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Biochemistry

Exposure of actin thiols by the removal of tightly held calcium ions

(actin conformation/divalent cation binding)

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ABSTRACT **The removal of bound metal ions from G-actin uncovered two thiols, Cys-10 and Cys-257. The uncovering of these thiols requires a free calcium concentration lower than 10 nM. Therefore, participation of one or both thiols in Ca^{2+} binding is suggested. Actin labeled with *N*-(5-fluoresceinyl)maleimide in the absence of calcium moves as a doublet in NaDodSO$_4$/PAGE. It is suggested that two conformers are induced by metal removal and labeling.**

Recently Sutoh showed that when myosin is crosslinked to actin with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), the actual surfaces joined are portions of the 20-kDa and the 50-kDa regions of myosin subfragment 1 (S-1) (1) and the N-terminal residues of actin (2). Therefore, in developing a three-dimensional map of acto–S-1 (3), it is of obvious importance to locate this "interface." A way to do this might be to label actin Cys-10 with a suitable probe. But there is little in the literature about reacting Cys-10, and previous efforts in this laboratory to do so have failed (4) even though the probe was especially targeted to a thiol neighboring on negative charges. Mornet and Ue (5), however, provided the necessary clue; they noted that Cys-10 and its neighbors constitute at least a partial sequence homology of a Ca^{2+}-binding site. If Ca^{2+} is held by Cys-10 and its neighbors, then the indifference of Cys-10 to thiol reagents is understandable, and a means of overcoming the indifference is obvious. We set out to find whether Cys-10 could be reacted if Ca^{2+} were removed from actin.

Ca^{2+} cannot be removed from F-actin, and conventional wisdom is that G-actin denatures on losing its "prosthetic" Ca^{2+}. The dilemma is surmountable, however, as was suggested to us in ref. 6. By maintaining the bound nucleotide of G-actin constant, it is possible to remove metal ions, react thiols, and still retain polymerizability and the ability of the polymer to stimulate myosin ATPase. It is thus possible to ascertain whether Cys-10 becomes reactive after Ca^{2+} removal.

MATERIALS AND METHODS

Actin was isolated from rabbit muscle as described (7) and converted to G-actin in 5 mM 2-[tris(hydroxymethyl)methylamino]ethanesulfonic acid (Tes), pH 7.6/0.2 mM ATP/0.2 mM $CaCl_2$ (buffer A) at 4°C. G-actin concentration was estimated using $A^{1\%}_{290} = 6.37$ (8). For a general exposure of actin to EDTA, 1 mM EDTA was added to buffer A. To prevent participation of the most reactive thiol (Cys-374) in certain reactions, we treated G-actin with a 10-fold molar excess of *N*-ethylmaleimide (MalNEt) for 30 min at 20°C, quenched the reaction with excess dithiothreitol, and removed excess dithiothreitol and quenching products by gel filtration on Sephadex G-25 (1 × 5 cm). Proteolysis degrades an N-terminal 9-kDa region of G-actin (5, 9), leaving a C-terminal 33-kDa "core"; core was prepared by digesting actin with trypsin at a weight ratio of 10:1 for 60 min at 20°C. A CNBr fragment of actin, CB-13 (44 N-terminal residues), was used as an electrophoretic standard; it was prepared as described (10), labeled with fluorescein isothiocyanate at Lys-18, and kindly donated to us by our colleague R. Takashi. Bound nucleotide is held tightly by G-actin in buffer A, but it was necessary to test its retention after exposure to EDTA. For this purpose, actin was incubated at 20°C in buffer A containing 1 mM EDTA. At various intervals aliquots were drawn, made 1 mM in $CaCl_2$ (to stop removal of actin-bound metal ions), and kept overnight at 0°C. The aliquots were filtered through 1 × 5 cm columns of Sephadex G-25 (5 mM Tes, pH 7.6/0.2 mM $CaCl_2$) to remove unbound ATP. The protein fractions eluted at void volumes from this filtration were collected, and each was analyzed for both protein and ATP as described by Faulstich *et al.* (6). Thiols of actin were titrated with Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid), DTNB], assuming $\varepsilon_{412} = 1.36 \times 10^4$ (11). [Ca^{2+}] was set by Ca^{2+}/EDTA buffer, assuming the formation constant of Ca-EDTA at pH 7.0 and 20°C to be 2×10^7 M^{-1} (12). For fluorescein labeling, the thiols of actin were reacted with *N*-(5-fluoresceinyl)maleimide (MalNFlu; Molecular Probes, Junction City, OR). Actin or actin-MalNEt was reacted in buffer A containing 1 mM EDTA, using a 2-fold molar excess of MalNFlu for 15 min at 20°C. The reaction was quenched by adding 1 mM dithiothreitol and 1 mM $CaCl_2$. The actin was rid of excess quenching reagents and their products by Sephadex G-25 gel filtration. The amount of bound label was estimated from its absorption spectrum, measured with a Cary 118-C spectrophotometer, assuming $\varepsilon_{495} = 9 \times 10^4$ (13). Fluorescence-emission spectra of labeled actin were recorded with a Hitachi/Perkin-Elmer fluorometer MFP-4 with an excitation wavelength of 480 nm. The fluorescence intensity of 1-anilino-8-naphthalenesulfonic acid (Ans) in an actin solution was measured with the same instrument, using an excitation wavelength of 400 nm and an emission wavelength of 480 nm. The competence of G-actin that had been exposed for 30 min to 1 mM EDTA at 20°C was tested in two ways. It was allowed to polymerize at 25°C for 1 hr after addition of 50 mM KCl and 2 mM $MgCl_2$. The extent of polymerization was estimated from the actin sedimented by centrifugation at 100,000 × *g* for 2 hr. The activation of myosin Mg^{2+}-ATPase also was measured. ATPase was assayed in 30 mM KCl/10 mM Tes, pH 7.6/1 mM Mg-ATP at 25°C. CNBr fragmentation of labeled actin was conducted as described (10), except that the thiols of actin were not blocked with monoiodoacetate. NaDodSO$_4$/PAGE was conducted in 15% polyacrylamide/6 M urea or in 10–18% gradient gels, both containing 0.1% NaDodSO$_4$. A stacking gel of 3% polyacrylamide was used (14).

