The Effect of Structure-disrupting Ions on the Activity of Myosin and Other Enzymes*

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SUMMARY

Neutral salts in high concentrations (0.3 to 3.0 M) inhibit the activity of myosin nucleoside triphosphatase, trypsin, lactate dehydrogenase, estradiol-17β dehydrogenase, and fumarase in an order of increasing effectiveness for anions: CH₃COO⁻ < Cl⁻ < NO₃⁻ < Br⁻ < I⁻ < SCN⁻ < ClO₄⁻, and in an order of increasing effectiveness for cations: (CH₃)₃N⁺ < Cs⁺ < K⁺ < Na⁺ < Li⁺. These orders, which are maintained in all of the salt concentrations tested, and with the widely different enzymes employed, are also the orders in which these anions and cations disrupt the organized structure of diverse macromolecules as established by physical methods. Furthermore, the anions listed above disrupt the structure of myosin with a similar order of effectiveness.

Because of the parallelism between general structure-disrupting effectiveness and activity-inhibiting effectiveness, it is considered that the various ions inhibit activity by disrupting structure. Given such a conclusion, it follows that the salt sensitivity of structurally different forms of the same enzyme should differ. This is the case when native and p-chloromercuribenzoate-modified myosin are compared. The adenosine triphosphatase activity of native and p-chloromercuribenzoate-modified myosin are similar when assays are carried out in the absence of salts. The p-chloromercuribenzoate-modified form, being less sensitive to salt inhibition, thus appears to be "activated" when assayed in 0.6 M KCl (or similar concentrations of the several salts studied) and compared with native myosin in 0.6 M KCl.

These observations provide additional evidence that the α and β forms of myosin correspond to different conformations despite the fact that optical rotatory dispersion cannot clearly indicate a difference in structure. They further suggest that salt sensitivity as evaluated by the activity parameter can be used as an extremely sensitive if only semiquantitative method for the evaluation of conformational change.

When anions or cations are observed to affect enzymatic activity, it is natural to think that they do so because of their proximity to the active site where they exert local effects on the electrical field directly involved in the enzymatic process. When the ion concentrations are low (i.e. 0.1 M or less) or when the same ions affect equally the corresponding nonenzymatic reaction, the foregoing explanations are undoubtedly correct. On the other hand, at considerably higher salt concentrations (i.e. 1 to 2 M), it is possible that activity effects arise because ions are disrupting organized structures in the enzyme which are necessary to carry on catalysis and in this way indirectly alter the enzymatic process.

Although the mechanism by which ions disrupt organized macromolecular structure is controversial (1-4), the fact that they do so is clear; their individual abilities in this respect fall into an ordered sequence of considerable generality (1). To date, the position of an ion in such a sequence has been established by its effectiveness in disrupting various macromolecules, effect being measured by some physical method such as optical rotatory dispersion.

This investigation was carried out to evaluate the possibility that "disruption" of a macromolecule which happens to be an enzyme might be measured even more sensitively by the parameter of enzymatic activity than by any physical method. In order to conclude that the effects on activity were indeed indirect (i.e. structural), we established the following criteria, at salt concentrations of 0.3 to 3.0 M: (a) that ions ordered by von Hippel and Wong (1) on the basis of physical methods remain in the same order on the basis of their capacity to inhibit a given enzyme, and (b) that the order of inhibitory effectiveness be the same for radically different enzyme systems. As judged by these two criteria, the following data show that various ions in these concentration ranges inhibit enzymes by disrupting their structure. Given this fact, it follows that if a given enzyme exhibits two activity forms because it exists in two structural forms, the two activity forms should differ in sensitivity to the same ordered sequence of ions. With the α and β forms of myosin nucleoside triphosphatase (5), we have obtained just this result, thereby providing additional evidence that the α and β activity states of myosin in salt solutions correspond to two different conformations.

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**EXPERIMENTAL PROCEDURE**

**Materials**—The majority of the salts used were reagent grade purchased from the J. T. Baker Chemical Company or Fisher Scientific Company and used without recrystallization. Sodium perchlorate was made by the neutralization of reagent grade perchloric acid with sodium hydroxide. Tetramethylammonium acetate was made by the neutralization of tetramethylammonium hydroxide (Matheson, Coleman, and Bell Inc.) with glacial acetic acid. Tetramethylammonium chloride was purchased from Eastman Organic Chemicals. ATP, estradiol-17β, and Tris base were obtained from Sigma. NAD⁺, NADP⁺, malic acid, sodium fumarate, CMB,¹ and BAE were obtained from Calbiochem.

