Cyanation of myosin with 6-thiol-14C-cyanato-9-β-ribofuranosylpurine shows a biphasic effect. During the incorporation of the first 4 mol of cyanide the Mg\(^{2+}\)-dependent ATPase activity increases approximately 8-fold, the Ca\(^{2+}\) activity rises only slightly, and the K\(^{+}\)(EDTA) activity falls to approximately 5% of its original value. These enzyme preparations can still be inactivated by N-ethylmaleimide/MgADP as is found for the native enzyme.

Further modification (4 to 9.7 thiol groups) does not inactivate the enzyme as is the case with other thiol reagents. Instead, there is a sharp increase in Ca\(^{2+}\)-ATPase activity together with a further increase in Mg\(^{2+}\) activity. No further change in K\(^{+}\) activity occurs, but a dramatic increase in protection against N-ethylmaleimide inactivation is observed.

At maximal cyanation (12.5 thiol groups) only 0.6 -SH groups can be determined with Ellman’s reagent in a myosin preparation which was initially found to contain 39.2/molecule. After treatment of the cyanated myosin with dithioerythritol almost all -SH groups can be detected again. This suggests that on maximal cyanation, 26 -SH groups have formed disulfide bridges due to the cyanation reagent. This is in agreement with the amount of reagent consumed during the cyanylation reaction and with the fact that addition of inorganic cyanide to the reaction mixture raises the number of incorporated cyanide groups to 26/molecule. The results show that cyanylated myosin retaining only 0.6 free sulfhydryl group is still active. This suggests that sulfhydryl groups are not directly involved in the mechanism of ATP hydrolysis, but that they affect the conformation of the active site.

Many enzymes lose activity on chemical modification of their sulfhydryl groups, but this in itself is generally insufficient evidence for assigning mechanistic significance to the

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In this paper we submit evidence that myosin activity can be retained after blocking all sulfhydryl groups, provided the blocking group is small. Even when only 0.6 of the total of 40 -SH groups/molecule is still free, the enzyme retains elevated levels of Ca\(^{2+}\)- and Mg\(^{2+}\)-ATPase activity and approximately 4% of its K\(^{+}\)-ATPase activity. It is also protected against N-ethylmaleimide inactivation. We conclude from these experiments that sulfhydryl groups are not directly involved in the mechanism of ATP hydrolysis by myosin.
Myosin, Cyanylation of Sulphydryl Groups

EXPERIMENTAL PROCEDURES

Materials

Myosin was extracted from the back muscle of rabbits by the procedure of Schaub et al. (18), but to all solutions 1 mM dithioerythritol was added. After the final precipitation step, the protein pellets were dissolved in a buffer solution of 0.6 M KCl, 10 mM phosphate, 10 mM EDTA, and 5 mM dithioerythritol at pH 6.5. This solution was mixed with an equal volume of glycerol and kept at −20°C. Thus stored, the myosin remained stable for at least 1 year. Portions of this solution were exhaustively dialyzed against a buffer of 0.2 M KCl and 50 mM Bicine/KOH, pH 7.9. These solutions retained their activity for at least 1 week at 2°C. Typical specific activities for the myosin used in these experiments in the assay system described below were (micromoles of P/ mg of enzyme/min): Ca2+, 37°C, 1.24; K+(EDTA), 37°C, 2.82; Mg2+, 37°C, 0.083. This myosin preparation contained 30.3 free SH groups/4.7 x 10^2 daltons, as determined with Ellman's reagent, and was used in all of the experiments described herein. [14C]KCN (61 Ci/mol), [14C]KSCN (60 Ci/mol), and [14C]ethylmaleimide (4 Ci/mol) were purchased from Amersham.

