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THE INFLUENCE OF CATIONS ON THE REACTIVITY OF THE SULFHYDRYL GROUPS OF ACTIN

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SUMMARY

1. The interaction of the sulfhydryl groups of G-actin with *p*-chloromercuribenzoate in the presence of chlorides of various cations has been studied. Calcium, in low concentrations, inhibits the reactivity of at least two of the 4 slowly reacting SH groups of actin, while magnesium slightly stimulates SH reactivity. Magnesium partially reverses the inhibition of SH reactivity due to calcium.

2. Zinc, cobaltous and nickel ions stimulate SH reactivity while manganous ions inhibit. Cadmium ions, though most active in precipitating actin, have no effect on SH reactivity. The alkali metal ions, lithium, sodium, potassium, and rubidium, in concentrations which normally induce polymerization of actin have no marked effect on SH reactivity.

3. ATP, which inhibits SH reactivity slightly, strongly increases the inhibition in the presence of calcium, but reduces the enhancing effect of magnesium.

4. These results are discussed in terms of a model in which calcium and magnesium have specific effects on the configuration of G-actin, the former acting to favor a "closed" configuration while substitution by the latter produces an "open" configuration. ATP appears to be intimately associated with the binding of calcium. Monovalent cations probably influence only the interaction between actin molecules as no effects on SH reactivity of the actin monomer were observed.

INTRODUCTION

The polymerization of actin has long been recognized to proceed in solutions of high ionic strength. STRAUB in his original work[1] emphasized the ionic nature of the required activation and MOMMAERTS[2,3] ascribed the action of salt, especially the cation, to its screening effect upon intermolecular repulsion due to the negative charge of the actin molecules. The activating effect of the bivalent cations calcium and magnesium, when added alone, is especially pronounced; while smaller amounts of magnesium accelerate polymerization induced by salts of monovalent cations and calcium inhibits this reaction[4-6]. It is possible that the pronounced polymerizing activity of higher

Abbreviation: PCMB, *p*-chloromercuribenzoate.

concentrations of these bivalent cations, in the absence of other salts, is entirely of an electrostatic nature, related to their high charge densities. By a similar mechanism, the apparent antagonism between monovalent cations and low concentrations of calcium in inducing polymerization might find an explanation in an antagonism between the ions in neutralizing the charge on the protein (*cf.* ref. 7). The present investigation, while not eliminating a contribution of antagonistic effects between ions or neutralization of charge, has shown that bivalent ions alter the reactivity of some of the sulfhydryl groups of actin.

As recently shown[8] there are approximately 6 such groups per 60000 g of actin, 2 of which react readily with mercurials and other reagents, while of the remaining 4, 2 are involved in the polymerization reaction. It was found that calcium decreased the reactivity of the slowly reacting SH groups while magnesium enhanced this. Higher concentrations of the alkali metals, Li^+, Na^+, K^+ and Rb^+, had no marked effect on SH reactivity while other bivalent ions had more complex relations to SH reactivity. ATP inhibited SH reactivity only slightly, but in the presence of calcium, a marked increase of the calcium inhibitory effect was seen. On the other hand, the enhancing effect of Mg^{2+} was reduced by ATP.

EXPERIMENTAL

Methods

Actin powder was prepared from the back and leg muscles of rabbits by the standard methods of the laboratory[8–10]. Extraction of the actin powder was carried out at 0° and polymerization induced with 0.1 M KCl. Two polymerizations were used in preparation and actin solutions were prepared in 0.2 mM ATP and 0.2 mM ascorbic acid. A final dialysis of G-actin was carried out overnight against 0.2 mM ATP–1.0 mM Tris-nitrate (pH 7.6–8.0).

Titrations of SH groups were carried out with PCMB utilizing the method of BOYER[11]. The spectral shift at 255 mμ was followed in a Hilger spectrophotometer, using a set of matched cuvettes. Solutions of PMB (2 × recrystallized) were made up weekly and the concentration determined from absorbance at 232 mμ. Reactions were carried out in dilute Tris-NO_3 buffer (approx. 5.0 mM) (pH 7.6) at room temperature. Reactions were started by adding actin to a solution containing the chloride salts of the cations and slightly less than two moles of PCMB/mole of actin sulfhydryl.