**Enzymes and Assays**—Myosin A was prepared by brief extraction of homogenized rabbit skeletal muscle in a manner previously described (5). After final purification, each of the preparations used (Preparations 70, 72, 73, and 74) tested negatively for myosin B contamination (6). The prepared myosins were stored in 0.6 M KCl, and their specific activities were such that when assayed in 0.6 M KCl as described below they liberated 4 to 5 μmoles of Pi per g sec. Assay of all of the enzymes studied was carried out at pH 8.0 at 25°C. Assay of myosin ATPase and ITPase was carried out either by use of the colorimetric technique of Fieke and Bubela Row described by Morales and Hotta (7) or in a pH-stat. The assay medium consisted of 20.0 ml of basic solution containing 10.0 mM calcium with or without salts in the molarity indicated. This quantity of calcium chloride was chosen after repeated assays revealed that the system was saturated with Ca⁴⁺ above 5 mM. In addition, this basic solution contained 0.05 M Tris-hydrochloride buffer at pH 8, and 2.0 mM NAD⁺, 50 mM Tris-hydrochloride, pH 8, with a total volume of 3.0 ml. The reaction was started by the addition of enzyme in the amounts indicated (usually 0.5 ml) in 0.6 M KC1.

Lactate dehydrogenase (crystalline from rabbit muscle) was obtained from C. F. Boehringer and Soens in 2.0 M ammonium sulfate as a suspension of 100 mg per ml. Measurement of activity was carried out in a Zeiss PMQ II spectrophotometer by recording the absorbance at 340 μM (due to the generation of NADH) on a Honeywell recorder wherein full scale deflection corresponded to 0.200 optical density unit. The assay solution contained 100 mM sodium lactate (prepared in a manner to destroy internal lactone formation (8)), 2.0 mM NAD⁺, and 50 mM Tris-hydrochloride buffer in a total volume of 3.0 ml. The stock enzyme was diluted 1000-fold in 0.05 M Tris buffer at pH 8, and the reaction was initiated by the addition of 0.1 ml of enzyme solution containing 0.1 μg of enzyme. In the absence of added salts, this quantity of enzyme generated 6.0 μmoles of NADH per min.

Estradiol-17β dehydrogenase was prepared by the method of Jarabak et al. (9) utilizing only the first six steps in their purification scheme. The enzyme, stored in 50% glycerol at a concentration of 4.3 mg per ml, was diluted 10-fold before use. The assay solution consisted of 1.0 μmole of NAD⁺, 40 μg of estradiol-17β added in 0.1 ml of propylene glycol, and 150 μmoles of Tris-hydrochloride buffer at pH 8.0, the mixture having a total volume of 3.0 ml. The generation of NADPH was followed in a recording spectrophotometer (see above). The reaction was started by the addition of 0.43 mg of enzyme in a total volume of 0.10 ml. This quantity of enzyme generated 12.5 μmoles of NADPH per min in the assay solution described above without added salts.

Crystalline fumarase from heart muscle was obtained from C. F. Boehringer and Soens in 2.0 M ammonium sulfate as a suspension of 10.0 mg per ml. The rate of conversion of malate to fumarate was measured spectrophotometrically at 295 μM. The assay solution consisted of 300 μmoles of sodium malate and 150 μmoles of Tris-hydrochloride, pH 8, in a total volume of 3.0 ml. The reaction was started by the addition of 0.10 ml of the stock enzyme which had been previously diluted 1:100 in 0.05 M Tris buffer. Thus, 10 μg of enzyme were used for assay, and under the above condition in the absence of added salts, this quantity of enzyme generated 2.8 μmoles of fumarate per min.

The conversion of fumarate to malate was followed by the disappearance of fumarate at the same wave length and with the same instrument. The assay solution consisted of 100 μmoles of sodium fumarate and 150 μmoles of Tris-hydrochloride, pH 8, in a total volume of 3.0 ml. The reaction was initiated by the addition of 10 μg of enzyme as described above. This quantity of enzyme generated 1.3 μmoles of maleate per min in the absence of added salts. It was impossible to study this reaction in the presence of nitrate ion which not only has a marked absorbance of its own at this wave length, but further decreases the absorbance of fumarate far below the 0.02 absorbance unit per μmole that was observed in water or the presence of the other salts.

