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Stabilizing Actions of Free Nucleotides on the Sulfhydryl Groups of G-Actin*

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ABSTRACT: The influence of a number of free nucleotides and related compounds upon the reactivity of the sulfhydryl groups of G-actin has been investigated. In the presence of low concentrations of endogenous adenosine 5′-triphosphate (ATP) (approx 0.01 mM), added ATP and inosine 5′-triphosphate (ITP) in concentrations of 0.1 mM were found to inhibit the reactivity of actin's slowly reacting sulfhydryl (SH) groups; guanosine 5′-, cytidine 5′-, and uridine triphosphates, adenosine 5′-mono- and diphosphates, adenosine, and adenosine 3′,5′-phosphate were without demonstrable effect. The inhibition of SH reactivity by ITP was less than that by ATP, and could be seen only in the presence of 0.1 mM $CaCl_2$. Adenosine tetraphosphate (0.1 mM) and higher concentrations of inorganic pyrophosphate (10 mM), but not of inorganic phosphate, enhanced the SH reactivity of G-actin. These findings can be interpreted in terms of specific structural requirements for both the nucleoside ring and the polyphosphate chain in the attachment of nucleotides to G-actin.

The role of ATP[1] as a functional group of G-actin has been recognized since the work of Straub and Feuer (1950) and Laki *et al.* (1950). However, the problem of the participation of actin-bound nucleotide in polymerization appears more complex than the stoichiometrically coupled dephosphorylation described by Mommaerts (1952). Thus, while the nucleotides ordinarily associated with G- and F-actin are ATP and ADP, respectively, G-actin containing bound ADP can be prepared and is able to polymerize (Hayashi and Rosenbluth, 1960; Grubhofer and Weber, 1961; Mommaerts, 1961; Katz, 1964), suggesting that dephosphorylation of ATP is not essential for polymerization. Furthermore, polymerization of nucleotide-free G-actin has recently been reported (Oosawa, 1964). The present investigation is directed to a characterization of the actions of added nucleotides and related compounds upon the SH reactivity of the G-actin monomer, it having previously been shown that free ATP protects some of the SH groups of actin from reaction with mercurials (Bárány, 1956; Strohman and Samorodin, 1962) in a manner that appears to reflect changes in the protein conformation (Katz, 1963).

Methods

Acetone-dried muscle powders were prepared from the back and leg muscles of rabbits by the standard methods of this laboratory (Katz and Mommaerts, 1962; Katz, 1963; Carsten and Mommaerts, 1963) and actin was extracted with 0.1 mM ATP at 1–3°. Twice-purified actin was dialyzed against 0.1 or 0.2 mM ATP and 1.0 mM Tris nitrate at *p*H 7.6; free ATP was not removed

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[1] Abbreviations used in this work: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-phosphate; GTP, guanosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; UTP, uridine triphosphate; ITP, inosine 5′-triphosphate; IDP, inosine 5′-diphosphate.

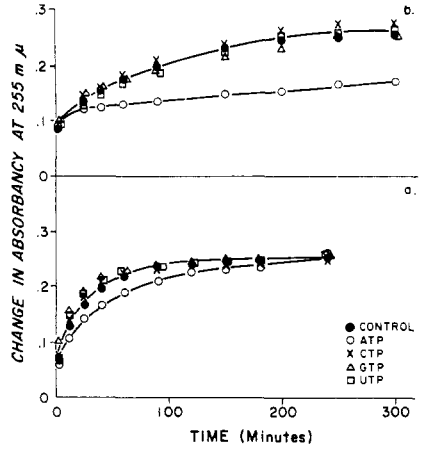

FIGURE 1: Effect of nucleoside triphosphates on the SH reactivity of G-atcin. The effects of 0.1 mM concentrations of ATP (○), CTP (x), GTP (△), and UTP (□) are compared to control reactions (●) in which no nucleotides were added; reactions were carried out in the absence (a) and presence (b) of 0.1 mM $CaCl_2$. Protein concentration was 0.5 mg/ml.

