to Schwartzenbach and Biedermann (1948). Mg was calculated from the difference between total divalent cation and Ca. Contamination of the KCl and imidazole solutions by Ca was below 4 μM, the limit of detection with the colorimetric method. The Ca contamination of ATP was determined by precipitating ATP with 5% $LaCl_3$ and examining the supernatant for atomic absorption. The ATP (crystalline sodium ATP, low in Ca, Sigma) contained 7.5 mmoles of Ca/mole of ATP.

The fraction of bound residual Ca which was exchangeable was measured by comparing the specific activity of Ca present in myofibrils to which ^{45}Ca had been added prior to washing with specific activity after the treatment for Ca removal had been completed; 0.21 μmole of the 0.5 μmole/g of protein residual Ca, *i.e.*, 40% was found to be exchangeable, a value similar to that found by others (Fuchs and Briggs, 1968).

Ca binding on readdition of Ca was calculated as previously described (Weber and Herz, 1963) from the distribution of ^{45}Ca between supernatant and precipitate (centrifugation at 3000*g* for 1 min) and the concentration of total Ca (sum of the added Ca, Ca present in the myofibrils, and Ca in the ATP).

ADP bound to myofibrils was extracted by 0.3 N $HClO_4$ and after neutralization was measured by an enzymatic method (Bergmeyer, 1965).

Exchangeability of Bound Myofibrillar ADP. Myofibrils were allowed to hydrolyze 1 mM [^{14}C]ATP to ADP for 5 min.

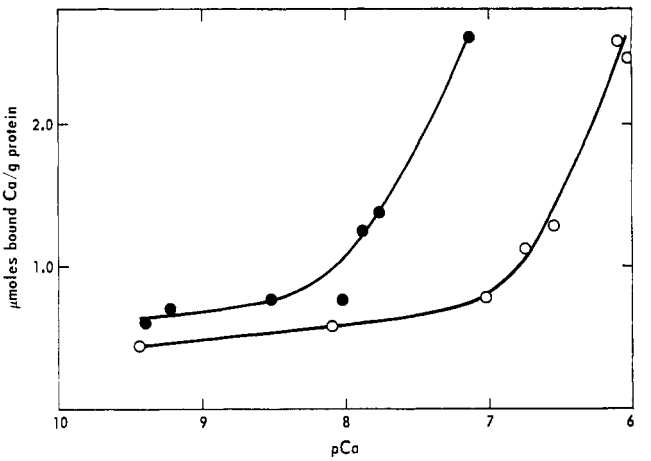

FIGURE 1: Ca binding as a function of pCa without and with added Mg present. Ionic strength, I = 0.1; 10 mM imidazole (pH 6.5); 5.4 mM ATP; EGTA varying from 13.5 to 0 mM; 13 mg of myofibrillar protein/ml; 3.7-ml total volume; temperature 24.8°. (●) No Mg added; (○) 5.4 mM Mg added; 0.4 mM Mg^{2+} and 5.0 mM MgATP calculated with the constants determined by Burton (1959).

The specific activity of the ADP present at this time was compared with the specific activity of the ADP after several washes with 0.1 M KCl; 2% of the bound ADP was found to have exchanged.

Syneresis and ATPase activity were determined under conditions that are described in the figure legends. After the addition of ATP to a vigorously stirred myofibrillar suspension, which was followed immediately by centrifugation at 3000*g* for 1 min, the extent of syneresis was estimated from the concentration of myofibrillar protein in the pellet. Syneresis was indicated by an increase in the protein concentration above 20–25 mg/ml (the concentration of protein found in the absence of ATP which was taken to represent O syneresis). Percentages of syneresis were calculated as previously described (Weber *et al.*, 1963). Assays for the determination of ATPase activity were started by the addition of ATP and terminated by trichloroacetic acid in a concentration of 4%. P_i in the filtrate was measured according to Taussky and Schorr (1953).

Calculation of the Concentrations of MgATP and Mg^{2+}. The concentration of MgATP was equal to the concentration of total Mg because ATP was added in such excess over Mg that the free Mg present was calculated to be only a few per cent of the total Mg. The calculation was based on the association constant for Mg and ATP determined by Burton (1959).

