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Nucleotide in Monomeric Actin Regulates the Reactivity of the Thiol Groups†

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ABSTRACT: A new thiol reagent, 2,4-dinitrophenyl glutathionyl disulfide, allowed the characterization of four thiol groups in monomeric actin by stoichiometric reaction. The number of thiol groups exposed to the reagent was found to depend on the nucleotide bound. In the absence of ATP, G-actin exposed four thiol groups (G_{4S}). On the addition of ATP (1 equiv), three of them were shielded. The resulting actin with one thiol group exposed (G_{1S}) is the form of monomeric actin normally produced by depolymerization of F-actin in buffers containing ATP. G_{1S} is stable over hours, while G_{4S}, i.e., monomeric actin in ATP-free solution, is not. This must be concluded from the fact that the shielding effect of thiol groups induced by addition of ATP was lost within ca. 30 min probably due to denaturation of G_{4S} to G_{4S}*. Therefore, denaturation of monomeric actin must be understood in terms of loss of thiol shielding, rather than by oxidation of the thiol groups. Addition of equimolar amounts of Ca^{2+} significantly retarded the denaturation process. ADP (50 equiv) shielded only ca. two of the four thiol groups but, similar to ATP, protected actin from denaturation. Three ATP analogues (10 equiv) were tested but had no shielding effect. In the presence of these analogues actin (G_{4S}) rapidly denatured (to G_{4S}*) as in the absence of added nucleotides. It was shown that the thiol-shielding activity and the protective capacity of a nucleotide are interrelated with its binding capability to monomeric actin. G_{1S} was found to be polymerizable as was $G_{\sim 2S}$ on the addition of ATP. No polymerization could be detected for G_{4S} or G_{4S}*. In general the ability to polymerize was found to be lost when, after addition of ATP to solutions of monomeric actin, the number of the exposed thiol groups was greater than two.

Rabbit muscle actin contains five cysteine residues (Elzinga et al., 1973) existing in the reduced form. Compared to the host of data available on structural and functional properties of actin [for a recent review see Korn (1980)], only a few studies of the last years dealt with the characterization of such thiol functions (Lusty & Fasold, 1969; Ishiwata, 1976; Knight & Offer, 1978). Most of the investigators describe a preferential reactivity of the cysteine moiety in the penultimate position of the protein chain, which, according to recent sequence corrections (Vandekerckhove & Weber, 1978), is cysteine-374.

While studying this question, e.g., by the preparation of mixed disulfides of rabbit muscle actin with glutathione, we detected a strong correlation between the accessibility of the various thiol groups in actin and the type of adenosine nucleotide bound. A detailed investigation of the effect was made possible by the finding that 2,4-dinitrophenyl glutathionyl disulfide (DNPSSG)[1] represents an excellent tool for probing the thiol groups of actin. The reagent, a mixed alkyl aryl disulfide, was superior to Ellman's reagent (Ellman, 1959) by showing more exactly the end point of reaction of distinct thiol groups. On the other hand, DNPSSG releases, like Ellman's reagent, 1 mol of nitrated thiophenolate anion per mol of thiol group reacted. By this stoichiometry the reaction can be followed in a similar way as with Ellman's reagent. The release of 1 mol of nitrated thiophenolate from DNPSSG provides

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[1] Abbreviations: DNPSSG, 2,4-dinitrophenyl glutathionyl disulfide; DNPSST, 2,4-dinitrophenyl thioglycoyl disulfide; APOPCP, adenosine (β,γ-methylenetriphosphate); APOPNP, adenosine (β,γ-imidotriphosphate); APCPOP, adenosine (α,β-methylenetriphosphate); NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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evidence that the reaction with thiol groups will not yield an undefined mixture of disulfides but results in the formation of exclusively dialkyl disulfides.

The usefulness of DNPSSG was proved recently with F-actin where one thiol group was shown to be readily accessible while a second one reacts slowly and can be shielded by ATP or phalloidin (Blackholm & Faulstich, 1981). Similarly, in the present study with monomeric actin, we were able to distinguish at least three different types of thiol groups in monomeric actin. The different reactivities as described for the thiol groups did not depend on the glutathione moiety (G) in the reagent (DNPSSG) but were obtained similarly with a reagent containing thioglycolic acid (T) instead (DNPSST).

