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The accessibility of the thiol groups on G- and F-actin of rabbit muscle

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The accessibility of the cysteine residues of actin from rabbit muscles to the thiol-targeted reagent 7-dimethylamino-4-methyl-(*N*-maleimidyl)coumarin (DACM) was investigated. Under conditions where the actin is in the unpolymerized form (G-actin), the most reactive thiol group was Cys-257, suggesting that it was located on the surface of the actin molecule. The selective modification of Cys-374 for this reagent as reported by Sutoh [(1982) Biochemistry **21**, 3654–3661] was not observed. Cys-10, Cys-217 and Cys-374 were much less reactive and only gradually became extensively modified when the concentration of DACM approached 5 molar equivalents of actin. Presumably these thiol groups were located further inward away from the surface or situated in a different environment that rendered them less reactive. On the other hand, Cys-285 was completely inaccessible and presumably was buried. The lack of preferential labelling of Cys-374 by DACM is incompatible with the finding with iodoacetic acid as the reagent as reported by Elzinga & Collins [(1975) J. Biol. Chem. **250**, 5897–5905]. This discrepancy, however, might well be due to the different reagents employed. The DACM–G-actin largely retained its competence for polymerization. Upon polymerization of G-actin, practically all the thiol groups became inaccessible to DACM, suggesting that a drastic change occurred in the conformation of actin units in the transition of monomers to filamentous actin.

INTRODUCTION

Actin, a globular protein of 42 kDa (G-actin), is a highly conserved protein in its primary sequence, varying little from species to species (Elzinga *et al.*, 1973; Collins & Elzinga, 1975; Lu & Elzinga, 1977; Vandekerckhove & Weber, 1978*a*,*b*,*c*, 1979). It has the ability to polymerize in the presence of suitable concentrations of bivalent cations, forming filamentous actin (F-actin), which is one of the principal components of contractile or motile systems of muscle or non-muscle cells respectively. In certain non-muscle cells up to 50% of the total actin may exist in its monomeric form (Korn, 1978). Because actin filaments are structural components of cells such as platelets, it is essential to understand their behaviour in terms of assembly and disassembly. In addition, the actin monomers are intricately constructed molecules with very specific functional sites, such as myosin-binding and ATPase-activating sites, as well as numerous other protein-binding sites, on its surface. Many of these binding sites only become accessible when actin is in its filamentous form. The degree of actin association may thus play a dynamic and vital role in a number of cellular processes, such as cell motility, cell shape change, cell division and chromosomal segregation. The elucidation of the conformational changes associated with its polymerization would undoubtedly help us to understand the dynamics of the cytoskeleton and other fundamental biological processes.

Actin contains five thiol groups, which are distributed in the linear sequence of the actin molecule (Elzinga *et al.*, 1973; Kuehl *et al.*, 1975). It is known that native actin can be specifically cleaved by proteinases, such as chymotrypsin or trypsin, into two distinct domains: a small *N*-terminal mass and a large *C*-terminal mass (Jacobson & Rosenbusch, 1976; Mornet & Ue, 1984). The *N*-terminal portion contains the residue Cys-10, whereas the other four thiol groups are all located in the large *C*-terminal mass, at positions 217, 257, 285 and 374. In addition, it has been reported that the thiol group of Cys-374 (previously referred to as Cys-373) is the most reactive among the five thiol groups in actin and could be selectively modified by reaction with thiol-targeted reagents (Elzinga & Collins, 1975; Sutoh, 1982). The characteristic property has been used by a number of investigators for various purposes (Lin, 1978; Tao & Cho, 1979; Frieden *et al.*, 1980; Kouyama & Mihashi, 1981), whereas the behaviour of the other cysteine residues has not been examined closely. There is thus a need for a systematic examination of the accessibility of each of these cysteine residues during or as a result of the transformation from G-actin into F-actin or vice versa. Such a study would enable us to deduce the dynamic changes of certain domains that, to a certain extent, may prove to be difficult to obtain even with X-ray crystallography. In the present study we have chosen 7-dimethylamino-4-methyl-3-(*N*-maleimidyl)coumarin (DACM) as a specific thiol-targeted reagent as an extrinsic marker.

We provide in this paper direct chemical evidence for

Abbreviations used: G-actin, globular actin; F-actin, filamentous actin; DACM, 7-dimethylamino-4-methyl-3-(*N*-maleimidyl)coumarin.

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major conformational changes of actin during its transformation into F-actin.