Titration of SH groups was originally carried out in solutions with approx. 0.03 mM ATP and ascorbic acid. However, it was found that ascorbic acid underwent a change in absorbancy during the reaction; it was therefore omitted. This also eliminated any possible binding of the cations by the ascorbic acid.

RESULTS

Inhibition of SH reactivity was seen with $CaCl_2$ at concentrations from $6 \cdot 10^{-6}$ to 10^{-3} M (Fig. 1). Higher concentrations of $CaCl_2$ caused precipitation of actin, as has been previously noted[1]. The initial reaction, amounting to approximately 1/3 of the total spectral shift, was not perceptibly affected by calcium, while inhibition of the slowly reacting SH groups was seen.

The stimulatory effect of magnesium on SH reactivity is seen in Fig. 2. The initial rapid reaction was again not perceptibly influenced while the slowly reacting

SH groups were seen to react more rapidly, when compared with the control curve where no $MgCl_2$ was added. This stimulation, in the absence of added calcium, was slight and not always seen. When both calcium and magnesium were added together, the curve obtained was between those where either bivalent cation was added alone. The amount of magnesium present in the actin preparations was about 10^{-5} M

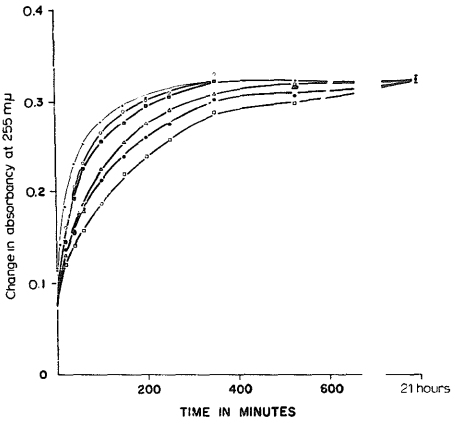

Fig. 1. The influence of calcium chloride on the SH reactivity of actin. The concentration of actin was $6.6 \cdot 10^{-6}$ M, the concentration of PCMB was 10^{-4} M, and the concentration of ATP was $2 \cdot 10^{-5}$ M. The inhibition of the slowly reacting SH groups by calcium chloride, in final concentrations of 10^{-2} M (□—□), 10^{-4} M (●—●), $4 \cdot 10^{-5}$ M (△—△), 10^{-5} M (■—■) and $6 \cdot 10^{-6}$ M (○—○) can be seen when compared with the control curve (·—·).

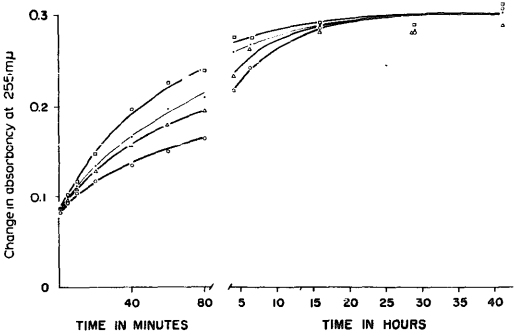

Fig. 2. The influence of magnesium on the SH reactivity of actin. The enhancement of SH reactivity in the presence of 10^{-4} M $MgCl_2$ (□—□) compared to the control values (·—·) can be seen along with reversal of the inhibition by 10^{-4} M $CaCl_2$ (○—○) when both $MgCl_2$ and $CaCl_2$ were added to give a final concentration of 10^{-4} M (△—△).

which, when diluted in the present experiments, gave a final concentration of Mg^{2+} less than 10^{-6} M. An attempt was made to remove this small amount of magnesium by dialysis of actin against Dowex-50, but some loss of sulfhydryl groups resulted. This procedure was then abandoned.