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Abbreviations: Ans, 1-anilino-8-naphthalenesulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); MalNEt, *N*-ethylmaleimide; MalNDAC, *N*-(7-dimethylamino-4-methylcoumarin-5-yl)maleimide; MalNFlu, *N*-(5-fluoresceinyl)maleimide; NTCB, 2-nitro-5-(thiocyano)benzoic acid; S-1, myosin subfragment 1.

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RESULTS

On the premise that the Ca^{2+} content, the nucleotide content, and the conformation of actin might all be interrelated, we inquired whether the Ca^{2+} vs. conformation relation could nevertheless be studied at constant nucleotide content. Fig. 1 shows that withdrawal of Ca^{2+} (and consequent changes in conformation, as we shall show below) for periods as long as 30 min can occur without loss of nucleotide. The figure also shows, incidentally, that if Ca^{2+} is restored to the actin after such periods, the actin remains competent to polymerize and then to activate myosin Mg^{2+}-ATPase.

How the number of titratable thiols per mole, n_{SH}, varies with Ca^{2+} content was of interest to us because of our eventual objective of labeling Cys-10 and also because n_{SH} is an indirect "conformation indicator" [Faulstich *et al.* (6)]. The experiment was conducted under conditions in which, as just described, the nucleotide content is maintained the same as it was when in equilibrium with buffer A. Fig. 2*A* shows the result of incubating actin in 5 mM Tes (pH 7.6) at 20°C with DTNB. When $[Ca^{2+}]$ is 0.2 mM, n_{SH} reaches ≈1 mol of SH reacted/mole of actin in ≈30 min. When actin was treated with 1 mM EDTA for 30 min followed by addition of 1 mM $CaCl_2$, n_{SH} reaches slightly more than 2 in 30 min. The increased n_{SH} on exposure to EDTA was partially reversed (to $n_{SH} \approx 1.5$) when actin was stored in ice-cold water overnight (data not shown). For comparison we show (Fig. 2*A*) the behavior of actin denatured by an overnight exposure to 1 mM EDTA at 20°C in the absence of ATP; in this case, n_{SH} reaches virtually the theoretical thiol content of actin (5 mol of SH/mole of actin). We may thus conclude that, under conditions in which actin is not irreversibly damaged, loss of bound Ca^{2+} results in the exposure of additional thiols. The thiol titratable independently of Ca^{2+} content is probably Cys-374 (10, 15). There are other accompaniments to Ca^{2+} loss under these conditions. For example, Fig. 2*B* shows that the fluorescence intensity from Ans in equilibrium with actin increases markedly after Ca^{2+} withdrawal from the actin.