EXPERIMENTAL

Materials

The following reagents were commercial products: DACM, urea, imidazole, *N*-acetylcysteine, ATP, 2-mercaptoethanol, formic acid, Nonidet P-40, EGTA and EDTA from Sigma Chemical Co.; acetone from Baker Chemical Co.; CNBr from Pierce Chemical Co.; Ampholines from LKB. All other reagents were the highest grade commercially available.

Methods

Preparation of actin. Actin was prepared from acetone-dried powder of rabbit leg and back muscle according to the procedures of Spudich & Watt (1971). The resulting F-actin pellet was suspended and stored in assembly buffer (10 mM-Pipes/NaOH buffer, pH 6.8, containing 40 mM-KCl, 1 mM-EGTA and 0.5 mM-NaN_3) at 4 °C in the presence (0.2 mM) or absence of ATP as specified for each experiment. The purity of actin was checked by means of SDS/PAGE under reducing conditions.

Preparation of G-actin. G-actin was prepared by dialysis of F-actin against a number of depolymerizing buffers as specified in each set of experiments.

Determination of protein concentration. The molecular concentration of G-actin was calculated on the basis of a molecular mass of 42 kDa (Collins & Elzinga, 1975) by using an absorbance of 1 mg of pure actin/ml (light-path 1 cm) at 290 nm as 0.62 or 0.64 for G- or F-actin respectively (Gordon *et al.*, 1976).

Reaction of DACM with thiol groups. The modification of the thiol groups of actin was carried out essentially by the procedures described by Sutoh (1982) as follows. (*a*) For native actin (G- or F-actin), unless stated otherwise, 1 mg of actin/ml was allowed to react with a prescribed amount of DACM for 1 min on ice. The reaction was terminated by adding 0.1 M-*N*-acetylcysteine (one-twentieth the volume of the reaction mixture). The solution was dialysed against distilled water (4 litres) with one change for a total of 20 h, and then freeze-dried. (*b*) For denatured actin, the final concentration of F-actin (initially determined before exposure to urea) in each reaction mixture was maintained the same as for G-actin unless stated otherwise. The DACM/actin molar ratio was as specified in each set of experiments. Reaction was carried out in 20 mM-sodium acetate buffer, pH 5.4, in the presence of 6 M-urea at room temperature for 3 min. Subsequent treatments were in the same manner as for G-actin.

To quantify the number of thiol groups/molecule accessible to the reagent, samples were processed as above. After isoelectric focusing, the fluorescent CNBr-cleavage fragments were excised and eluted by soaking in 1 ml each of lysis buffer (with the omission of Ampholine and a decrease in the concentration of urea to 6 M). The fluorescence intensity was measured with a model 204-A fluorescence spectrophotometer from Perkin–Elmer. The relative intensity of fluorescence in each CNBr-cleavage fragment was derived by comparing the reaction of denatured G-actin and native actin with the same concentration of DACM under identical conditions. The value of denatured actin was set as 100%.

The DACM was dissolved in acetone and kept in a freezer. The concentration of DACM was determined in 40 mM-sodium phosphate buffer, pH 7.0, spectrophotometrically each time just before modification of actin, by using the absorption coefficient 19800 $M^{-1} \cdot cm^{-1}$ (Yamamoto *et al.*, 1977).

Polymerization of DACM–G-actin. The DACM–G actin (0.62 mg/ml) was converted into DACM–F-actin by adding concentrated solutions of Mg^{2+} and KCl to final concentrations of 2 mM and 0.15 or 0.6 M respectively. It was allowed to stand for 3 h before centrifugation at 100000 *g* for 3 h.

CNBr cleavage of DACM-labelled actin. The dialysed DACM-labelled product was freeze-dried and then treated with an equal volume of 1 M-CNBr in 70% (v/v) formic acid for 18 h at 23 °C in the dark. The reaction mixture was dried under a stream of N_2 gas.

Isoelectric focusing. The procedures used were those described by O'Farrell *et al.* (1977). The solutions contained 2% (v/v) of a mixture of Ampholines of pH 3–10 and pH 5–7 in the ratio 5:1, unless otherwise stated. The lysis buffer contained 9.5 M-urea, 2% (w/v) Nonidet P-40, 2% (v/v) Ampholine and 5% (v/v) 2-mercaptoethanol. The dimensions of the gel tube were 2 mm × 13 cm. The electric focusing was performed at 610 V for 8–10 h.