Trypsin was obtained as a dry, crystalline powder from C. F. Boehringer and Soens and was freshly made up each day at a concentration of 0.5 mg per ml in unbuffered aqueous solution to which a single drop of hydrochloric acid had been added. The assay was carried out with the pH-stat in an assay solution of 20 ml of water with or without salts at the concentrations shown containing 200 μmoles of BAE which had been previously adjusted to pH 8. The second set of assays was carried out in the same solution which contained, in addition, 40 mM calcium chloride. Under the conditions described above and in the absence of added salts, the quantity of enzyme used (0.25 mg) hydrolyzed 6.0 μmoles of the synthetic substrate per min. When the assay solution contained 40 mM calcium chloride, the same quantity of enzyme hydrolyzed 8.0 μmoles of the synthetic substrate per min.

**Modification of Myosin**—CMB-modified myosin was prepared according to Rainford, Hotta, and Morales (5). The sulfhydryl content in a known quantity of myosin was measured (10) after the enzyme had incubated with the CMB overnight at 4°C. An aliquot of native myosin preparation was then incubated with a quantity of CMB sufficient to react with 45% of the total titratable sulfhydryl groups overnight at 4°C. After 24 and 48 hours of modification, aliquots corresponding to 3.0 mg of myosin were taken for assay of activity.

**Optical Rotatory Dispersion Studies**—Evaluation of α-helix content of myosin in various salt solutions was carried out in a Cary model 60 spectropolarimeter by two methods. With those salts which had sufficiently low absorbance, evaluation of the conformation-dependent Cotton effect from 233 to 300 μM (11, 12) was carried out with a 1.0-cm path quartz cuvette supplied with a water jacket for temperature regulation. These solutions contained 0.3 M KCl or 2.6 M sodium salts in 0.05 M Tris buffer at pH 8. Native or CMB-modified myosin was present in concentrations of 0.03%. For those salts which had large absorbance

¹ The abbreviations used are: CMB, p-chloromercuribenzoate; BAE, N-α-benzoyl-L-arginine ethyl ester hydrochloride.
at low wave length, evaluation of effect on myosin structure was carried out by determination of transition temperatures at 390 mp. The solutions contained 2.45 and 4 min for the reading to be taken. After the maximal change in rotation had been reached, observed rotation at each temperature was graphically evaluated. The temperatures at which the change in rotation had been reached, observed rotation at each temperature was graphically evaluated. 

RESULTS

Inhibition by Anions—The effect of various anions on the activity of myosin nucleoside triphosphatase activity is shown in Fig. 1. It is evident that all of the salts at 0.6 and 1.2 m concentrations caused profound inhibition as compared to the observed activity in water alone. The variation noted is that of the anions, except for tetramethylammonium acetate, which is included for comparison with potassium acetate. Furthermore, because of the insolubility of potassium perchlorate, comparison of 0.3 m sodium perchlorate and sodium thiocyanate was utilized to order the perchlorate ion. At this concentration, activity with sodium thiocyanate was 12.5% and with sodium perchlorate was 4% of the water control. Thus, the anions can be ranked in the following order of increasing effectiveness in reducing the activity of myosin ATPase: CH₃COO⁻ < Cl⁻ < NO₃⁻ < Br⁻ < I⁻ < SCN⁻ < ClO₄⁻.

The effect of the same anions on the activity of trypsin in the absence of added calcium is shown in Fig. 2. Fig. 3 shows similar data obtained in the presence of 40 mM calcium chloride, a cation which has been shown to stabilize trypsin (13). Exempt for reversal of NO₃⁻ and Br⁻ (in the presence of 40 mM CaCl₂), the order in which these anions affect activity is the same as with myosin. It should be noted that this enzyme is quantitatively considerably less sensitive to the inhibitory action of the salts than is myosin, and it is less sensitive to salt inhibition in the presence of calcium than in its absence.

The effect of the same group of anions on the activity of estradiol-17β dehydrogenase is shown in Fig. 4. It may be noted that the order of effectiveness is the same as with myosin, but this enzyme is intermediate in its sensitivity to the salts.

The effects on the activity of lactate dehydrogenase are shown in Fig. 5. Again the same order is established with one exception, which is the acetate ion. Unlike other anions, which with various enzymes essentially maintained their order with varying concentrations, acetate became a relatively better inhibitor as concentrations were raised, and it cannot be ordered. Because acetate ion resembles lactate in structure, it might be expected to be a competitive inhibitor even if not as effective as compounds with an electrophilic substituent on the α carbon (14). Neglecting this specific awkwardness, the order of effectiveness of the anions is that of the enzymes discussed above.