Although the Ca^{2+} vs. conformation relation at constant nucleotide content was of special interest to us, other conditions could be found in which conformation depended on nucleotide content, at constant Ca^{2+} content (Fig. 3). When the $[Ca^{2+}]$ in equilibrium with the actin is ≥0.2 mM, only one thiol is titratable, regardless of ambient [ATP]. When the actin is in equilibrium with essentially zero $[Ca^{2+}]$ (i.e., excess EDTA), at least one additional thiol is titratable, even when the ambient [ATP] exceeds 2 mM. In the zero-

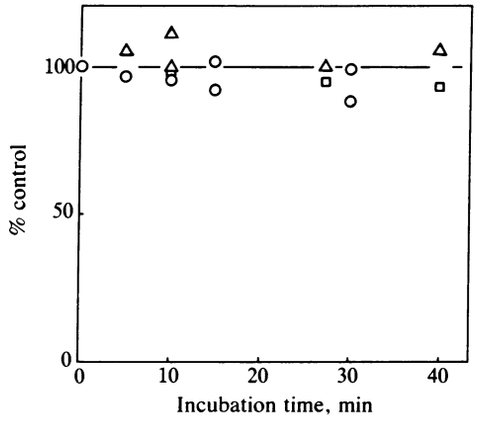

FIG. 1. Changes in the nucleotide content of actin (○), polymerizable actin content (□), and actin activation of myosin ATPase (△) after exposure of actin to EDTA (5 mM Tes, pH 7.6/0.2 mM ATP/1 mM EDTA at 20°C) for various times. All values were expressed relative to those obtained for untreated actin (100%).

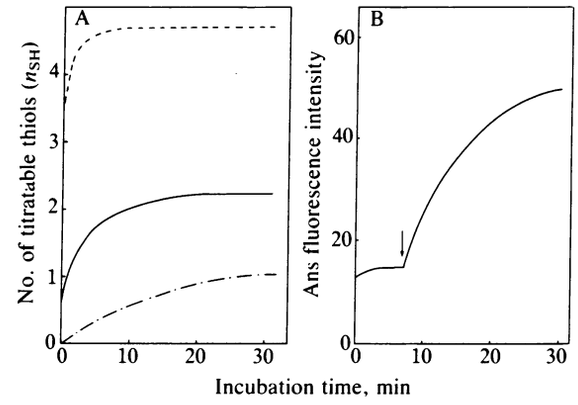

FIG. 2. Dependence of the titratable thiols of actin (n_{SH}) and the Ans-fluorescence intensity of actin solution on EDTA exposure. (*A*) DTNB titration of thiols of various actins. ----, Denatured actin (incubated overnight with 1 mM EDTA in the absence of ATP); —·—·, actin in the presence of 0.2 mM Ca^{2+}; —, actin exposed to EDTA for 30 min at 20°C. Conditions are the same as for Fig. 1; 0.25 mM DTNB was used. (*B*) Ans-fluorescence emission intensity of actin solutions equilibrated with Ans and excited at 400 nm. At the time indicated (arrow), 1 mM EDTA was added; other conditions are as in *A*; 25 μM Ans was used.

$[Ca^{2+}]$ situation, however, n_{SH} is a function of [ATP]; many—perhaps all—thiols became titratable as [ATP] approaches zero. Our data seem to agree, in general, with that of other workers, particularly Faulstich *et al.* (6); however, in this work we are concerned primarily with the situation in which Ca^{2+} is removed from the protein while nucleotide remains attached.

Fig. 3 indicates that 0.2 mM nucleotide maintains n_{SH} at the same low value as much higher concentrations, presumably because 0.2 mM nucleotide is sufficient to keep the nucleotide binding site filled. If we keep this concentration and progressively reduce the concentration of free Ca^{2+} ($[Ca^{2+}]_f$), we can assess the effect on n_{SH}—thinking of n_{SH} as an indicator of Ca^{2+} release from the protein (Fig. 4). The effect on n_{SH} of reducing $[Ca^{2+}]_f$ is virtually negligible from 0.2 mM down to 0.1 μM; it becomes clear at pCa 8 and reaches half its maximum at pCa 8.2. The same profile of n_{SH} exposure was observed when Ca/EGTA was used instead of Ca/EDTA (data not shown).