Unlabeled 6-thiocyano-9-β-thio-d-ribofuranosylpurine was prepared by a modification of the reported procedure (19). To an aqueous slurry of 1.93 g (6.8 mmol) of thioinosine at 0°C, 6.97 ml of 1 M NaOH were added with stirring. followed by addition over a 20-min period of 740 mg (7.0 mmol) of CNBr in 20 ml of ethanol. After a further 20 min at 0°C, the reaction mixture was evaporated in vacuo at 20°C to an oil which was chromatographed on silica gel using methanol/chloroform (1:9, v/v). The product fractions were collected and evaporated to an oil which was crystallized from ethyl acetate/methanol/chloroform (1:9:1) to give 1.63 (5.26 mmol, 78% yield) of large prisms, melting point 151–153°C. Ultraviolet λmax, nm (ε x 10^3), pH 1.275 (14.6), pH 7.275 (13.8), pH 12, unstable. Thin layer chromatography on silica gel, 9.1 (v/v) ethyl acetate/ethanol: Rr = 0.45.

C₈H₁₂N₂SO₄

Calculated: C 42.75 H 3.56 N 22.68 S 10.36
Found: C 42.65 H 3.54 N 22.04 S 10.47

6-Thiol[14C]cyano-9-β-thio-d-ribofuranosylpurine was prepared by two different methods.

Method A – 0.8 mg (25 μmol) of 6-bromo-9-β-thio-d-ribofuranosylpurine (20) were reacted with 1.9 mg (19.7 μmol) of [14C]KSCN in 7 μl of dry tetramethylenes in a sealed tube under N₂ at 115°C for 60 min. The solvent was removed at 40°C by a rotary evaporator linked to an oil pump. The residue was dissolved in 75 μl of methanol and applied to a preparative scale silica gel thin layer plate and chromatographed with a 9:1 (v/v) ethyl acetate/ethanol mixture. The product band was scraped off and applied to a small column, from which the product was eluted with fresh eluent. The product (yield 39% with respect to KSCN) had chromatographic and ultraviolet properties identical with 6-thiocyano-9-β-thio-d-ribofuranosylpurine and had a specific activity of 2.7 x 10^8 cpm/mmol.

Method B – To an aqueous slurry of 284 mg (1 mmol) of thioinosine 1 m NaOH (1 ml) was added and the resulting solution cooled to 0°C. Then 25 ml of 0.1 M NaHCO₃, 0.1 M NaI, 0.02 M I₂ were added dropwise with stirring. After standing overnight the precipitate was filtered and washed three times with 10 ml of cold water and then dried in a desiccator over P₂O₅. Yield 265 mg (0.84 mmol, 94%) of colorless crystals, m.p. 108°C. The ultraviolet spectrum (λmax = 290 nm) was identical with that reported (21) for the 5′-phosphorylated analogue.

Reactions between myosin and N-ethyl[14C]maleimide were also carried out in 0.5 M KCl and 50 mM Bicine/KOH, pH 7.9, at 0°C. The conditions used to block all four SH, and SS, groups were: incubation for 30 min with 220 μM N-ethylmaleimide followed by treatment for 20 min with 200 μM N-ethylmaleimide in the presence of 5 mM Mg²⁺ and 2 mM ADP. The mixture was transferred to dialysis chambers and dialyzed three times with 4 h for each treatment. The use of the Cary 18 spectrophotometer equipped with a cooling system. The thiocyano- purine nucleoside was found to be stable under the reaction conditions.

When not stated otherwise, cyanylation reaction conditions in 0.5 M KCl, 50 mM Bicine/KOH, pH 7.9, generally varied only in the concentration of reagent. Solutions of approximately 4.5 to 6 mg/ml of myosin in this buffer were incubated at 0°C with the appropriate concentration of 6-thio-['4Clcyanato-9-P-n-ribofuranosylpurine (30 μM to 2 mM) for 6 h. Reaction mixtures were then dialyzed against 0.1 M KCl, 50 mM MgCl₂, 1 mM ATP for Mg²⁺-ATPase; 0.6 M KCl, 50 mM EDTA, 1 mM ATP for [32P]ATP measurements and nitrogen analysis. The values obtained were in good agreement with those from the spectroscopic method as described by Taylor (25). For protein determination after cyanylation the Schaffner-Weissmann-procedure (24) was used, standardized against a myosin solution of known concentration (measured by the spectroscopic procedure).