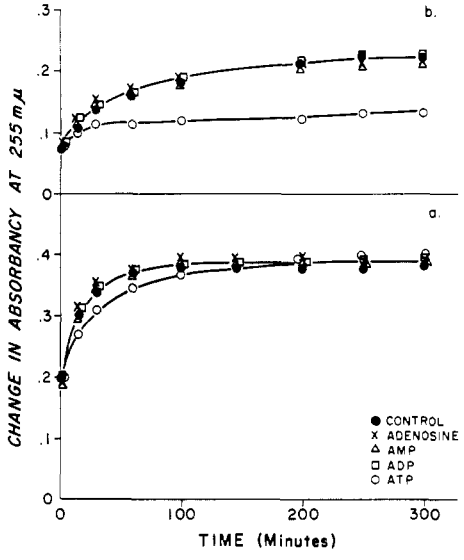

FIGURE 2: Effect of adenosine, AMP, ADP, and ATP on the SH reactivity of G-actin. The effects of 0.1 mM concentrations of adenosine (x), AMP (△), ADP (□), and ATP (○) are compared to control reactions (●) in which no nucleotides were added; reactions were carried out in the absence (a) and presence (b) of 0.1 mM $CaCl_2$. Protein concentration was 0.5 mg/ml.

from the actin solutions since this treatment may cause loss of SH groups (Bárány *et al.*, 1961).

Titration of SH groups with *p*-mercuribenzoate was carried out, utilizing the method of Boyer (1954) as previously described (Katz, 1963); the spectral shift at 255 mμ of the *p*-mercuribenzoate was followed in a

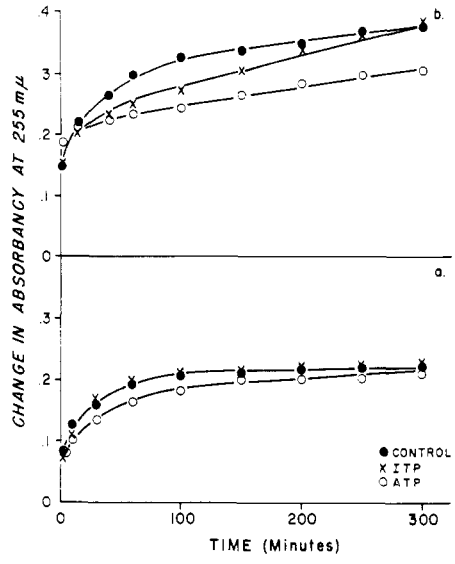

FIGURE 3: Effect of ITP on the SH reactivity of G-actin. The effect of 0.1 mM ITP (x) is compared with that of 0.1 mM ATP (○) and of a control reaction to which no nucleotide was added (●); reactions were carried out in the absence (a) and presence (b) of 0.1 mM $CaCl_2$. Protein concentration was 0.5 mg/ml.

Hilger or a Gilford spectrophotometer. The reactions described in this work were carried out at 25° at a protein concentration of 0.4 or 0.5 mg/ml in dilute Tris-nitrate buffer (approximately 0.1 M) at *p*H 7.6, with a molar ratio of *p*-mercuribenzoate to actin sulfhydryl groups of 1.5–2.0. Evidence for polymerization was not seen because inactivation of the protein by the mercurial was rapid compared with the rate of polymerization in the dilute actin solutions at low ionic strength. The free ATP concentration in the reaction mixtures was approximately 0.01 mM; the added nucleotides had absorbances between 0.8 and 1.7. Up to seven reactions were started simultaneously. In all cases rates were compared with a control reaction using the same actin preparation without added nucleotides because the curves obtained with different actin preparations differed slightly.

All chemicals used were reagent grade. *p*-Mercuribenzoate was reprecipitated twice before use, and nucleotides were obtained from the Pabst Laboratories or the Sigma Chemical Co.