Effects of the same group of anions on the conversion of malate to fumarate by fumarase are indicated in Fig. 6. Again, with the exception of nitrate, which could not be evaluated under the conditions of this assay, the order in which these anions produce inhibition is the same. Effects of these same anions on the conversion of fumarate to malate are shown in Fig. 7. In this instance there is one clear cut ionic reversal in that bromide is definitely less effective in activity inhibition than is chloride. It should also be noted that several of these anions at low concentrations are capable of actually activating fumarase, a situation

![Fig. 1 (left). Inhibition of myosin ATPase by anions. Assays were performed with Fiske-SubbaRow technique except for thiocyanates which were done in the pH stat. Reactions were started with 3.0 mg of myosin (Preparation 72) which in the absence of added salts (control) liberated 4.10 μmoles of Pi per min and equals 100% activity. In Figs. 1 to 7 the symbols are as follows: ●, potassium acetate; ○, potassium chloride; □, potassium nitrate; △, potassium bromide; Δ, potassium iodide; ○, potassium thiocyanate; X, sodium thiocyanate; ●, sodium perchlorate; ☐, tetramethylammonium acetate.](http://www.jbc.org/)

![Fig. 2 (middle). Inhibition of trypsin by anions. Assays were performed with the pH stat in the absence of added calcium. Reactions were started with 0.25 mg of trypsin which in the absence of added salts (control) hydrolyzed 6.0 μmoles of substrate per min and equals 100% activity.](http://www.jbc.org/)

![Fig. 3 (right). Inhibition of trypsin by anions in the presence of 40 mM calcium chloride. Assays were performed with the pH stat. Reactions were started with 0.25 mg of trypsin which in the absence of added salts (control) hydrolyzed 8.0 μmoles of substrate per min and equals 100% activity.](http://www.jbc.org/)
FIG. 4 (left). Inhibition of estradiol-17β dehydrogenase by anions. Reactions were started by addition of 0.43 mg of enzyme which in the absence of added salts (control) generated 12.5 μmole of NADPH per min and equals 100% activity. For the symbols for salts in Figs. 4 to 6 see the legend to Fig. 1.

FIG. 5 (middle). Inhibition of lactate dehydrogenase by anions. Reactions were started by addition of 0.10 μg of enzyme which in the absence of added salts (control) generated 20 μmole of NADH per min and equals 100% activity. For the symbols for salts see the legend to Fig. 1.

FIG. 6 (right). Effect of anions on conversion of malate to fumarate by fumarase. Reactions were started by addition of 10.0 μg of enzyme which in the absence of added salts (control) converted 28 μmole of malate per min and equals 100% activity.

Effect of Anions and CMB on Conformation of Myosin—At 40°C the order of increasing effectiveness of the 2.6 M salts studied in abolishing the conformation-dependent Cotton effect of native myosin at 233 and 240 μm was sodium acetate < NaCl < NaBr ≪ NaClO₄. Transition temperatures of native myosin in 2.45 M salt solutions were as follows: NaBr, 43.5°; NaNO₃, 43.5°; NaClO₄, 32°; and NaSCN, 30°. These data, when combined, establish an anion series in order of increasing effectiveness in disrupting the α-helix: CH₃COO⁻ < Cl⁻ < NO₃⁻ ≈ Br⁻ < ClO₄⁻ ≈ SCN⁻. Thus, the effects of the anionic series on disruption of the α-helix generally parallel those on activity. Although ClO₄⁻ was clearly more inhibitory on activity than SCN⁻, it only approximated it in disruption of the α-helix. Therefore, investigation of ClO₄⁻ as an agent with specific oxidation effects on titratable sulfhydryl groups was carried out by CMB titration of myosin in the presence of 3.0 M NaClO₄ and 0.6 M KCl (10), but no differences were found. The possibility of a specific reaction with histidine was investigated with the technique previously utilized to demonstrate its oxidation by peroxides in dioxane (5), but no such reaction was found.

Comparison of rotation as a function of wave length between 220 and 300 μm for native and CMB-modified myosin in 0.3 M KCl at 25° revealed their Cotton effects to be identical (Fig. 9). Comparison of the Cotton effects of native and CMB-modified myosin in 2.6 M NaClO₄ solution at 25° revealed them to be again identical, but rotation at 233 μm was approximately 43% less than in the dilute KCl solution, indicating an appreciable but identical (for native and modified myosin) decreased amplitude in ultraviolet conformational Cotton effect resulting from exposure to ClO₄⁻.

Effect of Cations on Activity—The effect of the chlorides of several monovalent cations on the activity of the various enzymes studied above is indicated in Table II. It can be seen that the cations utilized can be ordered in their degree of effectiveness in producing inhibition of activity as follows: (CH₃)₄N⁺ < Cs⁺ < K⁺ < Na⁺ < Li⁺. In the one instance where rubidium was utilized (with myosin) it can be seen that it falls between sodium and lithium. Thus, the cations also fall in the order of the von Hippel and Wong (1) cationic series.