The sulphydryl content of myosin preparations was determined with Ellman's reagent (26) as described by Buttkus (9). For low -SH contents the method was modified as follows: to 299 mg of urea were added: 40 μl of 0.1 M Ellman's reagent, 20 μl of 0.5 M EDTA (pH 6.5), and then 0.8 to 1.0 ml of 0.1 M L-cysteine. The urea was dissolved by warming this mixture to 37°C and bubbling argon through it. Finally, the solution was made up to 2 ml with water.

The hydrolysis of ATP by myosin was measured by following the release of inorganic phosphate from ['γ-32P]ATP prepared by the method of Glynn and Chapell (27). Aliquots of the assay mixture were transferred to disposable plastic test tubes containing a slurry of 20 to 60 mg of activated charcoal in 1 ml of a 0.2 M HCl solution in 20:80 (v/v) ethanol/water at 0°C. The tubes were centrifuged for 15 to 25 min at 2500 rpm and at 5°C, and aliquots of the supernatants tested in aqueous [32P]ATP.

Standard assays were performed at 37°C in 25 mM imidazole HCl, pH 7.3, containing: 0.1 M KCl, 5 mM CaCl₂, 1 mM ATP for Ca⁺⁺-ATPase; 0.1 M KCl, 5 mM MgCl₂, 1 mM ATP for Mg⁺⁺-ATPase; 0.6 M KCl, 50 mM EDTA, 1 mM ATP for [32P]ATPase. When not stated otherwise, anaerobic conditions were used for these as well as for the method of Weissmann procedure (24).

Reaction mixtures were transferred to dialysis sacks and dialyzed overnight at 4°C against 0.5 M KCl, 50 mM Bicine/KOH, pH 7.9.

Reactions were followed and final [14C]cytidine incorporation was determined by a modified membrane filter assay (28): 20 ml of the enzyme solution were added to a cold solution containing 0.2 M KCl and 20 mM Bicine/KOH, pH 7.9. The mixture was filtered over a nitrocellulose membrane filter (pore size 0.45 μm, 25 mm diameter, Schleicher and Schull) and the membrane washed three times with the same buffer. After drying the radioactivity was determined by standard liquid scintillation techniques. This method was found to give values identical with those obtained by precipitation with SDS-trichloroacetic acid prior to filtration according to the Schaffner-Weissmann procedure (24). The consumption of the cyanylation agent was monitored by the increase of the absorption at 312 nm arising from the liberated thioinosine (ε = 23,350) (29). It was measured under argon against a reference containing the same concentration of the reagent in the same buffer. The measurements were carried out on the scan Cary 18 spectrometer equipped with a cooling system. The thiocyano- purine nucleoside was found to be stable under the reaction conditions.

In all calculations, the molecular weight of myosin was taken as 470,000 (22). Several methods for protein determinations were compared and found to give identical results for the same sample of myosin solution. For example, the methods based on binding of Congo paper brilliant blue (23) and amidoschwarz 10B (24), although standardized with the same serum albumin solution, varied by as much as 100%. True standardization was achieved by precipitation with ultraviolet absorption identical with those of 6-thiocyano-9-β-thio-d-ribofuranosylpurine and had a specific activity of 1.2 x 10⁶ cpm/mmol.

Methods

Aqueous [14C]KCN and 150 μl of 100 mM aqueous NaCN were added. After 15 min the mixture was evaporated with 1 g of SiO₂ and chromatographed on a silica gel column using ethyl acetate/methanol (10:1) (v/v). The product fractions were collected and evaporated in vacuo. The resulting (yield 81% with respect to cyanide) had chromatographic and ultraviolet absorption identical with those of.

The abbreviations used were: Bicine, N,N-bis(2-hydroxyethyl)-glycine; TCRP, 6-thiocyano-9-β-thio-d-ribofuranosylpurine; CN-myosin, myosin of which SH groups are cyanlated; pCMB, para-chloromercuribenzoate; SDS, sodium dodecyl sulfate.
procedure previously used (11, 13) since they eliminated the cyanide groups.