Results

No alteration of the SH reactivity was observed in the presence of 0.1 mM GTP, CTP, or UTP, either in the absence of Ca^{2+} (Figure 1a) or in 0.1 mM $CaCl_2$ (Figure 1b). Similarly adenosine, AMP, and ADP had no effects in the absence or presence of 0.1 mM $CaCl_2$ (Figure 2a,b) and adenosine 3′,5′-phosphate was without effect. The highest final concentration of each nucleotide was 0.1 mM; higher concentrations proved

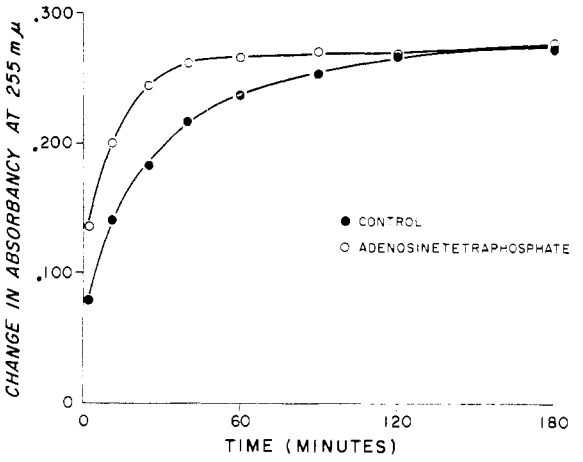

FIGURE 4: The effect of adenosine tetraphosphate on the SH reactivity of G-actin. The rate of reaction in the presence of 0.1 mM adenosine tetraphosphate (○) is compared to a control reaction in which no nucleotide was added (●), in the absence of added $CaCl_2$. Protein concentration was 0.5 mg/ml.

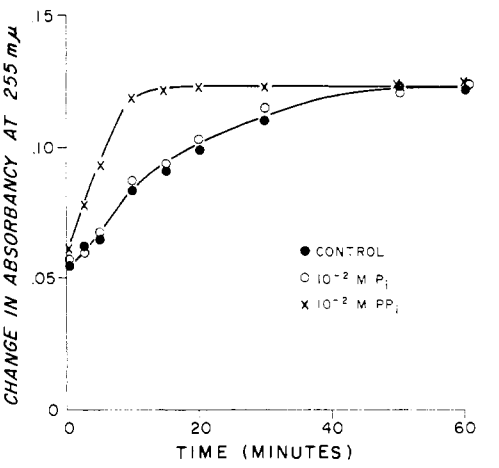

FIGURE 5: Effects of P_i and PP_i on the SH reactivity of G-actin. The rate of reaction in the presence of 0.01 M P_i (●) and 0.01 M PP_i (x) are compared to a control reaction (○) in the absence of added $CaCl_2$. Protein concentration was 0.4 mg/ml.

impractical because of the high absorbance of the nucleotides at 255 mμ. ITP was without demonstrable effect on actin SH groups in the absence of Ca^{2+} (Figure 3a). However, in 0.1 mM $CaCl_2$ slight but significant inhibition of SH reactivity was seen (Figure 3b). Adenosine tetraphosphate caused enhancement of the SH reactivity of G-actin in the absence, but not in the presence, of added Ca^{2+} (Figure 4).

Concentrations of P_i and PP_i equal to those of the nucleotides examined (0.1 mM) were without effect on SH reactivity. However at higher concentrations (10 mM) a significant enhancement of SH reactivity was seen with PP_i, but not with P_i (Figure 5). At the latter concentrations these compounds become the major buffer, requiring care in adjustment of the *p*H because of the *p*H dependence of the reaction (Katz and Mommaerts, 1962). Data were used only when the *p*H of the P_i and PP_i mixtures was 7.6 ± 0.1 at the conclusion of the experiments.

Discussion

These results, while demonstrating that ITP, like ATP (Bárány, 1956; Tonomura and Yoshimura, 1962; Katz, 1963), inhibited the reactivity of some of the slowly reacting SH groups of actin, fail to distinguish between two alternative mechanisms for the lack of inhibition by other nucleoside triphosphates: (a) that the absence of inhibition was owing to the failure of the nucleotide to exchange for actin-bound ATP in the presence of low concentrations of free ATP, or (b) that the nucleotide, when bound to actin, was unable to protect SH groups. The observation that UTP, CTP, and GTP have relatively low affinities for G-actin (Iyengar and Weber, 1964) and do not readily exchange for actin-bound ATP (Strohman and Samorodin, 1962; Martonosi, 1962) indicates that failure to bind to actin is the correct explanation. Therefore, the present results do not characterize the effects of these substances when bound to actin. ITP, which exchanges for actin-bound ATP (Martonosi and Gouvea, 1961; Martonosi, 1962), did inhibit the reaction with *p*-mercuribenzoate of actin's SH groups, although this inhibition was less than that produced by free ATP and could be exhibited only in the presence of added Ca^{2+}.