Effect of Anions on the Conversion of Fumarate to Malate by Fumarase—Reactions were started by addition of 10.0 μg of enzyme which in the absence of added salts (control) generated 1.3 μmole of fumarate per min and equals 100% activity. For the symbols for salts see the legend to Fig. 1.

Effect of Anions on the Conversion of Malate to Fumarate by Fumarase—Reactions were started by addition of 10.0 μg of enzyme which in the absence of added salts (control) converted 28 μmole of malate per min and equals 100% activity.

Effect of Anions on the Inhibition of Lactate Dehydrogenase by Anions—Reactions were started by addition of 0.10 μg of enzyme which in the absence of added salts (control) generated 20 μmole of NADH per min and equals 100% activity.

Effect of Anions on the Inhibition of Estradiol-17β Dehydrogenase by Anions—Reactions were started by addition of 0.43 mg of enzyme which in the absence of added salts (control) generated 12.5 μmole of NADPH per min and equals 100% activity.

Effect of Anions on the Conversion of Malate to Fumarate by Fumarase—Reactions were started by addition of 10.0 μg of enzyme which in the absence of added salts (control) converted 28 μmole of malate per min and equals 100% activity.

Effect of Anions on the Conversion of Fumarate to Malate by Fumarase—Reactions were started by addition of 10.0 μg of enzyme which in the absence of added salts (control) generated 1.3 μmole of fumarate per min and equals 100% activity.
TABLE I
Order of increasing effectiveness of anions in producing inhibition of enzymes studied

Assays were performed as described in text. Those anions not tested with a specific enzyme, except for acetate ion with lactate dehydrogenase which could not be ordered because of change in relative position with change in concentration, are designated by an asterisk (*).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Anionic order from left to right</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin, native</td>
<td>CH$_3$COO$^-$ Cl$^-$ NO$_2^-$ Br$^-$ I$^-$ SCN$^-$ ClO$_4^-$</td>
</tr>
<tr>
<td>Myosin, CMB-modified</td>
<td>CH$_3$COO$^-$ Cl$^-$ NO$_2^-$ Br$^-$ * SCN$^-$ ClO$_4^-$</td>
</tr>
<tr>
<td>Trypsin-30 nM Ca$^{++}$</td>
<td>CH$_3$COO$^-$ Cl$^-$ Br$^-$ NO$_2^-$ I$^-$ SCN$^-$ *</td>
</tr>
<tr>
<td>Trypsin-40 mM Ca$^{++}$</td>
<td>CH$_3$COO$^-$ Cl$^-$ Br$^-$ * SCN$^-$ ClO$_4^-$</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>CH$_3$COO$^-$ Cl$^-$ Br$^-$ * SCN$^-$ ClO$_4^-$</td>
</tr>
<tr>
<td>Estradiol-17β dehydrogenase</td>
<td>CH$_3$COO$^-$ Cl$^-$ Br$^-$ * SCN$^-$ ClO$_4^-$</td>
</tr>
<tr>
<td>Fumarase (malate = substrate)</td>
<td>CH$_3$COO$^-$ Cl$^-$ Br$^-$ * SCN$^-$ ClO$_4^-$</td>
</tr>
<tr>
<td>Fumarase (fumarate = substrate)</td>
<td>CH$_3$COO$^-$ Cl$^-$ Br$^-$ * SCN$^-$ ClO$_4^-$</td>
</tr>
</tbody>
</table>

* TMA$^+$, tetramethylammonium.
† These cases were not studied.

Because of the effects of divalent cations competing with calcium in the activation of myosin and producing stabilization of trypsin, only the monovalent cations were studied.

Effect of CMB Modification on Salt Sensitivity of Myosin—After 24 hours of modification as described above, aliquots containing equal amounts of native and modified myosin were assayed by the pH-stat method in an assay solution consisting of water alone and the sodium salts of the various anions in the concentrations as shown in Fig. 8. In the solution described as water (although it actually contained 15 mM KCl, 2 mM sodium ATP, and 10 mM calcium chloride), the activities of native and modified myosin were comparable (native = 3.9, modified = 4.4 µmoles of Pi per min). However, the salt sensitivities of the two enzyme forms differ markedly. Modified myosin is considerably less sensitive to the inhibitory effects of all of the anions tested at all of the concentrations. This sensitivity difference becomes particularly marked in 0.6 M salt solutions where in every case the native enzyme is at least twice as sensitive as is the modified form. Both native and modified enzymes were also assayed in the presence of a water assay solution and a 0.6 M potassium chloride solution. In the presence of this concentration of potassium chloride, the native enzyme demonstrated only 15% while the modified enzyme demonstrated some 65% of the activity seen in the absence of salt. These observations were carried out with Preparation 73.