Materials and Methods

$ATPNa_2$ and $ADPNa_2$ were from Boehringer, Mannheim, APOPCP and APOPNP were from Serva, Heidelberg, and APCPOP was from Sigma, München. NEM was from Serva, Heidelberg. 2,4-Dinitrophenyl glutathionyl disulfide (DNPSSG) was prepared as described by Fontana et al. (1968) with some modifications: to a solution of 1 mmol of reduced glutathione in 2 mL of formic acid (99%) 1.1 mmol of 2,4-dinitrophenylsulfenyl chloride was added and stirred at room temperature. After 1 h the reaction mixture was filtered and poured into 100 mL of diethyl ether. The precipitate was washed several times with the same solvent and dried in vacuo over P_2O_5. When dissolved in 30 mL of water, the hydrate of the compound crystallized spontaneously after a few minutes. The crystals were collected after several hours standing at 4 °C, filtered, and washed several times with water and ether: yield 80%; mp 187 °C dec. The analysis fits best with the monohydrate. Anal. Calcd for $C_{16}H_{19}N_5O_{10}S_2 \cdot H_2O$ (Found): C, 36.73 (36.71); H, 3.79 (4.04); N, 13.15 (13.38); S, 12.47 (12.25). Actin was isolated from rabbit muscle by the procedure of Löw & Dancker (1976) and was pure as proved by SDS electrophoresis. Mono-NEM–actin was prepared by reacting a 5-fold excess of NEM with a 2×10^{-5} M solution of F-actin in KCl (0.1 M)–Tris-HCl (1 mM), pH 7.4, according to Lusty & Fasold (1969).

The ratio nucleotide:actin was measured spectrophotometrically. Protein concentration was determined by the optical density at 290 nm using $\epsilon_{290} = 26\,460$ mol^{-1} cm^{-1} or $\epsilon_{290} = 28\,140$ mol^{-1} cm^{-1} for G- and F-actin, respectively. The adenine nucleotides were determined after precipitation of the protein by addition of 1 volume of 1 M perchloric acid to the actin solutions (10 min, 0 °C), followed by centrifugation (20000g, 2 min) and spectrophotometry using $\epsilon_{259} = 15\,000$ mol^{-1} cm^{-1}.

Excess nucleotide was removed from F-actin by gently homogenizing the pellet in KCl (0.1 M)–Tris-HCl (1 mM), pH 7.4 at 4 °C. After ultracentrifugation (140000g, 70 min) the nucleotide:actin ratio in the pellet was in all cases 0.9 to 1.0.

For depolymerization the actin pellet was gently homogenized in a Teflon homogenizer at 4 °C with buffers of Tris-HCl (1 mM) and adenine nucleotide (0.02–1.0 mM), pH 7.4. For the kinetic studies of actin denaturation the pellet was depolymerized in Tris-HCl (1 mM), pH 7.4, and after the pellet was allowed to stand for different times at 4 °C, the nucleotide was added in small volumes of millimolar solutions. Corresponding experiments were performed with the same buffer, but 0.02 mM in $CaCl_2$.

Reaction of 2,4-dinitrophenyl glutathionyl disulfide (DNPSSG) (or 2,4-dinitrophenyl thioglycoyl disulfide, DNPSST) with solutions of G-actin was followed spectrophotometrically using the extinction of the 2,4-dinitrothiophenolate ions ($\epsilon_{408} = 12\,700$ mol^{-1} cm^{-1}, at pH 7.4). The reaction was started by adding 20 μL of a 0.01 M solution of the reagents in 1% $NaHCO_3$ to 1 mL of the actin solutions. The increase in extinction at 408 nm was measured in an Aminco DW-2 double-beam spectrophotometer as the difference between the sample cuvette and the reference cuvette containing only reagent and buffer. Polymerization of actin was measured in an Ostwald viscometer (Canon 175) with an outflow time for water of 32 s (22 °C), after addition of a solution containing $MgCl_2$ (final concentrations 1.0–2.0 mM) or KCl (final concentration 0.1 M) directly into the viscometer. Viscometry was plotted as relative viscosity $\eta = t/t_0$. Binding of nucleotides to G-actin was studied by developing 1-mL mixtures of actin (4×10^{-5} M) and nucleotide (1–15 equiv) on a Bio-Gel P2 column (1 cm i.d. × 90 cm) in Tris-HCl (1 mM), pH 7.4. The elution was monitored by an LKB equipment at 254 nm. The recovery of nucleotides (total content in the two peaks) was in all cases >95%.