Reduction cleavage of thiocyanate groups and disulfides formed during the cyanylation reaction was achieved by incubation with 10 mM dithioerythritol at 0° for 24 h followed by dialysis against 0.5 M KCl and 50 mM Bicine/KOH, pH 7.9.

RESULTS

When a solution of myosin in 0.5 M KCl, 50 mM Bicine/KOH, pH 7.9 (4.5 to 6 mg/ml), containing 39.2 -SH groups/molecule is reacted at 0° for 6 h with 30 to 2000 μM concentrations of [14C]TCRP, four sulfhydryl groups (Fig. 1) react rapidly with the reagent in the concentration range up to 250 μM. Between 250 and 1000 μM another 5.5 -SH groups of moderate reactivity are cyanylated. Further increase in reagent concentration to 2000 μM causes essentially no further increase in cyanide incorporation.

During the incorporation of the first 4 mol of cyanide the Mg2+-dependent activity increases approximately 7-fold (Fig. 2), the Ca2+ activity rises only slightly (Fig. 3), and K+(EDTA) activity falls to approximately 5% of its original value. These enzyme preparations can still be inactivated with N-ethylmaleimide/MgADP (Fig. 3) as is found for the native enzyme.

Further cyanylation (4 to 9.7 thiol groups) causes a rapid increase in Ca2+-ATPase activity together with a further increase in Mg2+ activity. Whether the almost linear increase in Mg2+-ATPase activity with increasing modification reflects the oxidation of a specific -SH group which is not reacted in all myosin molecules until all -SH groups have reacted or whether it is due to a general increase in more -SH groups react is uncertain. Essentially no further change in K+ activity occurs but an increase in protection against N-ethylmaleimide/MgADP (Fig. 3) is found for the native enzyme.

When CN-9.7-myosin is treated with N-ethylmaleimide in the presence of MgADP, conditions which normally promote reaction of all four -SH, and -SH2 sulfhydryl groups with N-ethylmaleimide and concomitant loss of all ATPase activity (12, 18), only a very small decrease in the ATPase activity is observed (Fig. 3). Progressive cyanylation also decreases the amount of incorporated N-ethyl [14C]maleimide (Fig. 4) if that CN 8.7 myosin incorporates no N-ethylmaleimide. -SH determination shows that the remaining -SH groups are not affected by the N-ethylmaleimide treatment (Table I). Furthermore, the cyanide groups are retained in the protein during the N-ethylmaleimide reaction since no loss of radioactivity from the protein is observed after treatment with nonlabeled N-ethylmaleimide under the same conditions.

![Fig. 1. [14C]Cyanylation of myosin. Degree of incorporation of [14C]cyanide into myosin in 6 h at 0° by various concentrations of [14C]TCRP as described under "Methods."](image)

![Fig. 2. Specific activities of Mg2+-ATPase of cyanylated myosins. Mg2+ concentration, 5 mM.](image)

![Fig. 3. Specific activities of cyanylated myosins. O-O, Ca2+-ATPase (5 mM Ca2+); Δ-Δ, K(EDTA)-ATPase (0.6 M KCl, 1 mM EDTA). Also shown are the Ca2+-ATPase activities (O-O-O) of each cyanylated myosin preparation after reaction with 220 μM N-ethylmaleimide for 30 min at 0°, followed by 200 μM N-ethylmaleimide for 20 min at 0° in the presence of 2 mM MgADP.](image)

When CN-9.3-myosin is incubated with 10 mM dithioerythritol at 0° for 24 h all but 1.3 cyanide groups/molecule are removed. This enzyme preparation resembles native myosin in some respects (Table II). The Ca2+-ATPase activity returns to native levels and the K'(EDTA) activity returns to about one-half of the original value, but the Mg2+-ATPase activity remains at 11 times that of the native enzyme. Concomitantly with the removal of the cyanide groups by dithioerythritol the content of free sulfhydryls generally rises to a value of between 32 and 35 (Table I) -SH groups/molecule.