A different myosin preparation (74) was modified in a similar fashion with CMB and after 24 hours of modification, the activity of modified myosin was compared with native myosin in the absence of added salt and in the presence of potassium chloride.

![Fig. 8. Comparison of the salt sensitivity of native and CMB-modified myosin. Myosin (Preparation 73) was exposed to CMB for 24 hours. Assays were conducted with the pH-stat. A, reactions were started with 3.0 µg of native enzyme which in the absence of added salts (control) liberated 3.94 µmoles of Pi per min and equals 100% activity. B, reactions were started with 3.0 µg of modified enzyme which in the absence of added salts (control) liberated 4.40 µmoles of Pi per min and equals 100% activity.](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2162292/)

TABLE II
Order of increasing effectiveness of chlorides of monovalent cations in producing inhibition of enzymes studied

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cationic order from left to right</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin, native</td>
<td>TMA$^+$ Cs$^+$ K$^+$ Na$^+$ Rb$^+$ Li$^+$</td>
</tr>
<tr>
<td>Myosin, CMB-modified</td>
<td>TMA$^+$ Cs$^+$ K$^+$ Na$^+$ Rb$^+$ Li$^+$</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>TMA$^+$ Cs$^+$ K$^+$ Na$^+$ Rb$^+$ Li$^+$</td>
</tr>
<tr>
<td>Estradiol-17β dehydrogenase</td>
<td>TMA$^+$ Cs$^+$ K$^+$ Na$^+$ Rb$^+$ Li$^+$</td>
</tr>
<tr>
<td>Fumarase (malate = substrate)</td>
<td>TMA$^+$ Cs$^+$ K$^+$ Na$^+$ Rb$^+$ Li$^+$</td>
</tr>
</tbody>
</table>

* TMA$^+$, tetramethylammonium.
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Fig. 8. Comparison of the salt sensitivity of native and CMB-modified myosin. Myosin (Preparation 74) was exposed to CMB for 24 hours. Assays were conducted with the pH-stat. A, reactions were started with 3.0 µg of native enzyme which in the absence of added salts (control) liberated 3.94 µmoles of Pi per min and equals 100% activity. B, reactions were started with 3.0 µg of modified enzyme which in the absence of added salts (control) liberated 4.40 µmoles of Pi per min and equals 100% activity.

![Fig. 8. Comparison of the salt sensitivity of native and CMB-modified myosin. Myosin (Preparation 73) was exposed to CMB for 24 hours. Assays were conducted with the pH-stat. A, reactions were started with 3.0 µg of native enzyme which in the absence of added salts (control) liberated 3.94 µmoles of Pi per min and equals 100% activity. B, reactions were started with 3.0 µg of modified enzyme which in the absence of added salts (control) liberated 4.40 µmoles of Pi per min and equals 100% activity.](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2162292/)

TABLE III
Comparison of activity of native and CMB-modified myosin

Assays were carried out as described in text. Those marked with an asterisk (*) were done by Fiske-SubbaRow method, others were done in pH-stat. In all of the cases, the enzyme used was 0.5 ml of a 0.60% solution in 0.6 M KCl (3.0 mg) from Preparation 74. Modified enzyme was prepared as described in text and number of hours of exposure to CMB before assay are indicated. When ATP was used as substrate 80 µmoles were added.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>P$_i$ liberated in µmoles/min</th>
<th>Activity ratio of 0.6 M KCl to H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>ATP</td>
<td>4.38</td>
<td>0.88</td>
</tr>
<tr>
<td>Modified 24 hr</td>
<td>ATP</td>
<td>3.80</td>
<td>0.65</td>
</tr>
<tr>
<td>Modified 48 hr</td>
<td>ATP</td>
<td>4.25</td>
<td>0.65</td>
</tr>
</tbody>
</table>
Effects of 0.6 m salts on activity of native and CMB-modified myosin

Assays were carried out in pH-stat with ATP as substrate. In all of the cases, the enzyme used was 0.5 ml of a 0.65% solution in 0.6 M KC1 (3.0 mg) from Preparation 74. CMB modification was carried out for 24 hours as described in the text. In the absence of added salt, 3.0 mg of native and modified myosin hydrolyzed 3.80 and 4.20 ug of P1 per min, respectively, and these values equal 100% activity.