Results and Discussion

Actin is usually depolymerized in buffers containing ATP. The resulting monomer will persist, at low temperatures, without any signs of denaturation for hours. When reacted with excess of our reagent, DNPSSG, we found that this type of monomeric actin exposed one thiol group, thus confirming results of other laboratories reporting one fast reacting thiol group in G-actin (Lusty & Fasold, 1969; Martonosi, 1968). The most reactive thiol group of actin has been identified as the cysteine residue next to the carboxy terminus, and we expected that also for the reaction of DNPSSG with G_{1S} the thiol group of Cys-374 would be substituted. Evidence for this was given by preparing mono-NEM–F-actin as described by Lusty & Fasold (1969). After depolymerization in ATP-containing buffer, the corresponding monomer did no longer react with DNPSSG (Figure 1). Since the amino acid residue substituted in F-actin by NEM has been identified to be cysteine-374 (at that time cysteine-373) (Lusty & Fasold, 1969), we conclude that also in the reaction with DNPSSG this residue was the most reactive one.

The disulfide formation is complete after ca. 20 min (Figure 1). Since G-actin is usually prepared with an excess of ATP, we investigated whether actin (2×10^{-5} M) in the presence of only 1 equiv of ATP (2×10^{-5} M) also exposes only one thiol group. This was found to be true, showing that the affinity of ATP for monomeric actin is high and that an equimolar amount of ATP is sufficient to exhibit full protection of the protein.

When actin is depolymerized in the absence of ATP the protein exposes four thiol groups (G_{4S}). Since actin has five cysteine residues, this means that all thiol groups except one are accessible. All four thiol groups react with similar kinetics and similar to the thiol group in G_{1S} (complete reaction in ca. 20 min; Figure 1). We conclude that all four thiols are equally accessible to the reagent. G_{4S} denatures very rapidly, as proved by the loss of polymerizability. Even at low temperatures (4 °C), G_{4S} was found to be unpolymerizable after 30 min upon addition of ATP and Mg ions. While denaturation proceeds, all four thiol groups remain exposed and reactive.

The course of denaturation could be followed independently of the polymerizability by the addition of ATP to the solution of G_{4S} after various times and subsequent titration of the thiol groups in the presence of that nucleotide. For example, when actin was depolymerized in a buffer containing Tris only, (G_{4S}), and ATP was added instantly after the viscosity of the solution was down to buffer values (after maximum of 2 min), we found 1.2 thiol groups still reactive. We conclude that the