Following the course of the cyanylation reaction with 2 mM TCRP by the ultraviolet absorption of the thionines released reveals that 22 mol of reagent are consumed during the reaction (Fig. 5), indicating that in addition to the cyanylation of 9.7 -SH groups approximately 24.6 -SH groups have formed disulfides. This is in agreement with the observation that only 3.2 -SH groups are still accessible to Ellman's reagent in this myosin preparation. Further extension of the
FIG. 4. Degree of incorporation of N-ethyl[14C]maleimide of each cyanylated myosin preparation after reaction with 220 μM N-ethylmaleimide for 30 min at 0°C, followed by 200 μM N-ethylmaleimide for 30 min at 0°C in the presence of 2 mM MgADP.

**TABLE I**

Content of free —SH groups of modified myosin

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Mol of —SH/mol of myosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>39.2</td>
</tr>
<tr>
<td>CN-9.3</td>
<td>4.0</td>
</tr>
<tr>
<td>CN-9.5</td>
<td>4.2</td>
</tr>
<tr>
<td>CN-9.7</td>
<td>3.2</td>
</tr>
<tr>
<td>CN-9.3 + N-ethylmaleimide</td>
<td>3.9</td>
</tr>
<tr>
<td>CN-9.5 + N-ethylmaleimide</td>
<td>4.1</td>
</tr>
<tr>
<td>CN-9.7 + N-ethylmaleimide</td>
<td>3.3</td>
</tr>
<tr>
<td>CN-9.3 + dithioerythritol</td>
<td>32.6</td>
</tr>
<tr>
<td>CN-9.5 + dithioerythritol</td>
<td>34.8</td>
</tr>
<tr>
<td>CN-9.7 + dithioerythritol</td>
<td>32.5</td>
</tr>
<tr>
<td>CN-12.5</td>
<td>0.6</td>
</tr>
<tr>
<td>CN-18.0</td>
<td>5.5</td>
</tr>
<tr>
<td>CN-26.1</td>
<td>3.7</td>
</tr>
</tbody>
</table>

**TABLE II**

Relative ATPase activities of modified myosin

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Native</td>
<td>100</td>
</tr>
<tr>
<td>CN-9.3</td>
<td>290</td>
</tr>
<tr>
<td>CN-9.3 + dithioerythritol</td>
<td>95</td>
</tr>
<tr>
<td>CN-12.5</td>
<td>51</td>
</tr>
<tr>
<td>CN-18.0</td>
<td>266</td>
</tr>
<tr>
<td>CN-26.1</td>
<td>163</td>
</tr>
</tbody>
</table>

**TABLE III**

Incorporation of [14C]cyanide into myosin with 2 mM [14C]TCRP as described under "Methods" as a function of the pH of the reaction mixture

<table>
<thead>
<tr>
<th>pH</th>
<th>Mol of [14C]CN/mol of myosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>2.1</td>
</tr>
<tr>
<td>7.0</td>
<td>3.3</td>
</tr>
<tr>
<td>7.5</td>
<td>5.5</td>
</tr>
<tr>
<td>7.9</td>
<td>9.7</td>
</tr>
</tbody>
</table>

reaction time even to several days does not significantly decrease the content of free —SH groups.

Interruption of the cyanylation reaction after 6 h by dialysis and a second incubation for 24 h with fresh reagent generates a myosin preparation containing 12.5 cyanide groups and only 0.6 free sulfhydryl group/molecule. This highly modified enzyme still retains 51% of the Ca<sup>2+</sup>, 770% of the Mg<sup>2+</sup>- and 4% of the K<sup>+</sup>-dependent ATPase activity (Table II).