Accepting that the thiol of actin reactive at any nucleotide concentration was Cys-374, we set about to identify the thiols uncovered as $[Ca^{2+}]_f$ becomes very low. For these experiments a titrant far more visible than DTNB was chosen; namely, MalNFlu. The choice was accompanied by a com-

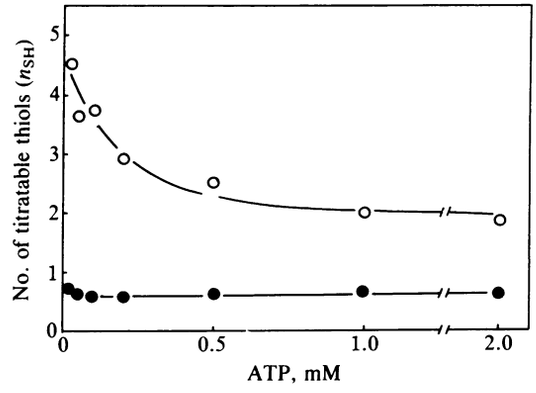

FIG. 3. Titratable thiols (n_{SH}) as a function of ATP concentration. ●, Actin in the presence of 0.2 mM $CaCl_2$; ○, actin in the presence of 1 mM EDTA. Other conditions were the same as for Fig. 2*A*.

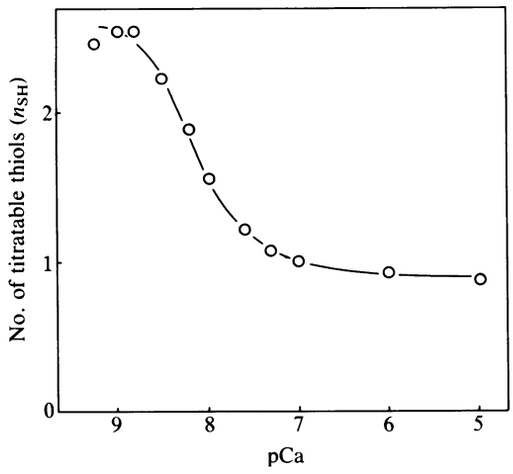

FIG. 4. Calcium requirement for exposure of actin thiols. Titratable thiols of actin were estimated at various concentrations of free calcium, set by Ca/EDTA buffers. Conditions: 5 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) (pH 7.0), 0.2 mM ATP, 0.1 mM $CaCl_2$, and the desired EDTA. Estimation of thiols was as for Fig. 2*A*.

plication peculiar to MalNFlu. Fig. 5 shows that labeling actin in the presence of a [Ca^{2+}] sufficient to saturate the binding sites produces one fluorescent band in PAGE, corresponding to the 42-kDa actin band detected with Coomassie blue; we presumed Cys-374 had been labeled. However, when actin was labeled at zero [Ca^{2+}], three fluorescent bands appeared—the original 42-kDa band plus two slower bands (44 kDa and 46 kDa, respectively) each of about the same intensity as the other; the same pattern was obtained with Coomassie blue staining. To ensure that the 42-kDa band arose from labeling Cys-374, and also to simplify the pattern, we labeled actin first with MalNEt in a Ca^{2+}-containing medium and then with MalNFlu in a Ca^{2+}-free medium. Now the 42-kDa band was no longer fluorescent, but the fluorescent 44- and 46-kDa bands persisted. Since MalNEt is known to react readily only with Cys-374 in a Ca^{2+}-containing medium (15), we considered our presumption confirmed, and thereafter we generally used actin preblocked (in a Ca^{2+} medium) with MalNEt. Labeling of actin with MalNFlu in the presence and in the absence of Ca^{2+} also had unexpected spectroscopic consequences. With