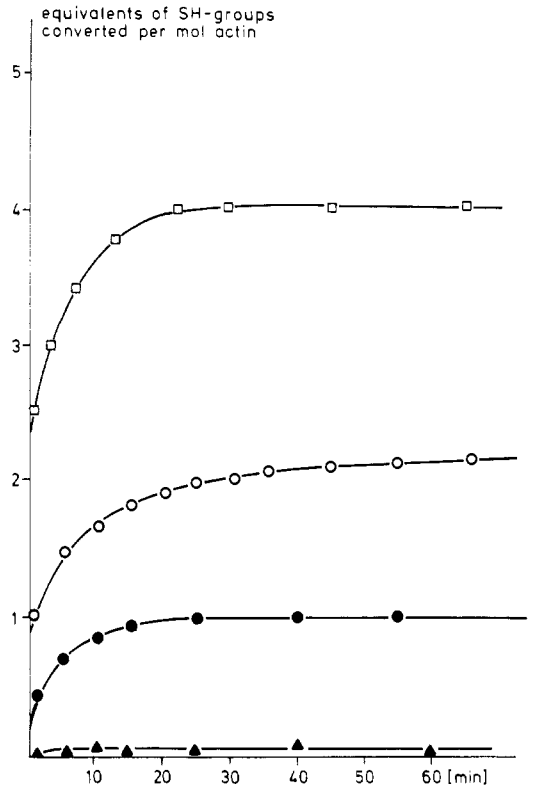

FIGURE 1: Reaction kinetics of different species of monomeric actin with DNPSSG at 30 °C as monitored by the absorption of 2,4-dinitrothiophenolate ($DNPS^-$) (408 nm). (Filled circles) Actin depolymerized in ATP-containing (2×10^{-4} M) buffer; (open circles) actin depolymerized in ADP-containing (mM) buffer; (squares) actin depolymerized in nucleotide-free buffer; (triangles) (mono)NEM–actin, depolymerized in ATP-containing (2×10^{-4} M) buffer. The buffer was Tris-HCl (1 mM), pH 7.4. Actin concentration was in all cases 2×10^{-4} M, and DNPSSG was applied in 10-fold excess over actin.

addition of ATP immediately after depolymerization in ATP-free buffer produces G_{1S} from G_{4S}. Without ATP, however, G_{4S} starts to undergo structural changes, soon after its release from the polymer. These structural changes can be followed by the concomitant loss of thiol-shielding capacity as revealed by titration with DNPSSG after the addition of ATP. The kinetics of the changes as monitored by the ATP-shielding effect are given in Figure 2: already after 10 min in ATP-free buffer, two thiol groups remain exposed when ATP is added. The changes continue logarithmically with time, and after 3 h four thiol groups are exposed, in the presence as well as in the absence of ATP. From this we conclude that at that time the changes leading from G_{4S} to a denatured form G_{4S}* are complete.

Parallel with the irreversible exposure of the thiol groups, we followed viscometrically the polymerization behavior of the actin initiated by addition of ATP and Mg^{2+} ions (Figure 3). The changes in the thiol groups of unprotected actin are paralleled by alterations in the polymerization behavior. During the first 10 min the rate of polymerization decreases continuously; also the final value of the relative viscosity (η) becomes lower. After 10–30 min the polymerizability of the actin is completely lost. This time interval has been marked in Figure 2, indicating that it corresponds to the exposure of greater than two thiol groups. We conclude that changes in the molecule affecting the first two thiol groups (available for the reagent) are not deleterious for polymerization. However, as soon as a third thiol group becomes involved, the polymerizability of the actin is lost. Although the position of the third thiol group is not known, it can be distinguished on the

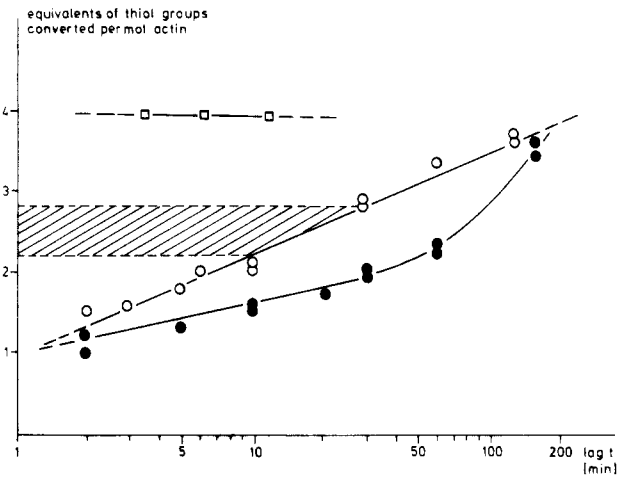

FIGURE 2: Increase with time of the number of thiol groups in actin exposed to DNPSSG when depolymerization was carried out in nucleotide-free buffer (Tris-HCl), and ATP was added after various periods of time (open circles). (Filled circles) The same experiment in the presence of 2×10^{-5} M Ca. For comparison, without the addition of ATP (squares). The shaded area represents the time interval between 10 and 30 min (corresponding to an irreversible exposure of 2.2–2.8 thiol groups), when actin in the absence of ATP loses polymerizability.