RESULTS

When G-actin was modified according to the procedure exactly as prescribed by Sutoh (1982), we unexpectedly experienced difficulties in duplicating the preferential labelling of Cys-374 with DACM. Since the thiol group of Cys-374 is reported to be the most reactive among the five thiol groups of G-actin (Bender *et al.*, 1976; Lin, 1978; Thomas *et al.*, 1979; Tao & Cho, 1979; Frieden *et al.*, 1980; Kouyama & Mihashi, 1981; Faulstich *et al.*, 1984), we examined a number of parameters, such as depolymerization or reaction buffer, dialysis time, concentration of reagent etc., that might affect the selective reaction of Cys-374, or any of the other thiol groups. Figs. 1(*a*)–1(*c*) show that none of the buffers used exerted any effect on the selective labelling of Cys-374 of native actin. Instead, the thiol group of Cys-257 appeared to be much more reactive than the others. The assignment of the cysteine-containing CNBr-cleavage fragments by Sutoh (1982) has been adopted (Fig. 1*d*). The uniform distribution of the label in the denatured actin suggests that these thiol groups were in a reduced state. However, Cys-285 was rarely detected even under the urea denaturing condition. The fact that G-actin derived by depolymerization in the presence or in the absence of a reducing agent yielded identical labelling patterns ruled out the possibility that it might exist in an oxidized state. These results are in agreement with the finding by Faulstich *et al.* (1984) that only four out of the five thiol groups could be titrated with 2,4-dinitrophenyl thioglycollyl disulphide.

We have made an attempt to quantify the extent of fluorescent labelling of each thiol group when G-actin was treated with various molar equivalents (0.8–10) of

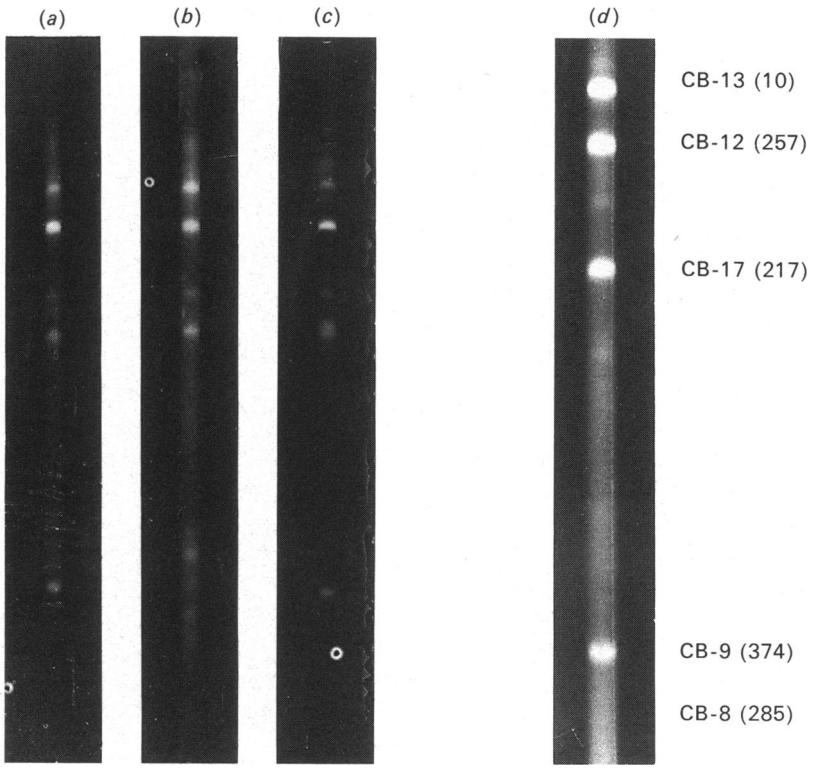

Fig. 1. Fluorescent peptides of complete CNBr-cleaved DACM-labelled G-actin

G-actin was derived from depolymerization of F-actin in (*a*) 1 mM-imidazole/HCl buffer, pH 7.0, containing 0.1 mM-$CaCl_2$ and 0.1 mM-ATP (Sutoh, 1982), (*b*) 1 mM-Tris/HCl buffer, pH 8, containing 0.2 mM-ATP (Elzinga & Collins, 1975) and (*c*) 2 mM-Tris/HCl buffer, pH 8, containing 0.2 mM-$CaCl_2$ and 0.2 mM-ATP. In each case actin was treated with 0.8 molar equivalent of DACM. (*d*) Denatured G-actin treated with 5 molar equivalents of DACM. CB indicates CNBr-cleavage fragment; the numerals in parentheses represent the cysteine residues in the primary structural sequence of actin.