The failure of ADP, AMP, and adenosine to inhibit the SH reactivity of G-actin probably reflects the absence of significant exchange of these compounds with ATP bound to G-actin (Martonosi *et al.*, 1960; Martonosi, 1962). As in the case of the nucleoside triphosphates studied, there was a close parallel between the affinities of nucleotide binding (Iyengar and Weber, 1964) and the ability to inhibit the reactions of actin's slowly reacting SH groups, favoring the interpretation that SH inhibition by ATP and ITP results from an interaction at the nucleotide-binding site of actin (Katz, 1963). The Ca^{2+}-induced inhibition of SH reactivity also appears to be related to binding at this site (Katz, 1963).

Direct binding of nucleotides to SH groups appears unlikely on the basis of several lines of evidence that have been discussed previously (Morales and Hotta, 1960; Katz, 1963). Further evidence that the nucleotides are not bound directly to SH groups can be obtained by comparing the rate at which *p*-mercuribenzoate reacts with actin's SH groups with the rate at which the mercurial causes release of actin-bound ATP. Loss of the ability to polymerize after addition of *p*-mercuribenzoate proceeds more rapidly than displacement of bound nucleotide (Drabikowski and Gergely, 1963), whereas *p*-mercuribenzoate renders G-actin unable to polymerize only after the mercurial is bound to the least reactive of actin's SH groups (Katz and Mommaerts, 1962; Tonomura and Yoshimura, 1962). The

apparent delay in displacement of actin-bound ATP after reaction of the SH groups favors the interpretation that the nucleotide was not bound directly to SH groups but, instead, that the reaction of actin with mercurial induced a conformational change in the actin monomer that greatly decreased the affinity between the protein and ATP.

Enhancement of SH reactivity by adenosine tetraphosphate suggests that this nucleotide replaces actin-bound ATP in a manner that reverses the ATP-induced masking of actin's SH groups. Similarly, the enhancement resulting from high concentrations of PP_i may be the result of its binding to G-actin in place of ATP. The reported failure to demonstrate displacement of actin-bound ATP in the presence of PP_i (Martonosi, 1962) may have resulted from use of relatively low concentrations of PP_i and from partial polymerization of the concentrated actin solutions that were studied. The effect described in the present report was obtained only at relatively high concentrations of PP_i in dilute actin solutions.

These results, along with previous studies of binding of nucleotides to G-actin, indicate that nucleotides are attached to actin through linkages to both the pyrophosphate chain and the nucleoside ring. Binding of polyphosphate per se is indicated by the observation that PP_i enhances the SH reactivity of G-actin in a manner compatible with displacement of actin-bound ATP without formation of additional bonds necessary to mask slowly reacting SH groups of actin (Katz, 1963). A specific bond between the nucleoside ring and actin is indicated by the finding that differently shaped rings induce different protective effects on actin's SH reactivity (Figures 1–3), as well as by direct studies of binding (Martonosi and Gouvea, 1961; Strohman and Samorodin, 1962; Iyengar and Weber, 1964). It is possible that the weak binding of UTP, CTP, GTP, ADP, and IDP to G-actin after isoelectric precipitation, without protection of ability to polymerize (Martonosi and Gouvea, 1961), reflects binding of the polyphosphate chain to the protein without a second attachment of the nucleoside ring.