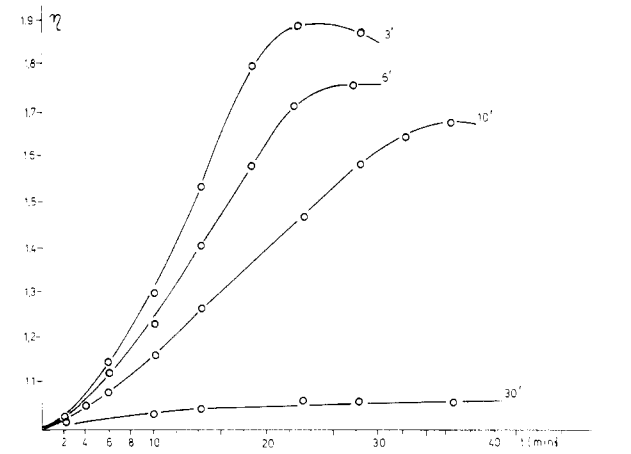

FIGURE 3: Polymerization of actin kept depolymerized for different periods of time in nucleotide-free buffer at 4 °C and started by the addition of ATP and Mg^{2+}.

basis of its involvement in the polymerization reaction.

We questioned further whether the damage leading to the irreversible exposure of greater than two thiols would be correlated to a loss of binding capacity for ATP. In this case the loss of polymerizability would be explained by the lost capacity for nucleotide binding. The problem was investigated by simple gel filtration experiments: On a Bio-Gel P2 column a mixture of monomeric actin with excess nucleotide separates into two peaks, one containing the protein–nucleotide complex and the other the free nucleotides (ATP, ADP) (Figure 4). When G_{1S}-actin with 2 equiv of ATP was assayed in this way, we found a ratio of ATP:actin of 0.8 in the first peak, in good accordance to the value of 0.7 reported by Mannherz et al. (1975). In the following experiments actin without ATP was kept for various periods of time, before again 2 equiv of ATP was added and the column was started. As the figures below indicate the ratio ATP:actin decreases rapidly with time: after 10 min, ratio 0.54; 20 min, ratio 0.38; 30 min, ratio 0.21. Obviously, the denaturation of monomeric actin, when stored without ATP, is accompanied by the loss of nucleotide binding capacity. Since denaturation involves also the changes in the accessibility of actin thiol groups as described above as well

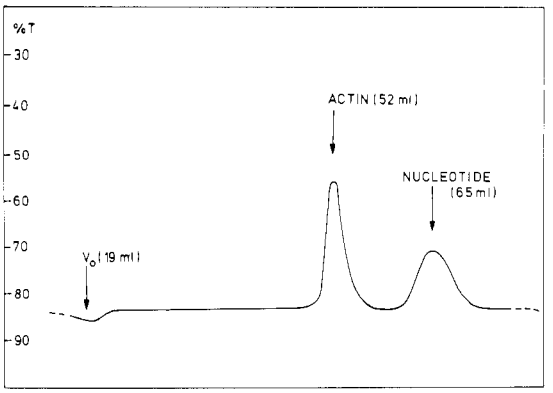

FIGURE 4: Typical elution diagram of monomeric actin and excess nucleotide from a Bio-Gel P2 column with 1 mM Tris-HCl, pH 7.4, monitored by OD at 254 nm. In the present experiment 1 mL (corresponding to 1.6 mg) of G-actin was stored in Tris buffer at 4 °C for 10 min; after the addition of 2 equiv of ATP, the mixture was applied to the column. The ATP:actin ratio in the protein peak was 0.54.

as the polymerization capability of actin, all three events must be interrelated and probably represent different aspects of the denaturation process.