DACM, with the values of denatured G-actin treated with corresponding molar equivalents as controls. Only the fluorescence of Cys-10, Cys-257 and Cys-217 could be estimated for the samples treated with up to 3 molar equivalents of DACM (Fig. 2). Cys-374 could not be accurately determined because of variable high background readings. Furthermore, no estimation could be made at all on samples treated with DACM beyond 3 molar equivalents. It is apparent from Fig. 2 that Cys-257 was most readily accessible. We realize that there are intrinsic drawbacks with this method of quantification; nevertheless, it is important to find that these results are in agreement with those made by visual observation.

We also found that duration of dialysis during depolymerization against a combination of buffers with adequate supply of ATP did not have any significant effect on labelling patterns (results not shown).

Effect of DACM concentration on the reaction with thiol groups of G-actin

The above results indicate that none of the depolymerization conditions examined could influence the selective reaction of any one of the five thiol groups. Because it would be extremely difficult to follow the kinetics of the reaction with this reagent to differentiate the reactivity of each thiol group of G-actin, we varied the concentration of DACM, while maintaining the concentration of actin constant.

Fig. 3 shows a representative set of results. At lower concentrations of DACM, the thiol groups of both Cys-

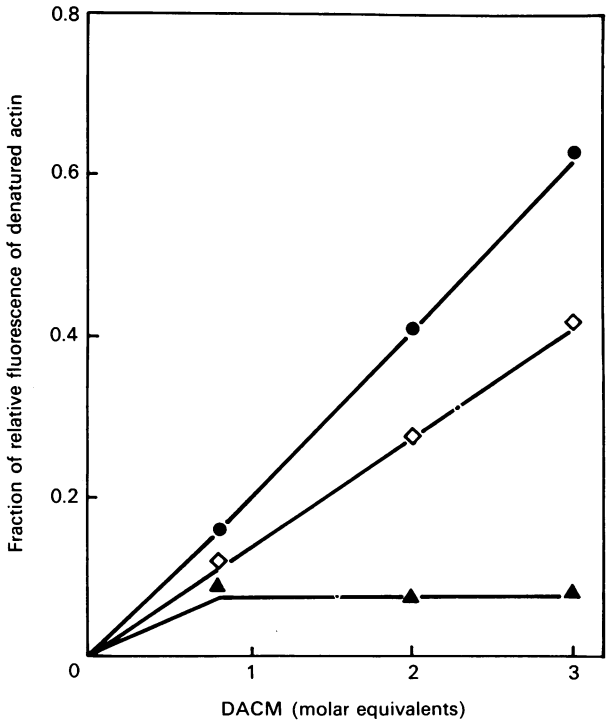

Fig. 2. Extent of labelling of various thiol groups on G-actin with various molar equivalents of DACM

●, Cys-257; ◇, Cys-217; ▲, Cys-10.

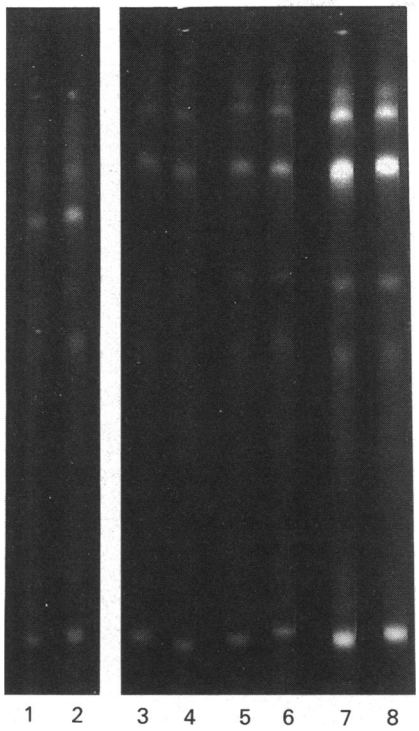

Fig. 3. Effect of DACM concentration on the reaction of thiol groups of G-actin

The molar equivalents of DACM for lanes 1–8 were 0.1, 0.4, 0.8, 1.4, 2.0, 3.0, 4.0 and 5.0 respectively.

257 and Cys-374 were most readily accessible to this reagent (Fig. 3, lane 1). As the concentration of DACM was increased, the thiol groups of Cys-10 and Cys-217 became increasingly labelled. We found that Cys-217 frequently gave a doublet and the ratio of distribution of the two bands often varied from experiment to experiment. None of the factors, such as the proportion of Ampholines (between pH 3–10 and pH 5–7), the amount of CNBr and the duration of the cleavage reaction, contributed to the ratio distribution of the doublets.

Effect of actin concentration on the reaction of DACM with thiol groups

When the modification reaction was carried out by varying the concentration of G-actin while maintaining the molar equivalent of DACM constant at 0.8, the DACM-labelled patterns were as shown in Fig. 4. At very low G-actin concentration, Cys-257 was rapidly labelled. As the concentration of G-actin was increased, Cys-10, Cys-217 and Cys-374 gradually became labelled. The thiol group of Cys-257 of G-actin has been consistently shown to be more reactive or more accessible to this reagent.