<table>
<thead>
<tr>
<th>Salt</th>
<th>Activity as percentage of that in absence of salt</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Native myosin</td>
</tr>
<tr>
<td>(CH2)6NCl</td>
<td>35</td>
</tr>
<tr>
<td>CsCl</td>
<td>16</td>
</tr>
<tr>
<td>KCl</td>
<td>15</td>
</tr>
<tr>
<td>NaCl</td>
<td>14</td>
</tr>
<tr>
<td>LiCl</td>
<td>10</td>
</tr>
</tbody>
</table>

solutions as shown in Table III and 0.6 m cation chloride solutions as shown in Table IV. Although the order of effectiveness in producing inhibition remains the same, the sensitivity of the native myosin is considerably greater than that of the modified form. After an additional 24 hours in the presence of CMB, the activity of modified myosin was compared with that of native myosin in the presence and absence of 0.6 m potassium chloride in the assay solution. In the absence of salt, the native ATPase and ITPase and modified ATPase activities were similar, while in 0.6 m KC1 with ATP as substrate the activity of the native enzyme was only 47% of that seen with the modified form. Thus, numerous observations with CMB-modified myosin from two separate preparations indicate clearly that although the order of effectiveness of both anions and cations in inhibiting activity is essentially the same, the sensitivity of native myosin to salt inhibition is markedly greater than that of modified myosin. In the absence of any salt (remembering of course that a small amount is always present, as indicated above), the CMB-modified enzyme shows little or no activation above the native form.

**DISCUSSION**

In high concentrations (0.5 to 2.0 m), the cations and anions studied demonstrate (with only two exceptions) a distinct and consistent order in their capacity to inhibit the activity of myosin, lactate dehydrogenase, trypsin, estradiol-17β dehydrogenase, and fumarase. Massey (15) has studied the inhibition of the conversion of fumarate to malate by fumarase at low concentrations (up to 0.1 M) of certain anions and established as an order of increasing effectiveness: Cl- > Br- > I- ≈ CNS-. Although this order follows that predicted by the von Hippel series, Massey’s data were obtained with the phosphate-activated enzyme. In this study, activating salts were, where possible, avoided in an effort to eliminate effects due to mere displacement of an activating ion. Studies by Fridovich (16) with acetoacetic decarboxylase from Clostridium acetobutylicum with salts from 10-4 to 10-2 M established an inhibition series of increasing effectiveness Cl- < Br- < ClO4- < CNS-. As explained in the introduction, however, we chose higher ranges of concentrations so that the activity effects might be unequivocally structural.

The enzymes were purposely chosen to be very different. Myosin is a protein with a high content of α-helix which hydrolyzes a polyanion substrate. Trypsin is a protein of low α-helix content which hydrolyzes (in this evaluation of its esterase activity) a ester substrate. Fumarase is a dehydrating enzyme which utilizes divalent anions as substrate, and lactate dehydrogenase is a protein of moderate α-helix content which uses a monovalent anion as substrate. NAD+ was used as substrate for lactate dehydrogenase while NADP+ was used as substrate for estradiol-17β dehydrogenase. The helical content of estradiol-17β dehydrogenase is not known, but the substrate is probably uncharged as the pK of estrone is 9.35, and studies by Langer and Engel (17) have indicated that the pH dependence of the enzyme is similar for estradiol and its 3-methyl ether.

Von Hippel and Wong (18) have shown that the anions and cations can be ordered by their capacity to alter the organized structure of diverse macromolecules, including DNA, collagen, gelatin, and ribonuclease. It seems pertinent that a similar order is demonstrated in the helix-coil transition of myosin which has a high (about 60%) α-helix content. The data reported in this manuscript establish an order of increasing effectiveness: Cl<sub>4</sub>COO- < Cl- < NO3- ≈ Br- < ClO4- ≈ CNS-. Previous studies by Tonomura, Sekiya, and Imamura (19) have established structure disruptive orders of increasing effectiveness: Cl- < Br-; I- < CNS-; and K+ < Li+. Thus the anions and two cations disrupt the α-helix of myosin in a manner generally similar to their effects on the more general macromolecular properties disclosed by von Hippel and Wong.