Remarkably, the denaturation of monomeric actin in the absence of ATP followed by polymerizability and thiol exposure was significantly retarded when Ca^{2+} was added. With a Ca^{2+} concentration of 2×10^{-5} M, i.e., equimolar with actin and far below the concentration inducing polymerization, the time course of irreversible exposure of thiols followed different kinetics (filled circles in Figure 2). In parallel, the polymerizability was maintained for a longer period of time (Figure 5). Polymerization capacity was lost after keeping the ATP-free actin for longer than 60 min (without Ca^{2+}, 10 min), and again, polymerizability disappeared after the irreversible exposure of thiol groups had reached values of 2. Interestingly, the rest of the denaturation process proceeded more rapidly, so that complete denaturation was again reached after 3 h, i.e., in the same time as in the experiments without Ca^{2+}. The data seem to allow the conclusion that polymerizability is generally lost when greater than two thiols remain exposed after the addition of ATP.

It is known that ADP, particularly in high concentrations, will protect monomeric actin from denaturation. We therefore assayed actin for its thiol exposure and its polymerizability in the presence of various concentrations of ADP. As expected, ADP shielded the thiols and conserved the polymerizability of monomeric actin but with two important differences: it shielded one thiol group less than ATP, and the effect depended strongly on the concentration of the nucleotide. The highest shielding effect we could achieve was that of approximately two thiol groups ($G_{\sim 2S}$). This effect required a large excess of ADP (10^{-3} M, molar ratio of ADP:actin = 50:1), and the end point of the titration was not so definite as with G_{1S} and G_{4S}. As Figure 1 shows, the reaction continued very slowly, with a rate of ca. 0.1 thiol group per h. Similar to G_{1S} the resulting $G_{\sim 2S}$ retained its polymerizability after storage for several hours at 4 °C when ATP and Mg ions were added.

The simplest explanation for the concentration dependence observed is that ADP binds to monomeric actin with much lower affinity. In fact, when monomeric actin with 15 equiv of ADP was assayed on the Bio-Gel P2 column, the protein peak had an ADP:actin ratio of 0.0. This means that the affinity of ADP to actin is so low that in equilibrium the portion of dissociated nucleotide is high enough to be completely separated from the protein by a single run through the gel filtration column. This finding is in line with the data of Neidl & Engel (1979) showing that the equilibrium association constant (K_A) of ADP and actin is 175 times lower than that of ATP.

Accordingly, at low concentrations of ADP the monomeric actin was found completely unprotected. For example, the equimolar concentration of endogenous ADP present in all actin solutions after depolymerization (in our experiments usually 2×10^{-5} M) as well as a 4-fold excess of ADP (8×10^{-5} M) had no protective effect. Only at concentrations of 2×10^{-4} M ADP did we observe shielding of one of the four thiols. For shielding of two of the four thiols a concentration of 10^{-3} M ADP was required ($G_{\sim 2S}$). There was no further shielding with ADP concentration $>10^{-3}$ M. We therefore believe that the monomeric actin with bound ADP characterized by two exposed thiol groups ($G_{\sim 2S}$) represents an actin species generally different from that with bound ATP characterized by only one thiol group exposed (G_{1S}).

The interrelation of nucleotide binding and thiol shielding prompted us to assay also three ATP analogues with nonhydrolyzable phosphate bonds, either in the α,β-position (APCPOP) or in the β,γ-position (APOPNP and APOPCP).