Reactivity to DACM of G-actin derived from ATP-exposed F-actin

It has been reported by Sleigh & Burley (1973) that F-actin stored in the presence of ATP showed a different conformation from that in the absence of ATP. We have examined the selective reactivity of the cysteine residues of both F-actin (see below) and G-actin derived from F-actin that had been exposed to ATP. When G-actin

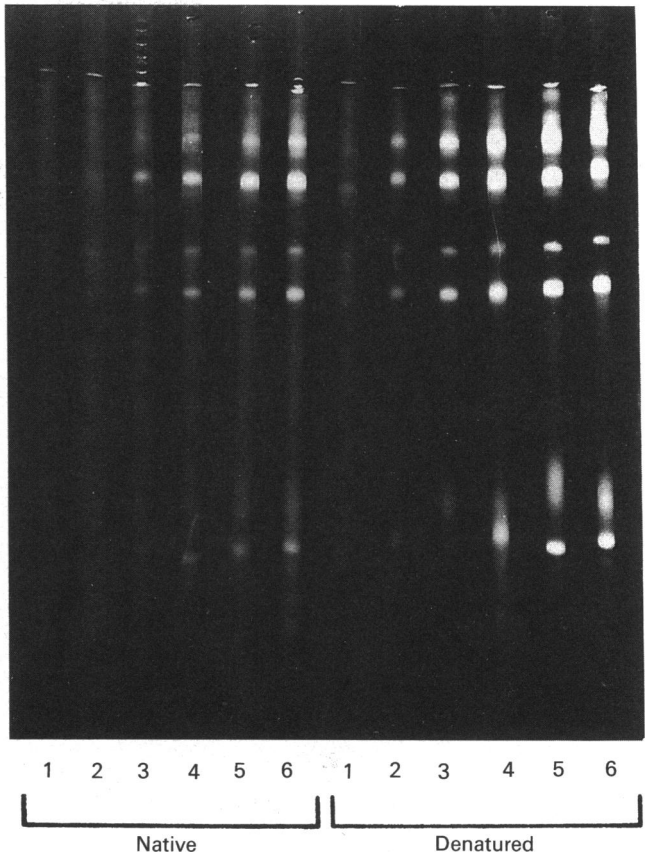

Fig. 4. Effect of G-actin concentration on the reaction of thiol groups with DACM

The concentrations of G-actin for lanes 1–6 were 0.07, 0.15, 0.30, 0.44, 0.59 and 0.74 mg/ml. The molar equivalent of DACM was maintained at 0.8.

preparations derived from F-actin that had been stored in the presence or absence of ATP were treated with different equivalents of DACM, they yielded labelling patterns similar to those shown in Fig. 3. These results suggest that no significant difference can be attributed to the exposure of F-actin to ATP. It is noteworthy that regardless of the status of F-actin the Cys-257 of G-actin was more reactive, whereas Cys-374 was less so.

Reaction of F-actin with DACM

When F-actin was treated with 5 molar equivalents of DACM, a considerable degree of labelling of the thiol groups could be detected (Fig. 5*a*, lanes 1 and 2). The degree of conjugation of these thiol groups varied from experiment to experiment. Since such samples were usually modified in Sutoh's buffer, it is possible that some of the F-actin was converted into G-actin. If such is the case, it would explain the low degree of labelling (as compared with total label in the denatured F-actin; Fig. 5*a*, lanes 3 and 4). Because of this uncertainty, we carried out the modification of F-actin in assembly buffer instead of Sutoh's buffer. The extent of substitution was approximately the same. The amount of label in the native F-actin is significantly less if it is compared with that in the native G-actin that had reacted with an equal molar equivalent of DACM. However, in all cases the