When a myosin solution in 0.5 M KCl, 50 mM Bicine, pH 7.9, is incubated simultaneously with 2 mM [14C]TCRP and 2 mM [14C]KCN of the same specific activity for 2 days at 0°C, 19 cyanide groups are incorporated and 5.5 —SH groups remain free. A second incubation for another 2 days results in the incorporation of a total of 26.1 cyanide groups and a decrease of free —SH groups to 3.7. This indicates that the disulfide bridges formed by the action of TCRP can be cleaved by cyanide. Thus, even after reaction for 4 days the enzyme still retains elevated Ca<sup>2+</sup>- and Mg<sup>2+</sup>-ATPase activities (Table II). The cyanylation reaction also strongly depends upon the pH of the reaction mixture (Table III). Decrease of the pH from 7.9 to 6.5 decreases the number of incorporated cyanide groups under otherwise identical conditions from 9.7 to 2.1/molecule. The K<sub>a</sub> for Ca<sub>a</sub> remains unchanged after cyanylation and thus for both native myosin and CN-9.7-myosin a value of 2 × 10<sup>10</sup> M.

**DISCUSSION**

When myosin is isolated under conditions which minimize disulfide bond formation, it contains approximately 40 free sulfhydryl groups/4.7 × 10<sup>5</sup> daltons (9). Of these approximately 30 are located in the globular head regions which possess the ATP hydrolysis function, and 10 are located in the helical tails (8, 30).

When the —SH groups, the two fastest reacting sulfhydryl groups of the heads in the absence of ligands (18), are blocked by N-ethylmaleimide the Ca<sup>2+</sup>- and Mg<sup>2+</sup>-ATPase activities reach a maximum, whereas the K<sup>+</sup>- (EDTA) activity is almost abolished. Reaction of a further two sulfhydryl groups, the —SH<sub>2</sub> groups, results in complete loss of both activities. The same biphasic response to sulphydryl reagents occurs when myosin is treated with p-chloromercuribenzoate (31), phenylmercuric acetate (32), iodoacetamide (33), or Cu<sup>2+</sup> (34).

Because modification of these specific sulfhydryl groups eliminates ATPase activity and because, conversely, the binding of ligands seems to affect the reactivities of the —SH, and —SH<sub>2</sub> groups toward sulphydryl reagents (17, 18), it has been...
assumed that these two groups of sulphydryls are 1) at the site catalyzing ATP hydrolysis (16, 17) and 2) essential for this activity (14, 15). On the other hand, Gaetjens et al. (35) reacted myosin with dithioglycolic acid dimethylester and obtained an enzyme preparation which contained less than 0.3 free —SH group but still had 60% of the Mg<sup>2+</sup>-ATPase activity. Bagshaw and Reed (36) showed by magnetic resonance investigations that the divalent cation (Mn<sup>2+</sup>) at the active site and the —SH<sub>H</sub> groups are not always attack from each other for direct interactions as postulated by Reisler et al. (17). Thus, there are conflicting reports on the importance of myosin sulphydryl groups, in particular the —SH<sub>H</sub>—SH<sub>H</sub> groups for ATPase activity.

Led by the observation of others (2-7) that inhibition of enzyme activities by —SH reagents is often the result of introducing a bulky group rather than blocking —SH groups of mechanistic significance, we tried to react the —SH groups of myosin with the small cyanide group using TCRP which became available during our investigation on purine ribonucleosides modified in the 6-position (37). The cyanylation reaction of myosin with TCRP is apparently accompanied by the formation of disulfide bridges, a conclusion based on the observation that after incorporation of 9.7 CN/molecule of myosin initially containing 39.2 —SH groups, only 3.2 —SH groups remain free, while a total of 22 eq of the reagent are consumed (Fig. 5). If one assumes that one equivalent of the reagent causes the formation of one S—S bond, the —SH balance is reasonably well preserved (3.2 SH free + 9.7 —SH cyanylated + 24.0 —SH oxidized to S—S = 37.5 —SH). The cyanylation reaction as well as the disulfide formation by the TCRP reagent is reversed by treatment with dithioerythritol (Table I). A further indication for the formation of protein disulfides is the fact that they can slowly be cleaved by cyanide ion resulting in the formation of thiocyanato groups and regeneration of —SH groups, which can subsequently be cyanylated by TCRP. Simultaneous incubation with 2 mM ['<sup>4</sup>C]KCN and 2 mM ['QTCRP for 2 days activities as CN-9.7-myosin (Table II). The drop in all three ATPase activities observed on repeating the reaction with 37.5 preparation is approximately 6.3 (44) and that at pH 6.5 only 2.1 sulhydryl groups/molecule are cyanlated.