Results and Discussion

Residual-Bound Myofibrillar Ca and Actin-Bound Cations. Pretreatment of the myofibrils with Mg and EGTA removed a large part of the bound Ca but not all of it. A residual amount of bound Ca of about 0.4–0.5 μmole/g of protein could not be removed even in the presence of added Mg and a concentration of ionized Ca below 10^{-9} M (Figure 1). When the treatment by EGTA and Mg was followed by five washes with KCl instead of two, the residual Ca was higher (about 1 μmole/g of protein; Table I), suggesting that KCl contained some contaminating Ca. Ca that was removed was probably replaced by Mg since the total amount of bound cation remained roughly constant (Table I).

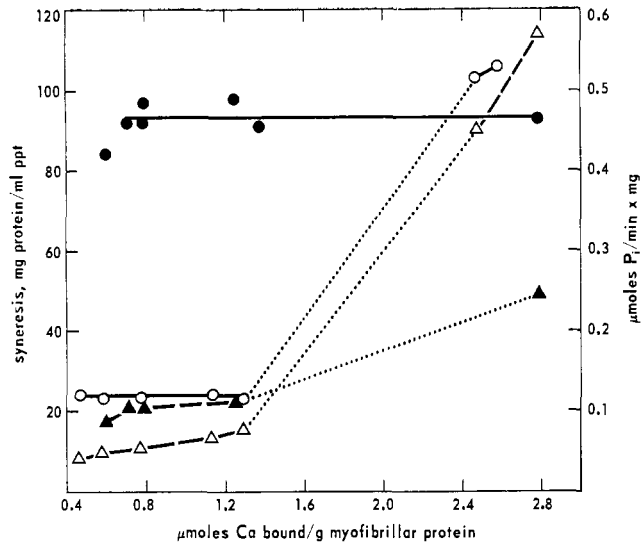

FIGURE 2: Syneresis and ATPase activity with and without added Mg as a function of bound Ca. (●, ○) Syneresis; (▲, △) ATPase activity; (●, ▲) no Mg added; (○, △) 5.4 mM Mg. Conditions as in Figure 1. For syneresis and determination of bound Ca at 3000*g* centrifugation for 1 min.

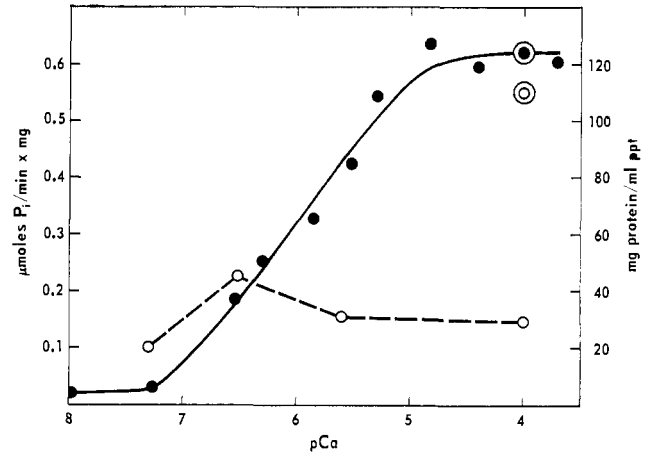

FIGURE 3: Syneresis and ATPase activity as a function of pCa with myofibrils washed as free of Mg as possible. I = 0.1; 10 mM imidazole, pH 7.0; EDTA varying from 1.1 to 0 mM; CaEDTA from 0.1 to 15 mM; 1 mM ATP; 2.2 mg of myofibrillar protein/ml; 10-ml total volume; 24.8°; (○) syneresis; (●) ATPase activity; (◎) maximal syneresis with 2 mM Mg; (◉) maximal ATPase activity with 2 mM Mg.

We are not certain where the residual Ca is bound. It does not represent the unexchangeable cation bound to fibrous actin (Barany *et al.*, 1962; Oosawa *et al.*, 1964) for the following reasons. First, 40% is exchangeable. The percentage may even be higher because back-exchange with traces of cold Ca in the washing KCl may have caused the final specific activity to be too low. Secondly, the concentration of the residual myofibrillar Ca was much smaller than the concentration of actin monomer, a finding which was recently confirmed (Fuchs and Briggs, 1968). Fibrous actin prepared from acetone powder contains as much unexchangeable Ca (Barany *et al.*, 1962) as unexchangeable ADP (Martonosi *et al.*, 1960), *i.e.*, one per monomer. By comparison myofibrils with an amount of bound Ca of 0.5 μmole/g of protein contained 4 μmoles of unexchangeable ADP/g of protein.[1]