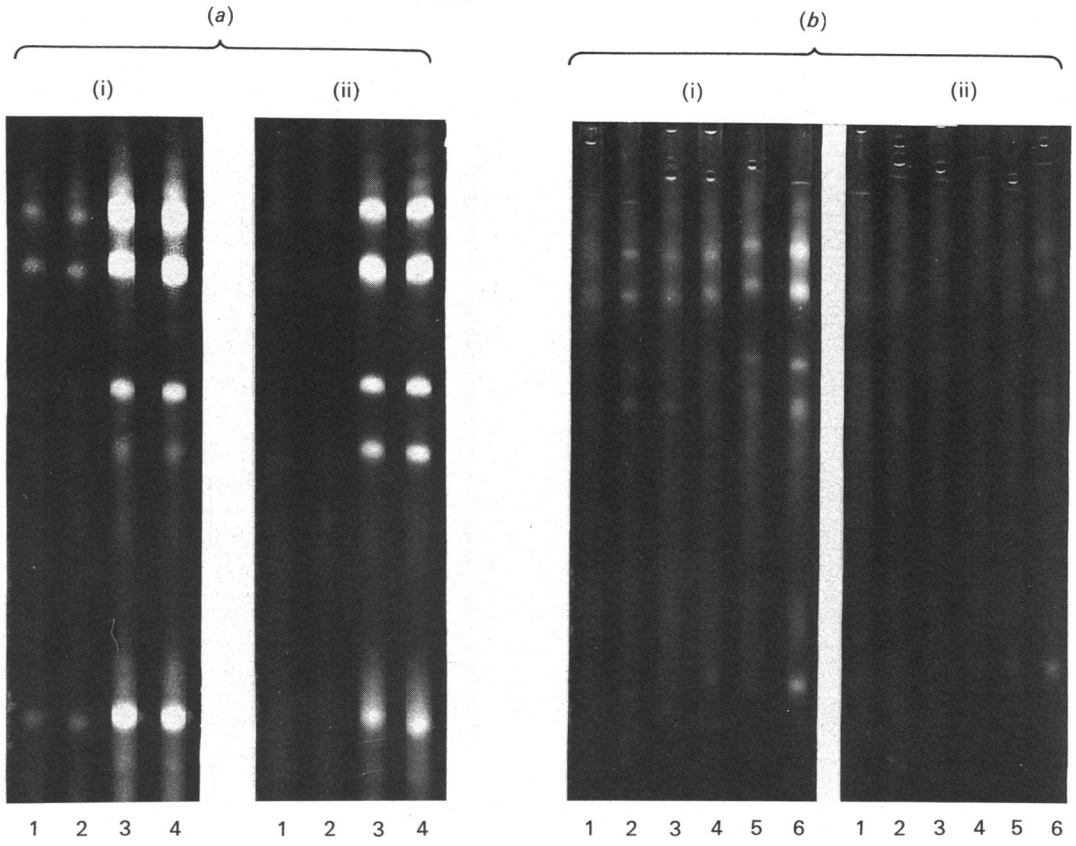

Fig. 5. (*a*) Reaction of F-actin with DACM and (*b*) the effect of ATP on the reaction of F-actin with DACM

(*a*) Reaction of F-actin with DACM. The reaction was carried out in Sutoh's buffer (i) or in assembly buffer (ii). Five molar equivalents of DACM were used. Lanes 1 and 2, native F-actin; lanes 3 and 4, denatured F-actin. (*b*) Effect of ATP on the reaction of F-actin with DACM. F-actin was stored in the absence (i) or in the presence (ii) of ATP. The molar equivalents of DACM were 0.1, 0.3, 0.6, 0.8, 2.0 and 5.0 for lanes 1–6 respectively.

labelling pattern was strikingly similar to that of the G-actin.

Effect of ATP on accessibility of thiol groups on F-actin

Since the labelling pattern of G-actin was similar to that of F-actin, the labelling of thiol groups on F-actin might be due at least partly to the presence of G-actin in the F-actin solution. In fact, we found that when freshly prepared F-actin was treated with 5 molar equivalents of DACM, it showed much less labelled CNBr-cleavage fragments (results not shown). This observation was at variance with reports that Cys-374 was preferentially labelled in F-actin (Bender *et al.*, 1976; Lin, 1978; Thomas *et al.*, 1979; Kouyama & Mihashi, 1981; Faulstich *et al.*, 1984).

When F-actin was treated with different molar equivalents of DACM, there were always certain amounts of thiol groups accessible to the reagent, depending on the way F-actin was stored. The degree of labelling was always greater in the absence than in the presence of ATP (Fig. 5*b*). However, the labelling patterns were very similar. These results suggested that ATP may influence the equilibrium between G-actin and F-actin, and further suggested that conjugation had taken place on G-actin rather than on the actin units of F-actin (for further evidence see below).