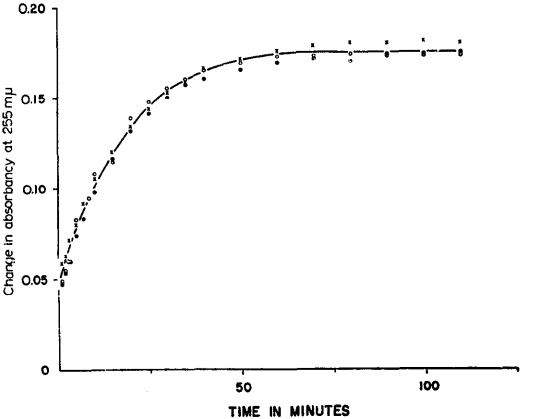

Fig. 3. The influence of barium and strontium chlorides on SH reactivity of actin. No significant effect of 10^{-3} M $BaCl_2$ (○—○) or $SrCl_2$ (●—●) can be seen when compared with the control values (×—×).

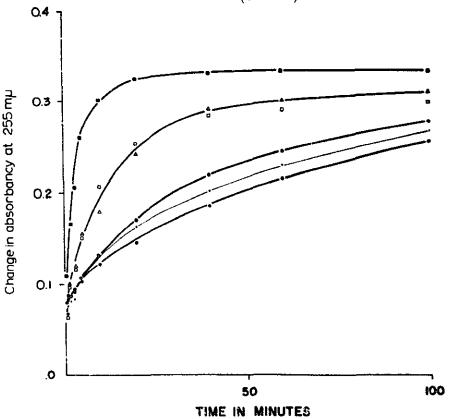

Fig. 4. The effects of several bivalent cations on the reactivity of the SH groups of actin. The stimulatory effect of 10^{-4} M $ZnCl_2$ (■—■) is greater than that of $CoCl_2$ (△—△) and $NiCl_2$ (□—□) at the same concentration. $MgCl_2$, 10^{-4} M, shows much less stimulation (○—○) while $MnCl_2$, 10^{-4} M, inhibits slightly (●—●) when compared with the control curve (·—·).

Barium and strontium chloride had no significant effect on SH reactivity (Fig. 3), while Mn^{2+} weakly inhibited the reaction of the slowly reacting SH groups (Fig. 4). This inhibition was less marked than that of Ca^{2+} in the same concentration.

Zinc, nickel and cobaltous chloride at concentrations of 10^{-4} M stimulated SH reactivity to an extent greater than Mg^{2+} in the same concentration (Fig. 4). The stimulatory effect of Zn^{2+} was the greatest, while that of Ni^{2+} and Co^{2+} were approximately equal. Cadmium chloride, at concentrations of $2 \cdot 10^{-5}$ M, had no effect on SH reactivity, while calcium chloride had an inhibitory effect at the same concentration (Fig. 1). All of the PMB titrations in the presence of Cd^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+} were carried out with concentrations of cation slightly less than that causing precipitation of the actin.

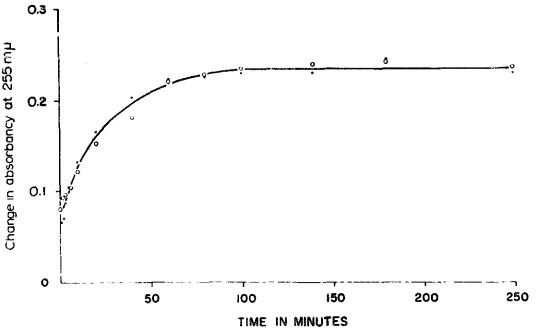

Fig. 5. The influence of KCl on the SH reactivity of actin. No difference is seen between the reaction rates in the presence of 0.1 M KCl (●—●) and a control curve (○—○).