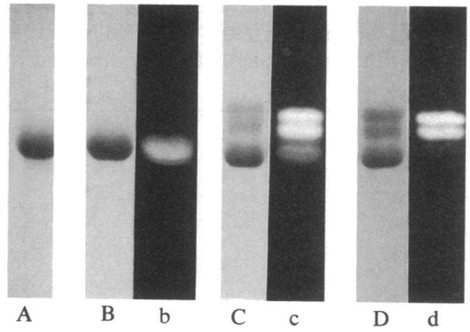

FIG. 5. Labeling of actin with MalNFlu. Lanes: A, unlabeled actin; B and b, actin labeled with MalNFlu in the presence of calcium; C and c, actin labeled in the presence of 1 mM EDTA; D and d, actin-MalNEt labeled in the presence of EDTA. Labeling was conducted at 20°C for 15 min in 5 mM Tes (pH 7.6)/0.2 mM ATP/1 mM EDTA. Lanes A–D show gels stained with Coomassie blue, and lanes b–d show the fluorescent bands revealed under UV light before staining. As standard proteins, actin (42 kDa), S-1 heavy chain (95 kDa), three fragments of S-1 (50 kDa, 27 kDa, and 20 kDa) were used (not shown).

or without Ca^{2+}, MalNFlu labeling generates an absorption spectrum peaking at 495 nm, but the spectrum obtained with MalNEt-blocked actin in the zero-[Ca^{2+}] case is much broader and has a distinct shoulder at 460 nm. Likewise the fluorescence-emission spectrum of MalNEt-blocked actin labeled with MalNFlu in the absence of Ca^{2+} is red-shifted by ≈4 nm, relative to that of actin labeled in the presence of Ca^{2+}. That reaction with MalNFlu generates two (rather than one) new bands in PAGE, as well as a broadened spectrum, is not attributable to the existence either of two forms of actin or of two forms of label, since anomalies do not occur when actin is labeled in the presence of Ca^{2+}. Further, since anomalies after reaction in zero [Ca^{2+}] do occur with iodoacetamidofluorescein as with MalNFlu but do not occur with *N*-(7-dimethylamino-4-methylcoumarin-5-yl)maleimide (MalNDAC) (data not shown), we have to conclude that the fluorescein chromophore may be one of the requirements for anomalous behavior; the other requirement is inherent in the thiols made available by Ca^{2+} removal.

Since the procedure of removing Ca^{2+} and then labeling appeared to generate two labeled species (the unlabeled, MalNEt-blocked actin constituting a third), we tried to see whether the process of actin polymerization could "filter out" one species or another. These experiments also tested whether labeled actin was functionally competent. In a typical set of experiments, a control showed that ≈90% of MalNEt-blocked actin polymerized under the conditions employed. MalNEt-blocked actin then was labeled to 0.78 mol of MalNFlu/mol of actin. About 82% of this actin polymerized. The polymer, however, contained only 0.47 mol of MalNFlu/mol of actin. On the other hand, the electrophoretogram of the polymer showed the same fluorescent-band patterns as G-actin. The control and the labeled polymers activated myosin Mg^{2+}-ATPase equally well. Thus it seems that polymerization discriminates somewhat against labeled monomers but that once incorporated, such monomers are active; polymerization does not discriminate between the two labeled species.

The amino acid sequence of actin is such that after CNBr cleavage, no fragment contains more than one thiol (10). By denaturing actin in the presence of MalNFlu and then cleaving with CNBr, we have realized the expected five fluorescent bands in PAGE. The strategy in the present experiments was to study the CNBr cleavage pattern of the electrophoretic species displayed in Fig. 5. In lane d of that figure is shown the two-band pattern resulting from MalNFlu-labeling the "additional" (not Cys-374) thiols exposed by the zero-[Ca^{2+}] condition. These two bands were electrophoretically separated from one another, and then each was subjected to CNBr fragmentation; the fragments were then electrophoresed. In Fig. 6, lane d shows the fluorescence-labeled fragmentation pattern of the upper band of the two-band pattern, and lane e shows the fragmentation pattern of the lower band. Surprisingly, the two bands give essentially the same fragmentation pattern—a pattern showing that two thiols are labeled by MalNFlu when Ca^{2+} has been withdrawn from the protein. This pattern results irrespective of the increment in n_{SH}; for example, if under certain conditions titration shows that n_{SH} has increased by 1, that unit increment may have resulted from reacting each of two thiols by the extent of 1/2.