When a sample of F-actin was centrifuged before the reaction with DACM, the F-actin recovered yielded no detectable labelled CNBr-cleavage fragments (Fig. 6*a*). This finding is in agreement with the observation made with freshly prepared F-actin. The recovered G-actin (in the supernatant), when treated with DACM, yielded patterns similar to those of the uncentrifuged F-actin as shown in Fig. 6(*b*). In addition, if the recovered F-actin was stored for some time in assembly buffer and then treated with the reagent, it again yielded a labelled pattern (Fig. 6*c*) similar to that of uncentrifuged F-actin. These results strongly suggest that the labelling in F-actin was occurring on G-actin and not on the actin units of F-actin. These results further suggest that the thiol groups of various cysteine residues on the actin units of F-actin are completely inaccessible to this reagent. The different degrees of labelling observed were mainly due to the amount of free G-actin in equilibrium. This was confirmed by the results from centrifuged F-actins that had been stored in the presence or in the absence of ATP, as shown in Fig. 5(*b*).

Polymerizability of DACM-labelled G-actin

When G-actin was treated with 0.8 molar equivalent of DACM, it yielded a product that was polymerized by added KCl and Mg^{2+}. The F-actin was recovered by centrifugation. Such recovered F-actin was depolymerized by dialysis against various depolymerization

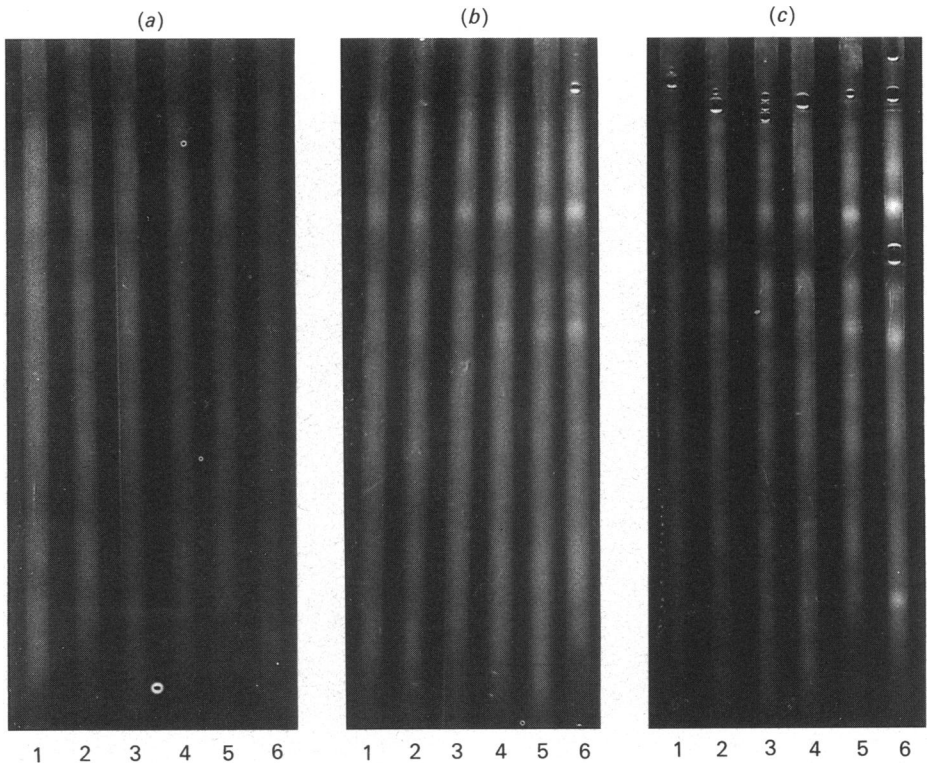

Fig. 6. Effect of ultracentrifugation on the reaction of F-actin with DACM

(*a*) F-actin after centrifugation at 100000 ***g*** for 3 h; (*b*) supernatant from (*a*); (*c*) F-actin from (*a*) but modified after a 9-day storage. The molar equivalents of DACM were 0.1, 0.4, 0.6, 0.8, 2.0 and 5.0 for lanes 1–6 respectively.

buffers. It was found that the F-actin was not as readily depolymerized as unmodified F-actin. Prolonged dialysis was necessary to achieve the completion of depolymerization. When the resulting G-actin was again polymerized by adding salts, only about two-thirds was recovered. The labelling pattern of such a final product is practically the same as the starting labelled G-actin, suggesting the DACM derivative of G-actin retained its competence for polymerization.

CONCLUSION

In the G-actin, among the five thiol groups Cys-257 is most reactive in our study, Cys-374, Cys-217 and Cys-10 being much less so. The accessibility of Cys-257 and Cys-10 was not due to a lack of bound Ca^{2+} on G-actin as reported by Konno & Morales (1985), who suggested that these two cysteine residues were uncovered only after the removal of the metal ions. As to the thiol group of Cys-285, it is completely inaccessible to DACM, presumably buried, as was also observed by Faulstich *et al.* (1984) and Lusty & Fasold (1969). The relatively fast reactivity of the thiol group of Cys-374 observed in our study is consistent with the findings by Elzinga & Collins (1975) and Sutoh (1982). However, preferential labelling of this thiol group with DACM could not be readily achieved. On the other hand, we cannot rule out the possibility that different alkylating agents react somewhat differently with these thiol groups. Other factors, such as neighbouring effect and/or reaction conditions, might also affect the accessibility of a given thiol group and points to the urgency for further investigation on the reactivity of various thiol groups towards reagents that have different functional groups.