It is interesting to consider briefly how cation binding by myofibrillar actin differs from that of isolated actin according to our data. It is possible that the myofibrillar actin originally contained bound Ca that was exchanged for Mg during the Mg + EGTA treatment. That would mean that F-actin-bound Ca is exchangeable in myofibrillar actin but not in isolated actin; and thus that differences exist in the conformation of myofibrillar and isolated actin. The other possibility, that the bound cation of the myofibrillar actin is also unexchangeable, but that it is Mg instead of Ca seems more likely, because it was not possible to reduce the concentration of bound Mg below 4 μmoles/g of protein (Table I). The myofibrillar content of strongly bound Mg which could not be replaced by Ca equaled that of the unexchangeable ADP just as unexchangeable Ca equals ADP in isolated actin. Since experiments with isolated actin showed that polymerization in the presence of a high ratio of Mg/Ca results in F-actin containing mostly bound Mg instead of Ca (Drabikowski and Strzelecka-Golszewska, 1963; Oosawa *et al.*, 1964), myofibrillar actin may contain bound Mg because of the high ratio of Mg/Ca in the muscle cell.

Mechanism of the Relaxing Action of Mg. Mg displaced Ca from myofibrils, possibly by direct competition for the same sites. Thus a reduction of the bound Ca to 1.4 μmoles/g of protein required that the concentration of Ca^{2+} in the medium was lowered to 0.01 μM when Mg was absent as compared with 0.2 μM in the presence of 5 mM MgATP and 0.4 mM free Mg ion (Figure 1).

However, Figure 2 shows that Mg has still another function in relaxation (*i.e.*, inhibition of syneresis and ATPase activity) because without added Mg, relaxation did not take place when nearly all except the residual Ca had been removed. The residual Ca of 0.45–0.5 μmole/g of protein, which could not be removed by Mg and EGTA (Figure 1), did not cause any syneresis in the presence of Mg whereas in its absence syneresis was 85% complete with 0.6 μmole of Ca/g of protein. In the absence of Mg the extent of syneresis did not increase on increasing the bound Ca; apparently syneresis was independent of Ca. In the presence of Mg syneresis depended upon bound Ca and increased from 0% when 1.4 μmoles of Ca was bound per g of protein to 100% with 2.5 μmoles/g of protein. In the absence of added Mg, syneresis remained below 100% because the concentration of Mg introduced as a contaminant was not high enough to satisfy the well-known Mg requirements for syneresis (Szent Györgyi, 1947; Weber and Portzehl, 1952).

[1] This value for bound ADP is similar to that reported by Szent Györgyi and Prior (1966) and between the values measured by Perry (1952) and those measured by Seraydarian *et al.* (1962). From these data and a more recent molecular weight of 45,000 (Rees and Young, 1967; Tsuboi, 1968) one calculates that 18% of the myofibrillar protein is actin. This value is much higher than the 11% found by Corsi *et al.* (1966) by the difficult method of differential extraction without the use of a specific marker for actin. It agrees well, however, with the value (20%) we calculated (following a suggestion by A. G. Szent Györgyi) from the number of thin filaments and the amount of myofibrillar protein per ml of muscle (Huxley, 1960) and the length of an actin monomer (2 monomers/53 Å). It is also similar to the value calculated on the basis of the finding by Huxley and Hanson (1957; Huxley, 1960) that the I filament protein minus the Z line substance amounts to 31% of myofibrillar protein and subtracting one-fourth of the protein for tropomyosin and troponin (Ebashi and Endo, 1968).

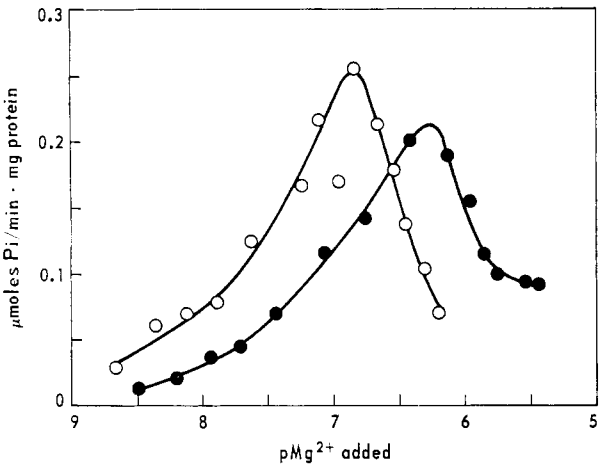

FIGURE 4: ATPase activity in the absence of Ca^{2+} as a function of ionized Mg. I = 0.1; 10 mM imidazole, pH 7.0; 2.2 mg of myofibrillar protein/ml; 10-ml total volume; 24.8°; 1.0 mM EGTA; MgATP from 10^{-7} to 4×10^{-4}; unchelated ATP: (○) 5 mM; (●) 1 mM; 30-sec incubation with ATP.