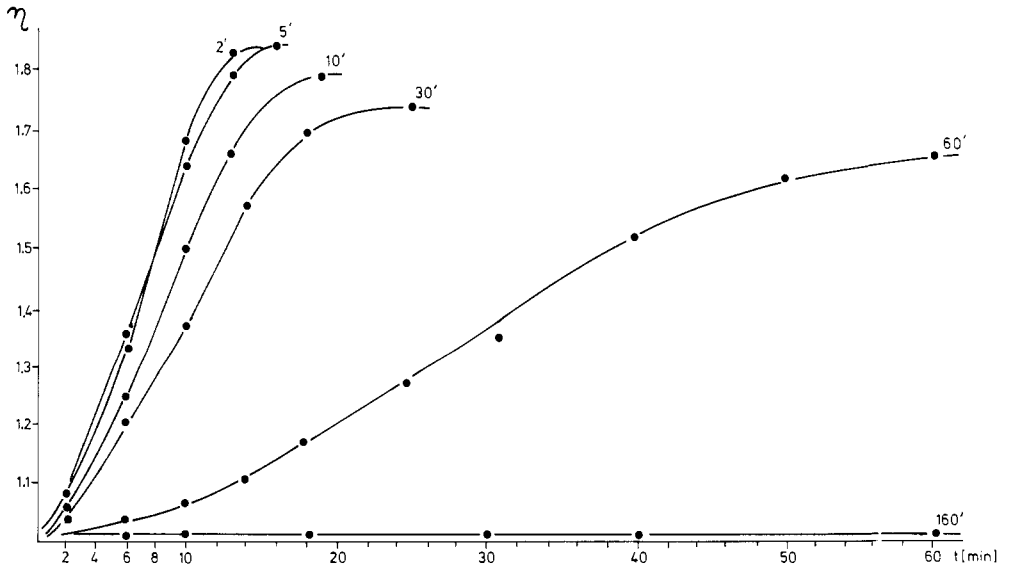

FIGURE 5: Same as Figure 3, except in the presence of 2×10^{-5} M Ca^{2+}.

None of them exhibited any shielding effect on the thiol groups of monomeric actin, when added in concentrations of 2×10^{-4} M (=10 equiv). Under these conditions ADP shielded at least one of the four thiol groups (see above). This means that all three ATP analogues are less effective than ADP, and by no means comparable with ATP, of which one equivalent exhibits the full shielding effect. Accordingly, the binding assay as described above yielded a nucleotide:actin ratio of 0.0 for each of them, indicating that the affinity to actin of each of the three analogues is as low or even lower than that of ADP. Accordingly, none of the three analogues exhibited any protective effect on monomeric actin in a concentration of 2×10^{-4} M. When polymerizability was tested after storage for various periods of time (by addition of ATP and Mg ions), the denaturation rate was found to be similar to that of G_{4S}.

In summary, we distinguish three species of monomeric actin differing in their number of thiols exposed in the presence of ATP and ADP. One is the monomeric actin normally prepared, G_{1S}, representing the complex G_{1S}·ATP. The second is the corresponding complex existing in the presence of large excess ADP, $G_{\sim 2S}$, or probably $G_{\sim 2S}$·ADP. The third is G_{4S}, the actin obtained by depolymerization in the absence of nucleotides, or, in the presence of ATP analogues with very low affinity, and probably without the endogenous ADP complexed to it.

There is no evidence for the existence of actin species other than the three described. For example, during denaturation of G_{4S} we found the number of exposed thiol groups increasing from 1.2 to 4 (Figures 2 and 5), obviously representing mixtures of G_{1S} and G_{4S}*. Similarly, we feel that the fraction of actin exposing three thiols in the presence of 2×10^{-4} M ADP (see above) is not an individual species but a 1:1 mixture of $G_{\sim 2S}$ and G_{4S}*. Furthermore, when actin, after being kept in ATP-free buffer for 10 min, exposes two thiol groups, it probably represents a 2:1 mixture of G_{1S} and G_{4S}*, rather than a distinct species, even though it is formally identical with $G_{\sim 2S}$, the actin species existing in large excess of ADP.

The data presented here suggest that the nativeness of monomeric actin can be measured by the number of thiol groups exposed, relative to the number of thiol groups shielded on the addition of ATP. Evidence of atmospheric oxidation of actin thiols was rarely seen, and only after many hours of standing. We therefore believe that the native state of monomeric actin is not lost through the oxidation of thiol groups. Instead, an irreversible structural change occurs, affecting the accessibility, or function, but not the state of oxidation, of the thiol groups. Correspondingly, there is no evidence that this structural change can be prevented by the addition of mercaptoethanol to actin solutions.

Registry No. 5′-ATP, 56-65-5; ADP, 58-64-0; DNPSSG, 20197-08-4; DNPSST, 88510-78-5; Ca, 7440-70-2.

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