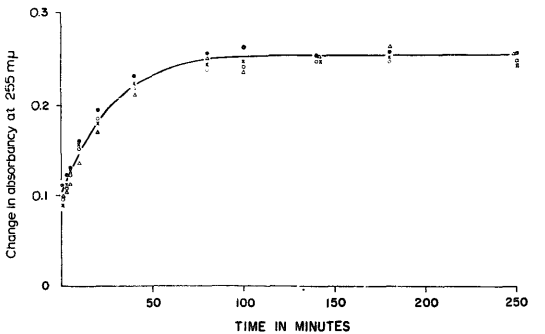

Fig. 6. A comparison of the effects of monovalent cations on the SH reactivity of actin. No difference is seen between the reaction rates in the presence of LiCl (○—○), NaCl (●—●), KCl (×—×) and RbCl (△—△) all at a concentration of 0.1 M.

The study of the influence of higher concentrations of potassium chloride on SH reactivity was complicated by the effect of 0.1 M solutions of this salt on the absorbancy of PCMB. It was found that, while the absorbancy at 255 mμ of PCMB bound to actin SH in 0.1 M KCl did not differ significantly from that in dilute buffer, the absorbancy of free mercurial was slightly higher in the salt. Sulfhydryl titrations, performed in both dilute buffer and 0.1 M KCl, gave values from 5.8 to 6.4 per 60000 g of actin in accord with previous results[8]. When suitable corrections for the increased absorbancy of the unbound PCMB were made, no major alteration in SH reactivity

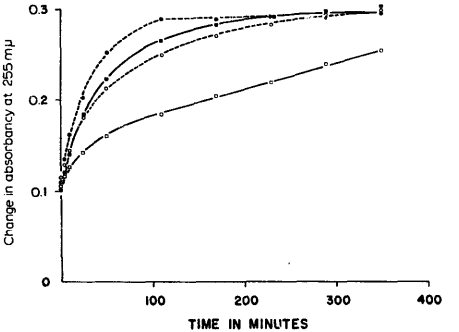

Fig. 7. The effect of ATP on the SH reactivity of actin in the presence of Ca^{2+}. ATP, when added to give a final concentration of $3.5 \cdot 10^{-5}$ M (■—■) slightly inhibits SH reactivity when compared to a control reaction where the ATP concentration was $1.7 \cdot 10^{-5}$ M (●---●). The inhibitory effect of 10^{-4} M $CaCl_2$ is considerably greater in the presence of $3.5 \cdot 10^{-5}$ M ATP (□—□) than in $1.7 \cdot 10^{-5}$ M ATP (○---○).

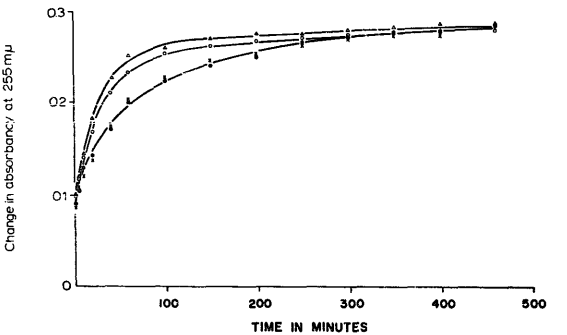

Fig. 8. The effects of ATP on the SH reactivity of actin in the presence of Mg^{2+}. In the presence of $1.7 \cdot 10^{-5}$ M ATP, $MgCl_2$ (10^{-4} M) enhances the reactivity of the SH groups (△—△) when compared to a control curve without added Mg^{2+} (○—○). The same concentration of $MgCl_2$, in the presence of $3.5 \cdot 10^{-5}$ M ATP (×—×), has no effect on SH reactivity when compared to control values at the same ATP concentration (●—●).

was seen in 0.1 M KCl* (Fig. 5). With some preparations of actin a slow increase in absorbancy at 255 mμ was seen in the presence of 0.1 M KCl. This reaction continued for several hours after the control reaction had stopped and it appears likely that this was due to a change in the spectrum of one of the other groups in the reaction mixture, possibly the bound nucleotide. This additional reaction was not influenced by either Ca^{2+} or Mg^{2+}. Because of this complication, these results cannot exclude a slight enhancement of SH reactivity by 0.1 M KCl. The inhibitory effect of calcium was seen in the presence of KCl. Tenth molar solutions of LiCl, NaCl and RbCl had the same effect on the absorbancy of PCMB as 0.1 M KCl, and these salts did not differ from KCl in their effects on the reactivity of actin SH groups (Fig. 6).