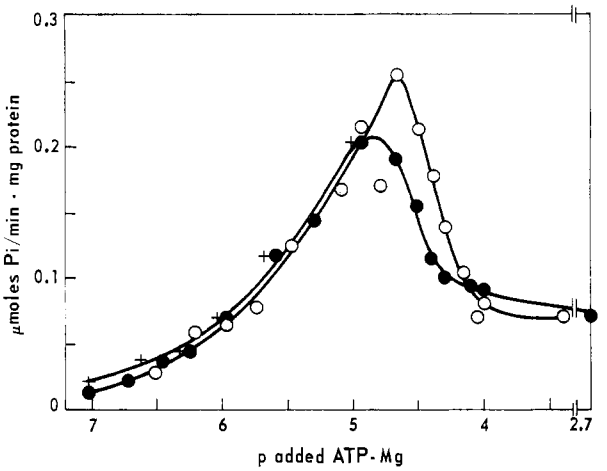

FIGURE 5: ATPase activity in the absence of Ca as a function of MgATP. Same experiment as in Figure 4. (●) (+) 1 mM unchelated ATP; (○) 5 mM unchelated ATP.

Ca cannot replace Mg for contraction (Hasselbach, 1956); on the contrary, syneresis was suppressed when Mg was replaced by Ca (Watanabe and Yasui, 1965).

In the absence of added Mg, the ATPase activity was twice as high as in its presence, even when all but the residual Ca had been removed (Figure 2). This activated rate amounted to about 15% of the maximal rate. It was presumably limited by the low concentration of Mg present.

Thus Mg is required for both contraction and relaxation; and the dependence of both processes upon the Mg concentration was such that increasing the Mg concentration, in the absence of Ca, resulted first in the activation of ATPase activity (and syneresis, not shown here) followed by inhibition (Figure 5). One may ask whether myofibrils are also relaxed in the complete absence of Mg and Ca (Figures 3–5) when syneresis does not occur and ATPase activity is close to zero. Experiments which determine only syneresis and ATPase activity cannot supply the answer, because they show only that there is no ATP-activated interaction between actin and myosin, but they do not indicate whether actin and myosin are dissociated (= relaxation) or whether they have formed a stable complex (= rigor). Inhibition of syneresis and ATPase activity *in the presence of Mg* is known to be caused by dissociation of actin and myosin from a variety of experiments other than measurement of syneresis and ATPase activity (*cf.* Weber, 1966). Measurements of stretch resistance by Hasselbach (1956) after removal of divalent ions by 2 mM EDTA show it to be lower in the presence of 5 mM ATP than in its absence when rigor exists. Stretch resistance, however, was higher than under conditions of complete dissociation of actin and myosin. Thus it cannot be decided whether in the complete absence of divalent ions myofibrils are relaxed.

Whereas without added Mg, syneresis was not influenced by changes in the bound myofibrillar Ca, ATPase activity increased the more Ca was bound. However, it is doubtful that this increased rate of ATP hydrolysis was related to contraction; and it is more likely that it represented the Ca-activated ATPase activity which, in contrast to the Mg-activated ATPase, is not coupled to energy transfer (Weber and Portzehl, 1962; Hasselbach, 1956; Watanabe and Yasui, 1965). (The Mg-activated ATPase may require Ca as a second ion if a complete relaxing system is present.) The Ca-dependent uncoupling of syneresis and ATPase activity is illustrated in Figure 3. Although ATPase activity increased considerably between 1 and 100 μM Ca^{2+} syneresis was 90% inhibited (*cf.* Experimental Section). The small extent of syneresis (25%) at 0.1 μM Ca^{2+} was probably due to the presence of some residual Mg whose effect was antagonized at higher Ca concentrations. The Ca concentrations which caused activation of ATPase activity in this experiment were considerably lower than some of the previously reported values (Perry and Grey, 1956; Weber, 1959; Watanabe and Yasui, 1965) but quite similar to the values for free Ca ion found by Maruyama and Watanabe (1962). Consistent with previous data (Weber, 1959) this difference may be related to the unusually low Mg concentration in this experiment where a special effort had been made to remove as much Mg as possible.