The reaction of the thiol groups of Cys-10 and Cys-217 depends on the concentration of the reagent DACM. Thus at higher concentration of the reagent all four thiol groups could be substituted. Lusty & Fasold (1969) also observed that only three thiol groups reacted with 2,2′-dicarboxy-4′-iodoacetamidoazobenzene. On the other hand, Tao & Cho (1979) reported that besides a major labelling site there is a minor labelling site (or class of labelling sites) of unknown location. According to Faulstich *et al.* (1984), the number of thiol groups exposed to reagent depends on the nucleotide bound. In the absence of ATP G-actin exposed four thiol groups, but only one in the presence of ATP. However, in our study ATP, which was always present in all samples, showed no such effect with native G-actin. The differences observed in accessibility among these thiol groups are probably due to shielding effects and/or changes in microenvironment in the vicinity of each cysteine residue, which could probably modulate the rate of its reaction.

In the case of F-actin, Kasai & Oosawa (1963) reported that 2 mol of thiol groups/mol of 60 kDa actin in the normal F-actin is rapidly titratable by *p*-chloromercuribenzoic acid. By the addition of EDTA, the number of rapidly titratable thiol groups increased to about four without depolymerization of F-actin. Lusty & Fasold (1969) also reported that only one thiol group in F-actin reacted with this reagent.

Our results on F-actin, however, showed (*a*) that

F-actin, when centrifuged before reaction with DACM, yielded practically no detectable label, (*b*) that the G-actin, recovered from supernatant derived from the F-actin sample, gave DACM-labelled patterns similar to the one normally observed with G-actin, and (*c*) that the centrifuged F-actin, after being stored in assembly buffer for some time, yielded a labelled pattern similar to that of uncentrifuged F-actin. Thus normally we would expect a certain amount of G-actin to coexist with F-actin. The amount of G-actin in a given sample of F-actin must therefore be in equilibrium with F-actin and dependent on the concentration of actin and its critical concentration under certain conditions. In the presence of ATP the amount of free G-actin would be somewhat lower in concentration than in the absence of ATP. The rate of depolymerization (the 'off' rate) is known to be lower than that of polymerization (the 'on' rate) (Korn, 1982; Oosawa, 1983). The 'on' rate in our case would be expected to be relatively faster in the presence than in the absence of ATP.

We have provided evidence to indicate strongly that the thiol groups on F-actin do not react with the reagent DACM. It is therefore reasonable to assume that the labelling of F-actin normally observed might have taken place on G-actin rather than on the actin units of F-actin.

The lack of reactivity of F-actin thiol groups could be readily explained if these residues became buried in the actin units as a result of conformational changes occurring as a consequence of polymerization. We cannot, however, exclude the possibility that at least some of them were located in the monomer–monomer contact area and/or between neighbouring monomers on the opposite two strands of the helix, although it is highly unlikely that all of these cysteine residues are in these contact areas. The finding of such an extensive change in the conformation of G-actin is indeed very interesting and important in our understanding of the transformation of G-actin into F-actin, particularly in terms of why some of the actin-binding proteins, such as myosin, bind only to the actin units of F-actin but not to G-actin. Presumably, these sites on G-actin are sterically hindered for these actin-binding proteins, or unavailable possibly because they are either completely or partly buried.

The DACM derivative of G-actin largely retained its competence for polymerization. However, the resulting F-actin could not be readily depolymerized and could do so only after prolonged dialysis.

In conclusion, we found that Cys-257 is most reactive to DACM among the five cysteine residues of G-actin. We also obtained conclusive evidence for the inaccessibility of all the thiol groups on the actin units of F-actin. On the basis of our results, we suggest that the labelling of F-actin reported in the literature is highly likely to be due to the labelling of free G-actin existing in equilibrium with F-actin. The study on the reactivity of cysteine residues of actin by alkylation with DACM has shown that Cys-257 is on the surface of the molecule, whereas Cys-10, Cys-217 and Cys-374 are partially shielded. Cys-285 is the most unreactive. The immediate environment of these cysteine residues is drastically changed in the transition of monomers to filamentous actin.

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