Accepting that withdrawal of Ca^{2+} exposes thiols which then react with MalNFlu, we sought to find out whether one of these thiols was Cys-10. It has been shown by Jacobson and Rosenbusch (9) and by Mornet and Ue (5) that proteases digest rapidly an N-terminal region of ≈9 kDa, leaving undamaged a C-terminal core of ≈33 kDa. After trypsinolysis, we isolated and purified this core, labeled it with MalNFlu in the absence of Ca^{2+}, and subjected it to CNBr fragmentation and PAGE. From comparison of the fragmen-

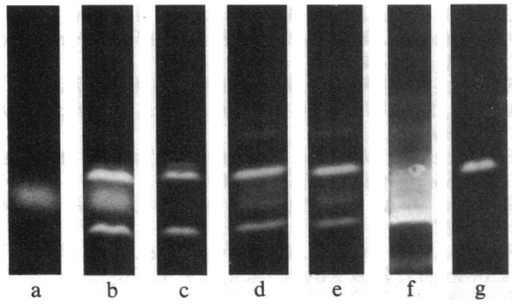

FIG. 6. CNBr fragmentation patterns of actin labeled with MalNFlu. Lanes: a, actin labeled in the presence of calcium; b, actin labeled in the presence of EDTA; c, actin-MalNEt labeled in the presence of EDTA; d, slower-migrating band in lane d of Fig. 5; e, faster-migrating band in lane d of Fig. 5; f, 33-kDa tryptic fragment (core) labeled with MalNFlu in the presence of EDTA; g, purified CB-13 whose lysine was labeled with fluorescein isothiocyanate. For cleavage, proteins were incubated with 1 M CNBr in 70% formic acid overnight at room temperature.

tation pattern (Fig. 6, lane f) with the fragmentation patterns (lanes d and e) of the two actin bands, we conclude that the thiol resident on the faster-moving fragment of either labeled actin is in the core of actin, is therefore C-terminal, and is therefore not Cys-10. Of the CNBr fragments of actin the most N-terminal is CB-13: this fragment contains Cys-10, and methods are available for obtaining it pure and separate from all other CNBr fragments (10). Its electrophoretogram is shown in lane g; its mobility is identical to that of the slower-moving fragment in lane d or e. From this last comparison, we conclude that this slower-moving fragment is CB-13 and that its resident thiol is Cys-10.

We tried to identify the other thiol (besides Cys-10) that becomes reactive after EDTA treatment; it must be Cys-217, or Cys-257, or Cys-285 (10). Fig. 7 shows the results of fragmentation of MalNDAC-labeled actin with CNBr and with 2-nitro-5-thiocyanobenzoic acid (NTCB; cleaves on the

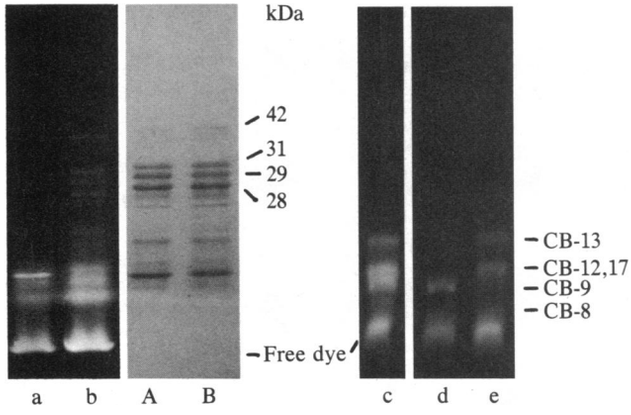

FIG. 7. NTCB (*Left*) and CNBr (*Right*) fragmentation patterns of actin labeled with MalNDAC. Lanes A and B were Coomassie blue-stained; all the other lanes were photographed under UV light. Lanes a, A, and d: fragmentation patterns of actin exposed to MalNDAC in the presence of Ca^{2+}, so label at Cys-374 can be presumed. Lanes b, B, and e: fragmentation patterns of actin whose Cys-374 had been blocked with MalNEt and which then had been exposed to MalNDAC in the absence of Ca^{2+}. Lane c: fragmentation pattern of actin exposed to MalNDAC while in 8 M urea. For NTCB cleavage, the protein was incubated at 37°C for 2 hr in 8 M urea containing 20 mM NTCB at pH 9.0. CNBr cleavage was as described for Fig. 6. MalNDAC appears to generate fluorescent bands having no counterpart in the Coomassie blue display; these are due to unattached, possibly aggregated, dye (unpublished observation).