The tendency of bivalent cations to cause precipitation of actin, at pH 7.6 in dilute Tris-nitrate buffer, paralled the stimulation of SH reactivity, with the exception of cadmium and manganous ions. The order of precipitation-inducing activity was $Cd^{2+} > Zn^{2+} > Ni^{2+}$, $Co^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$.

Free ATP was present in all reaction mixtures at a concentration of approx. 10^{-5} M. When the concentration of ATP was doubled, a slight inhibition of SH reactivity was seen (Fig. 7). However, in the presence of the higher concentration of ATP, the inhibition of SH reactivity due to Ca^{2+} was increased to an extent greater than the sum of the inhibitory effects due to either ATP or Ca^{2+} alone (Fig. 7). On the other hand, the extent of enhancement of SH reactivity due to Mg^{2+} was reduced by added ATP (Fig. 8).

DISCUSSION

When viewing these effects of various bivalent cations upon the reactivity of the "slow" sulfhydryl groups, it must be stressed that corresponding effects upon the "fast" groups are not excluded; they were not detected, but might conceivably become evident upon the application of suitable rapid methods of study. Our findings, and therefore our considerations, apply only to the slow groups. This is no disadvantage, since it is among these groups that the ones essential to polymerization are found. The number of SH groups "protected" by calcium cannot be accurately determined from these data. It is likely that at least two are involved since reaction of slow SH groups is 55 % of the control at 200 min in the presence of 10^{-3} M $CaCl_2$ (Fig. 1).

The described results might be ascribed to a direct interaction between the cations and either the reagent, PCMB, or the sulfhydryl groups themselves. The former possibility can be excluded since SH inhibition by Ca^{2+} is seen when there is a 10-foid excess of mercurial (Fig. 1). The observed effects do not correlate with what is known regarding the reactivities of these ions with SH groups[12,13]; for calcium, especially, such a strong interaction would be unprecedented. It is far more probable that these ions cause modifications of the conformation of the actin molecule, and thus influence the accessibility of the SH groups indirectly, as is the case, more drastically, for agents like urea. The extent of this configurational change has not been estimated; it may be small, affecting only the immediate surroundings of the sites, or may be more widespread.

* Polymerization of the actin did not occur in these experiments because of the low concentration of the protein.

The effects of calcium are of particular interest since this ion is likely to play a role in the physiological activity of actin. The inhibition of SH reactivity by calcium parallels the protection of trypsin against heat denaturation[14] and self-digestion[15]. An interaction of calcium with SH groups in the case of this enzyme is excluded since trypsin has 6 disulfide bridges but no free cysteine[16,17]. On the basis of a shift in the titration curve of trypsin at low pH, it has been postulated that calcium is bound to the enzyme through carboxyl groups[16]. This is in accord with observations on model ligands, where oxyanions, such as carboxyl and hydroxyl groups and the phosphate of bound ATP in the case of actin, are sites in proteins to which calcium would be expected to bind most strongly[19,20]. Titration curves of actin at low pH in the presence and absence of calcium would be of interest, but such data will be complicated by the precipitation of actin at slightly acid pH, and the possibility of complete denaturation at greater acidity. Binding of calcium to an "essential" amino group[21], is unlikely[22] as is binding to an imidazole[13,22] which has been suggested to participate in the polymerization of actin[23]. The stabilization of actin SH groups cannot be attributed to the replacement of lost calcium which is normally bound to each molecule of actin[24-26], since the actin used in the experiments was polymerized without added Mg^{2+}, and preliminary observations on these actin preparations confirmed the presence of approx. 1 mole of calcium bound/mole of actin[27].