There are two lines of evidence which suggest that the —SH<sub>H</sub>, —SH<sub>H</sub> pairs are nonessential for Mg<sup>2+</sup>- and Ca<sup>2+</sup>-ATPase activity. Our initial experiments showed that the ATPase activity of cyanlated myosin which contained only 3.2 to 4 free —SH groups/molecule could not be altered by N-ethylmaleimide treatment under a variety of conditions (Figs. 3 and 4; Table I). Although it appears likely that the —SH<sub>H</sub>, —SH<sub>H</sub> pairs have been either cyanylated or oxidized to disulfide bonds in this still active myosin preparation, it is remotely possible that the four remaining free —SH groups are the —SH<sub>H</sub>, and —SH<sub>H</sub> groups which are now somehow sheltered from N-ethylmaleimide.

When, however, the cyanylation with 2 mM TCRP is performed for 6 h, dialyzed, and reacted with fresh 2 mM TCRP for an additional 24 h, an enzyme preparation is obtained that contains 12.5 cyanide groups and only 0.6 free —SH group/molecule. This myosin preparation has 61% of the Ca<sup>2+</sup>, 770% of the Mg<sup>2+</sup>, and 4% of the K<sup>+</sup>-dependent ATPase activities. We believe that the decrease in specific activities of this CN-12.5 preparation compared to CN-9.7 is at least in part due to the long time the modification procedure required (a total of 60 h including two dialyses). The fact that this preparation still retains Ca<sup>2+</sup> and Mg<sup>2+</sup>-ATPase activities, we feel, is unequivocal proof that no sulphydryl groups including the —SH<sub>H</sub>,—SH<sub>H</sub> pairs are essential for ATPase activity.

In their extensive study of the enzymatic properties of —SH<sub>H</sub>, N-ethylmaleimide-myosin, Sekine and Kielley (45) concluded that the effects of sulphydryls modification seem to be mainly conformational in origin, pointing out that similar disturbances can be achieved merely by adding a particular organic solvent to solutions of native myosin during the assay. Our results, which rule out mechanistic significance of the —SH<sub>H</sub> and —SH<sub>H</sub> groups, may favor this interpretation. When a bulky group reacts at —SH<sub>H</sub>, a favorable conformation of Ca<sup>2+</sup>- and Mg<sup>2+</sup>-ATPase is created; when the —SH<sub>H</sub> group is likewise modified the active site is further disrupted, this time with unfavorable consequences for the ATPase activity. A smaller group (CN) on —SH<sub>H</sub>, or disulfide formation seems to produce a smaller, but still favorable change with respect to the Mg<sup>2+</sup>-ATPase while not affecting the Ca<sup>2+</sup>-ATPase. At the same time the K<sup>+</sup>-EDTA-ATPase is reduced approximately 5% of the original value, but is never completely abolished as is the case with the other sulphydryl reagents. Subsequent blocking of —SH<sub>H</sub>, and further reaction of other sulphydryl groups have no additional effect on the K<sup>+</sup>-ATPase and enhance further the Mg<sup>2+</sup>-ATPase activity as well as producing a favorable conformational state for the Ca<sup>2+</sup>-ATPase. The K<sub>e</sub> for CaATP remains unchanged during the modification procedure, also indicating that the —SH groups are not essential for catalysis or binding of the substrate.

This myosin preparation which contains no free —SH groups but still shows Ca<sup>2+</sup>- and Mg<sup>2+</sup>-ATPase activities might be useful for affinity labeling studies which, with native myosin, tend to be complicated by the high reactivities of the —SH<sub>H</sub> and —SH<sub>H</sub> groups. Exhausitively cyanylated myosin might also be used to study the role of the sulphydryl groups in the function of myosin in muscle.

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