In these experiments Mg was present as the free ion and as MgATP chelate. To determine which of the two species is responsible for relaxation, we measured the ATPase activity at various concentrations of MgATP from 0.1 to 100 μM while keeping the concentration of unchelated ATP constant at 5 mM in one series of experiments, and at 1 mM in another. Thus, according to the equation $[Mg^{2+}] = K([MgATP]/[ATP])$, for each concentration of MgATP the ATPase activity was measured at two different concentrations of Mg^{2+} ranging in one series from 2×10^{-9} to 6×10^{-7} M and in the other from 3×10^{-9} to 4×10^{-6} M. Figure 4 shows that the rate of ATP hydrolysis was not determined by the concentration of free Mg ion since for each Mg^{2+} concentration two quite different rates were obtained, depending upon the MgATP concentration. Thus at 10^{-8} M Mg^{2+}, ATP was hydrolyzed at a rate of 0.03 μmole/min mg of protein when the MgATP concentration was 0.03 μM; and at a rate of 0.07 μmole/min mg with 1.2 μM MgATP (Figure 4). By contrast, at a constant concentration of MgATP of 1.0 μM the rate of 0.065–0.07 μmole/min mg of protein was not altered when the concentration of free Mg ion was raised from 7.6×10^{-9} to 3.6×10^{-8} M (Figure 5). These results agree well with earlier data by Watanabe and Yasui (1965). The concentration of MgATP apparently completely defined the activation of ATPase activity; but relaxation, *i.e.*, the inhibition of ATPase

activity, was not determined solely by the concentration of MgATP (Figure 5). With higher concentrations of unchelated ATP or lower concentrations of free Mg ion slightly more MgATP was necessary for inhibition. Thus a reduction of the ATPase activity to 0.15 μmole/min mg of protein required a concentration of MgATP of 30 μM when the concentration of Mg^{2+} was 1.1 μM which was increased to 46 μM MgATP when the Mg^{2+} was lowered to 0.3 μM. Inhibition was complete at about 0.1 mM MgATP. The dependence of relaxation upon MgATP as demonstrated here is consistent with the finding that relaxation cannot take place at very low ATP concentrations (Weber and Herz, 1963; Endo, 1964; Levy and Ryan, 1965), and that complete inhibition of ATPase activity requires ATP concentrations of about 0.04 mM (Weber, 1969). The difference between 0.1 and 0.04 mM may be explained by the very large difference in the concentration of ionized Mg (10^{-6}–10^{-3} mM *vs.* 1.0 mM) in the experiments described here and those quoted for which the total concentration of ATP was limiting for relaxation.

Whereas these experiments show that ATP exerts its relaxing effect as the Mg chelate they do not explain its mechanism of action. The data are compatible with the widely held assumption (Weber *et al.*, 1964; Levy and Ryan, 1965) that ATP causes relaxation when it binds to a special inhibitory site.

The finding that in the presence of very low Mg concentrations syneresis is independent of bound Ca implies that Ca cannot be an obligatory cofactor in actin–myosin interaction as proposed by Davies (1963). The best evidence for this conclusion is the finding by Ebashi *et al.* (1967) and by Fuchs and Briggs (1968) that Ca does not bind to actin or myosin when Ca activates contraction but that it binds to troponin instead. Thereby Ca abolishes the relaxing action of the system troponin–MgATP. Without troponin, relaxation is impossible and contraction occurs without bound Ca. With troponin but very low MgATP, relaxation is also prevented and contraction also proceeds without Ca as shown by these results.

Conclusions

Mg displaced Ca from myofibrils; but the experiments do not show whether it displaced Ca from sites regulating contraction or whether it displaced Ca from other sites.

MgATP was necessary for relaxation even after the removal of almost all of the bound Ca. It appears that MgATP is part of the relaxing system of the myofibril together with tropomyosin and troponin. In the absence of MgATP in sufficiently high concentrations, just as in the absence of tropomyosin and troponin (Ebashi and Endo, 1968), ATP caused a permanent state of contraction and relaxation could not occur even when nearly all of the bound Ca had been removed.