The present findings in regard to the SH reactivity of actin can be compared to the comparable properties of myosin in which, like actin, the reactivities of different SH groups toward *p*-mercuribenzoate are dissimilar (Gilmour and Gellert, 1961; Hotta, 1961) and in which both ATP (Stracher, 1964) and PP_i (Morales and Hotta, 1960) inhibit the reaction of some SH groups with *p*-mercuribenzoate. Recent studies of Rainford *et al.* (1964) indicate that there are at least three classes of SH groups of myosin, as has been reported in the case of actin (Katz and Mommaerts, 1962). The interaction of nucleotides with myosin appears to be less specific than that with actin because a wide variety of nucleoside triphosphates can be hydrolyzed by myosin (Hasselbach, 1956; Ikehara *et al.*, 1961), whereas inhibition of actin's SH reactivity and exchange with actin-bound ATP could be demonstrated only in the case of ATP and ITP. As is the case for actin, it is likely that ATP is bound to myosin both through the nucleoside ring and the polyphosphate chain (Blum, 1955; Hotta, 1961; Yount and Koshland, 1963; Dempsey *et al.*, 1963). The occurrence of dual binding sites of ATP to both actin and myosin and demonstrations of changes in binding characteristics that would follow dephosphorylation of actomyosin-bound ATP, coupled with evidence for conformational changes in F-actin (Asakura *et al.*, 1963), G-actin (Katz, 1963, 1964), and myosin (Iyengar *et al.*, 1964) that could occur under such conditions, suggest a number of intriguing mechanisms to account for the molecular basis of muscular contraction. However, selection of any of these possible mechanisms would be premature at the present time.

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Studies on β-Lactoglobulins A, B, and C. I. Comparison of Chemical Properties*

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ABSTRACT: β-Lactoglobulins A, B, and C have been isolated, purified, and examined for amino acid composition. The previously described difference between β-A and β-B in content of aspartic acid, glycine, valine, and alanine is confirmed, and β-C is found to differ from β-B only in content of glutamine and histidine. β-Lactoglobulin C has 2 residues more of histidine and 2 residues fewer of glutamine than β-B per molecule of ~36,000 molecular weight. DEAE-cellulose column chromatography, using a phosphate buffer containing a NaCl gradient, has been extended to provide a separation of the three proteins. Photooxidation data and ultraviolet spectra are in agreement with the amino acid analyses.

An examination of the amino- and carboxyl-terminal sequences of the three variants yielded identical qualitative results; but a difference in the rate of release of amino acids from the carboxyl end of β-C as compared with β-A and β-B was observed. The conclusion is drawn that the amino acid substitutions among the β-lactoglobulins do not occur at or near the terminal portions of the peptide chain.

The genetically different bovine β-lactoglobulins A and B, discovered by Aschaffenburg and Drewry (1955, 1957a), have been chemically characterized by Gordon *et al.* (1961) and Piez *et al.* (1961). They found that the proteins differed in the content of four amino acids, β-A having 2 more aspartic acid and valine residues and 2 fewer glycine and alanine residues per mole than β-B. By studying peptides from chymotryptic digests of β-A and β-B, Kalan *et al.* (1962) showed that the differing amino acids are linked in pairs, aspartic acid being substituted for glycine and valine for alanine. The isolation and composition of the peptides containing the aspartic acid for glycine substitution were described but the valine for alanine substitution was not located. Townend *et al.* (1960a) have shown that each β-lactoglobulin molecule (~36,000 mw) consists of two identical chains of approximately 18,000 mw. Fraenkel-Conrat (1954, 1956) and Niu and Fraenkel-Conrat (1955) found, by chemical methods, 2 amino-terminal leucine residues and 2 carboxyl-terminal isoleucine residues per mole for mixed β-lactoglobulin. Neurath *et al.* (1954), using carboxypeptidase A, showed that histidine is the penultimate amino acid. Davie *et al.* (1959) investigated the kinetics of the release of isoleucine and histidine from a mixed preparation. Later work (Kalan and Greenberg, 1961) reported on the action of carboxypeptidases A and B on β-lactoglobulins A and B and their *S*-sulfo derivatives. These results revealed an identical C-terminal sequence, -Leu-His-Ileu, and identical rates of hydrolysis for both protein variants.

Bell (1962) reported the discovery of a third genetically determined β-lactoglobulin, β-C. This variant was shown to have a slightly slower mobility than β-B in starch-gel electrophoresis at *p*H 8.6. The immediate question arose as to the relationships in amino acid composition and structure of β-C to the previously analyzed A and B proteins. The opportunity to investigate the chemical properties of β-lactoglobulin C was

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