N-terminal side of unlabeled cysteine residues) (16, 17). Were Cys-217 the thiol in question, the shortest NTCB fragment (with Cys-10 and Cys-217 labeled) would contain 257 residues. Lanes b and B, however, show a fluorescent peptide that moves well ahead of myosin light chain 3 (comparison not shown) and so should contain <140 residues. Thus Cys-217 is excluded. Lane A shows that NTCB generates large peptides, of 28–31 kDa, but these must be N-terminal since they have no fluorescent counterparts in lane a. The single fluorescent peptide in lane a should therefore be the shortest containing the C-terminus (the label is at Cys-374). This shortest peptide should have 91 residues (285–375). For lane b, the expectations depend on whether (in addition to Cys-10) Cys-257 or Cys-285 was labeled. If Cys-257 were labeled the shortest fluorescent peptide should contain 68 residues, whereas if Cys 285 were labeled it should contain 119 residues. Since the fluorescent peptide in lane b appears to run slightly faster than that in lane a, we can assume that it contains fewer than 91 residues. This result tends to favor (though not strongly) the hypothesis that Cys-257 was labeled. Experiments with CNBr fragmentation further strengthen this deduction. Ideally, lane c should reveal five fluorescent fragments, each containing one thiol. Lane d identifies the fragment among the five that bears Cys-374 (CB-9); this fragment will not appear again in lane e, wherein the starting material had Cys-374 blocked by MalNEt. From comparison of lane e with lane d, it is clear that the withdrawal of cations has generated two fluorescent bands. The slower of the two is CB-13, which, as already explained, bears Cys-10 and contains 44 residues. Therefore the next slowest fluorescent band could not be CB-8, bearing Cys-285, as CB-8 contains only 15 residues and must run faster than CB-9, which contains 20 residues. Also it could not be CB-17, bearing Cys-217, as above we have shown that Cys-217 is not labeled. It must, therefore be CB-12, consisting of 42 residues and bearing Cys-257. So, once more we conclude that "the other thiol" labeled after EDTA treatment is Cys-257. In summary, removal of calcium ions exposes Cys-10 and Cys-257.

DISCUSSION

In showing that actin conformation (as indirectly reported by n_{SH} and by the fluorescence of bound Ans) is a function of both bound Ca^{2+} and bound nucleotide, we are mainly confirming what previous workers have recognized. However, it is of some importance that we have realized a situation in which n_{SH} is a function of $[Ca^{2+}]$ at constant bound nucleotide. This relationship (Fig. 4) suggests that the affinity of Ca^{2+} for actin may be of an order of $10^8\ M^{-1}$; much greater than previously thought (18–20).

Our work has revealed an interesting conformational difference in actin after metal removal; it is distinguishable with a particular label (fluorescein). There seem to be two conformers of actin after removal of metal. On metal removal we could detect a conformational change in actin by measuring an increase in n_{SH} and Ans fluorescence intensity. However, the presence of two conformers was not detectable by these methods; this could be done only by labeling with fluorescein. Once the thiols exposed were labeled, the two conformers were separable by their mobilities on $NaDodSO_4$/PAGE. We do not know the basis of the difference between these two conformers; however, it is neither a difference in dye content nor a difference in species of labeled thiol.

Mornet and Ue (5) pointed out that the homology to a Ca^{2+}-binding site that they found around Cys-10 was only partial, suggesting that the rest of the "cage" might be provided by other regions of the primary sequence. The symmetries between the two thiols that become exposed (Cys-10 and Cys-257) and the (fluorescein) ligand lead us to

speculate that both thiols interact directly with one Ca^{2+}, but at this stage it remains a logical possibility that only one interacts and that the second thiol is exposed by a conformational change propagating out from the first one.

We come finally to what was our central objective—to find out whether Cys-10 is exposed upon removal of Ca^{2+} from actin. This now seems clearly so, and the puzzle of why Cys-10 is so hard to label now seems solved. Our results are entirely consistent with the speculation that a Ca^{2+}-binding site exists near the N-terminus of actin (5); the surprise is only that a second thiol (Cys-257) participates as well. The significance of a divalent cation placed at the same point at which S-1 binds to actin is an exciting issue for future research.

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