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Effect of *N*-Bromosuccinimide on Dihydrofolate Reductase*

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ABSTRACT: Dihydrofolate reductase from chicken liver contains four tryptophan residues per mole (mol wt 22,000), as determined by: (a) the absorbancy ratio at 280 and 288 mμ at pH 6.5 in the presence of 6 M guanidinium chloride; or (b) the decrease in absorbancy at 278 mμ after treatment of the enzyme with excess *N*-bromosuccinimide at pH 4.0 in the presence of 5.3 M urea. Between pH 6 and 8, and in the absence of urea, the action of *N*-bromosuccinimide on the enzyme occurs in two stages. Relatively low levels of *N*-bromosuccinimide (up to 4 moles/mole of enzyme) cause a two- to threefold *increase* in catalytic activity without any appreciable destruction of the tryptophans. This activation is probably due to oxidation of a sulfhydryl group on the protein since it can be reversed by dithiothreitol. Higher concentrations of *N*-bromosuccinimide (*ca.* 10 moles/mole of enzyme) result in tryptophan oxidation with a concomitant *decrease* in enzymatic activity.

Complete loss of activity, however, corresponds to the oxidation of only one tryptophan residue. Incubation of the enzyme with either reduced triphosphopyridine nucleotide or dihydrofolate affords protection against the effects of *N*-bromosuccinimide. These results indicate that at least one tryptophan residue, and a sulfhydryl group as well, are located at or near the substrate binding sites of the enzyme, and that oxidation of these moieties by *N*-bromosuccinimide leads to conformational changes in the protein, either at the active site or at some other location which, in turn, can affect the active site.

Recent studies from this laboratory[1] have shown that the binding of substrates (TPNH and dihydrofolate) or inhibitors (aminopterin and amethopterin) to highly purified chicken liver dihydrofolate reductase is accompanied by a decrease in fluorescence of the protein. Tryptophan residues[2] are believed to be responsible for the observed fluorescence. Similar observations and interpretations have been made for dihydrofolate reductase from L1210 cells (Perkins and Bertino, 1966; Hillcoat *et al.*, 1967), as well as for a number of other pyridine nucleotide dependent enzymes (Boyer and Theorell, 1956; Velick, 1958; Winer and Schwert, 1959; Theorell and McKinley-McKee, 1961; McKay and Kaplan, 1964).

The present study was undertaken in order to investigate more directly the role of tryptophan residues in substrate binding and in the catalytic mechanism of the chicken liver enzyme. *N*-Bromosuccinimide, a reagent which attacks tryptophyl residues[3] in proteins (Viswanatha *et al.*, 1960; Viswanatha and Lawson, 1961; Okada *et al.*, 1963; Green, 1963; Hayashi *et al.*, 1965; Davidson and Westley, 1965; Steiner, 1966; Spande *et al.*, 1966), was allowed to react with the enzyme under various conditions. Changes in catalytic activity and loss of tryptophan residues (*i.e.*, conversion of the indole ring into an oxindole derivative) were followed as a function of the molar ratio of *N*-bromosuccinimide to enzyme.

Experimental Section

Materials. Dihydrofolate reductase was isolated from chicken liver according to the procedure of Mathews and Huennekens (1963), as modified by Mell *et al.* (1966). All preparations used in these studies had specific activities of 6–8 μmoles of dihydrofolate reduced per min per mg of protein. As discussed elsewhere (Kaufman and Gardiner, 1966[2]), preparations having a specific activity in this range are essentially pure (*i.e.*, free from extraneous proteins), although the enzyme may be resolved electrophoretically into multiple forms (Mell *et al.*, 1968). The molecular weight of the enzyme, as determined by passage through a standardized column of Sephadex G-100 (Mell *et al.*, 1966) or by sedimentation equilibrium analysis,[2] is 22,000. A 0.1% solution of the enzyme at pH 7.0 has an absorbance of 1.55 at 278 mμ.

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[1] G. P. Mell and F. M. Huennekens, in preparation. See also Huennekens *et al.* (1967).

[2] G. P. Mell, J. H. Freisheim, F. M. Huennekens, and K. Dus, in preparation.

[3] Although *N*-bromosuccinimide is generally considered to be selective for tryptophan residues, effects on other functional groups have also been observed (Ramachandran and Witkop, 1959; Schmir and Cohen, 1961; Ramachandran, 1962).