A further parallel between the stabilization of trypsin and the inhibition of SH reactivity of actin by calcium is seen in the effects of Mn^{2+}, Ba^{2+}, and Sr^{2+}. As in the case of actin, Mn^{2+} has a stabilizing effect on trypsin slightly less than that of Ca^{2+}, while Ba^{2+} and Sr^{2+} are without effect[14,15]. On the other hand, the stimulatory effect of Mg^{2+} was not seen in the case of trypsin[14,15]. Zinc enhances the heat inactivation of trypsin[14], while cobalt enhances self-digestion[15] but not heat inactivation[14] of this enzyme; both of the latter enhance SH reactivity of actin.

The partial reversal by magnesium of the calcium inhibition of the SH reactivity of actin (Fig. 2) suggests that magnesium and calcium may be bound at the same site. A formal kinetic analysis to test this hypothesis was not undertaken because (a) there is no obvious way to separate the observed reaction curves into reaction rates for each of the four slowly reacting SH groups, (b) the sequence of the reactions of the four slowly reacting SH groups may be affected by the cations, and (c) the spectral shift of PCMB with the individual SH groups need not be identical. It should be pointed out that the Mg^{2+} stimulation seen with actin was slight, and not always observed. It could be that this apparent stimulation is actually the reversal of the inhibitory effects of small amounts of Ca^{2+} in the actin solutions.

The stimulatory effects of Zn^{2+}, Ni^{2+}, and Co^{2+} on actin SH reactivity are probably due to a different mechanism since these ions are more likely bound to the proteins through nitrogen than oxygen[22]. The order of binding to glycine, alanine, and glycyl glycine[28,29] and to several other amino acids[30] is $Ni^{2+} > Zn^{2+} > Co^{2+}$, and the binding to imidazole is $Ni^{2+} > Zn^{2+}$ (see ref. 22). This order is not that observed for the stimulation of actin SH reactivity, but in contrast to the experiments on model substances, the binding of zinc to insulin is greater than that of cobalt[31], analogous to the present results. The binding of zinc to bovine[32] and human[33] serum albumin, appears to depend exclusively on a bond between the metal ion and an imidazole group. This binding of zinc to insulin has also been demonstrated to take place through the imidazole of histidine, although chelation to carboxyl groups

appears likely in this protein[34]. Cadmium, which binds more strongly than zinc to imidazole[35], has had no effect on actin SH reactivity. The reason for this apparent discrepancy may lie in the observation that Cd^{2+} forms weaker bonds than either Zn^{2+}, Co^{2+} or Ni^{2+} with amino acids other than histidine[22,30]. Of the ions studied, cadmium had the greatest tendency to precipitate actin, and the order for this activity, $Cd^{2+} > Zn^{2+} > Ni^{2+}$, is similar to the precipitating effects of these ions on human serum albumin[36]. The strong stimulation of actin SH reactivity seen with Zn^{2+}, Co^{2+} and Ni^{2+}, but not with Cd^{2+}, may be due to secondary binding of the first three cations from an imidazole to another site on the protein, while cadmium binding to an imidazole is without effect on SH reactivity due to failure to form bonds between two parts of the actin molecule.

These many effects of various bivalent cations could be explained by several models. It seems worthwhile to formulate a model to explain the interactions of greatest physiologic interest, those of actin with calcium and magnesium. This tentative model is illustrated in Fig. 9. The striking difference in behavior between calcium and magnesium is attributed to the tendency of calcium to form tridentate chelates;

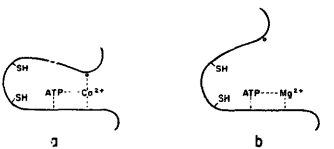

Fig. 9. Schematic representation of a possible mechanism for the interaction of bivalent cations with actin. (a) The stabilizing of a "closed" configuration of actin by calcium. The Ca^{2+}, through three bonds, serves to approximate two portions of the actin molecule and block the reactions of some SH groups (arbitrarily represented as two in number). (b) The replacement of Ca^{2+} by Mg^{2+} results in strong attachment of the cation to only one portion of the actin molecule; the absence of third bond to the other part of the actin molecule results in an "open" configuration. The previously blocked SH groups become more accessable (discussed in text).