In this work no attempt was made to study the time dependence or the reversibility of the salt effects (it is implicit, of course, that the effect of KC1 on myosin is reversible, since stock protein dissolved in 0.6 M KC1 was transferred into weaker salt solution with a resulting increase in activity). However, the results of Tonomura, Sekiya, and Imamura (18) on myosin bear importantly on this point. In general they showed that the structural effects of such salts as LiBr, KCl, and CNS- were reversible; the enzymatic effects, on the other hand, were sometimes time dependent, and reversal after short exposure was variable; for instance, from LiBr it was slight but from CNS- it reached 46%. Reversal from the effects of exposure to CNS-; merely on dialysis against KC1 was substantial, and this probably precludes the otherwise interesting possibility that CNS- might thio carbamylate NH₃ groups.

It is of interest that the salts inhibit enzymic activity in the same order, although generally in lower concentrations. That this effect is on the catalytic step (i.e., k₅) is assured by having the substrates at levels several times Kₗ and on several occasions doubling the concentrations of Ca<sup>2+</sup> or ATP, BAE, and estradiol-17β without any change in the degree of salt inhibition. The parallelism between ionic order in structure and activity alterations strongly suggests that conformational changes may be responsible for activity changes and that the latter, which occur at lower salt concentrations may result from subtle conformational changes, undetectable with presently available physical methods. Whether such conformational changes result from changes in water lattice structure (1–3) is yet another problem. Jencks (4) has pointed out that this hypothesis, in its simplest form, cannot be correct. Although the effects of anions on activity generally follow their water disruptive capacity as evaluated by the B-coefficients of viscosity and unitary partial molal entropy, this is not the case for the cations. Regardless of whether these effects...
are solvent mediated, it is evident that the final result is a disruption of organized protein structure which appears to be of a general nature rather than specific at an active site.

Because the quantitative effects of the salts in inducing conformational change are imposed upon a basic structure, a change in this basic structure induced by modification might well result in a change in salt sensitivity. With activity as a parameter, this is indeed the case with trypsin in the presence of calcium and more importantly with CMB-modified myosin.

It has long been known that myosin dissolved in 0.6 M KCl has two adenosine triphosphatase activity states (α, native; β, activated) and that the α → β transformation is caused by many agencies, among which is partial blockage of sulfhydryl groups by CMB. Recent work of Rainford, Hotta, and Morales (5), Sekine and Kielley (19), and Hartshorne and Morales (20) has provided indirect evidence that the transformation corresponds to some structural change. Attempts at getting direct physical evidence made in many laboratories have thus far failed except for a report by Tonomura, Sekiya, and Imamura (21) indicating marginal differences between native and CMB-modified myosin when α-helix contents were determined by the Moffitt-Yang (22) treatment. With a much improved spectropolarimeter, we have sought these differences with negative results (Fig. 9). On the other hand, it has been shown above that the sensitivity of enzymatic activity to an ordered sequence of ions is indeed quite different for the α and β forms of the enzyme. By the criteria set forth in the introduction, these effects occur under conditions in which the ions are disrupting structure. Therefore, the most natural inference is that the two myosin forms being attacked by the ions differ in structure. That optical rotatory dispersion does not distinguish between the forms means merely that these observations average over the entire molecule of myosin, most of which is invariant to the α → β transformation.

Somewhat unexpectedly, the foregoing myosin experiments illuminated another feature of myosin modification. When, as is the custom, myosin adenosine triphosphatase is studied in 0.6 M KCl at pH 8, enzyme modified to the β state has a higher activity than native (α) myosin, so one says that myosin is activated by this modification (i.e. CMB treatment). Fig. 8 and Table III show that in the absence of salt, the activities of native myosin as an adenosine triphosphatase or an inosine triphosphatase and those of modified myosin as an adenosine triphosphatase are very nearly the same. Although various salts inhibit all of these activities, they inhibit native ITPase and modified ATPase less than native ATPase. Therefore, perhaps a better description of the consequences of modification is to say that the activity of either CMB- or ITP-modified myosin is more resistant to salt inhibition than native myosin or, more speculatively, that ions disrupt the conformation of native myosin more easily than that of modified myosin. No ion seems to be inert in respect to native myosin, even tetramethylammonium acetate inhibits it at low concentrations (Fig. 1).

That CMB modification does not produce conformational changes of a magnitude gross enough to be recognized by optical rotatory dispersion, although changes in salt-induced inactivation are obvious, suggests this approach as an extremely sensitive, if only semiquantitative method for evaluation of conformational change.

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Addendum—While this manuscript was in review, there appeared the important paper of Robinson and Jencks (23) reporting the effect of various ions on the activity coefficient of a model peptide. The correlation (pointed out to us by Professor Jencks) between inhibiting activity (our results) and salting-in NH₃ groups (23) is indeed very suggestive.

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