whereas magnesium, because of its smaller ionic radius, tends to form bidentate chelates[20,37]. From previous results, in which it was found that removal of bound calcium led to a slow, rather than immediate release of bound nucleotide[38], it has now been postulated that the nucleotide (ATP in this example) is bound to actin directly at one point, and through calcium at another (Fig. 9a). In distinction to magnesium, calcium, which has three binding sites, can associate with oxyanions at two parts of the actin molecule, producing a "closed" configuration. In this "closed" configuration, some of the SH groups (arbitrarily represented in the figure as being two in number) become less accessible to reaction with PCMB. The stimulation of SH reactivity by magnesium, and its reversal of the inhibition by calcium, according to this model, is due to its replacement of calcium. Such replacement has been demonstrated[39]. Since magnesium is not likely to form tridentate chelates, this ion converts the "closed" configuration, represented in Fig. 9a, to an "open" configuration in which SH groups are no longer protected from interaction with PCMB (Fig. 9b). However, these groups are still unable to react with *N*-ethylmaleimide[8]. Barium and strontium ions, possibly because of their larger ionic radius, do not interfere with this binding.

The inhibition of SH reactivity by ATP, previously noted*,[21,40], can also be understood in terms of this model where the nucleotide can stabilize the "closed" configuration by reducing the exchange of Ca^{2+}. This is evidenced by the inhibition of SH reactivity, particularly in the presence of added Ca^{2+}, and the reduction of the enhancing effect on SH reactivity by Mg^{2+} in the presence of higher concentrations of ATP. The protection by ATP against inactivation by EDTA[41] is also accounted for by the model. The requirement for Mg^{2+} in polymerization can be explained if bound Ca^{2+} must be replaced in order to stabilize the "open" configuration. (Bound magnesium may also play a role in the binding of the actin monomers to one another.) Furthermore, the slight and variable Mg^{2+} stimulation of SH reactivity indicates that magnesium increases the rate of release of bound calcium little, if at all, and may act only to replace Ca^{2+} when the latter is released. The action of monovalent cations, such as K^+, in inducing polymerization is not explained by the model; this action appears to be due to dissipation of the electrostatic repulsion between individual actin molecules as has been suggested[2,3]. The approach of the second actin molecule may, with Mg^{2+}, convert the configuration to the "open" form, thus permitting the formation of bonds between the molecules. These bonds may involve SH groups since 2 of these become unreactive in the case of F-actin[8]. Finally, the slow release of bound nucleotide[40,42] and bound Ca^{2+} (see refs. 26, 40) observed when SH groups are reacted with one of a number of inhibitors, may be due to the steric action of the inhibitor forcing the configuration of actin into the "open" form, thus reducing the stability of the binding of ATP and Ca^{2+} to the protein.

The effects of the other bivalent cations on SH reactivity can be understood in terms of one of a number of extensions of this model. Thus, Mn^{2+} may replace Ca^{2+} and form a weaker tridentate bond to maintain the "closed" configuration, while Zn^{2+}, Ni^{2+} and Co^{2+} may act to maintain the "open" configuration by binding at adjacent sites. Cadmium appears to be bound with no effect on this configuration. Some pertinent data concerning the interactions of these cations with proteins, in the context of the present results, have already been discussed; however, the addition of further detail to this model to explain the various effects observed does not appear warranted.

NOTE ADDED IN PROOF

Further evidence favoring a common binding site for bivalent cations and ATP (Fig. 9) is found in the recent work of STROHMAN AND SAMORODIN[43] and BÁRÁNY *et al.*[44]. The former authors have demonstrated stabilization of actin ATP binding by Ca^{2+}, and to a lesser extent, Mg^{2+}; the latter have presented evidence favoring binding of ATP to actin through a bond involving Ca^{2+}.

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* TONOMURA AND YOSHIMURA[40] failed to note an inhibitory effect of Ca^{2+} on SH reactivity; this is explained since they studied the effect of Ca^{2+} in the absence of free ATP. However, their published data suggest that a low level of inhibition occurred in their experiments.

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