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Interchain and intrachain crosslinking of actin thiols by a bifunctional thiol reagent

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From 1,9-nonylenedithiol and Ellman's Reagent the bifunctional asymmetric disulfide *n*-nonylene-1,9-bis-[5-dithio-(2-nitrobenzoic acid)] (NBDN) was prepared. By monovalent reaction with cysteine-374 the crosslinker could be introduced into monomeric actin, with release of one equivalent of yellow 2-nitro-5-thiobenzoate (NTB). From the monovalent actin derivative we prepared a crosslinked actin dimer (Cys-374–Cys-374') as well as a monomer with a crosslink between Cys-374 and Cys-10. Neither crosslinked actin species was able to polymerize the crosslinked monomer even in the presence of phalloidin. The crosslinked monomer polymerized on the addition of dithiothreitol, thus providing the first unpolymerizable actin species whose polymerizability can be restored under mild conditions. We suggest the use of NBDN as a thiol-specific crosslinker that reacts under spectrophotometric control and can be removed by the addition of thiols.

Unpolymerizable actin; Disulfide exchange reaction; Protein thiol; Reversible thiol-specific crosslink

1. INTRODUCTION

Recently we have shown that the disulfide exchange reaction can be used to introduce aliphatic, low-molecular-weight thiol components into thiol-exposing proteins. For example, using glutathionyl-2,4-dinitrophenyldisulfide, the tripeptide could be attached through a disulfide bond to cysteine in position 374 of actin. Because of their low mechanical stability the filaments formed from this actin derivative provided an assay system for studying the effects of filament-stabilizing agents [1,2]. By using [^{14}C]cysteinyl-2,4-dinitrophenyldisulfide a hitherto unknown conformational change in actin monomers was detected, in which cysteine in position 10 becomes uncovered [3,4]. The same reagent was used for studying the complete unfolding of actin after removal of the tightly bound nucleotide [5]. More recently, we were able to demonstrate that the same type of reaction can be used to attach even large molecules such as a thiol derivative of phalloidin (M_w ca. 1000) to the two accessible thiol groups of actin [6]. Encouraged by these results, we prepared a bifunctional reagent of this type for potential use in crosslinking experiments. Using actin as model protein we showed that this reagent is indeed a useful tool for crosslinking protein thiols with high specificity and under spectrophotometric control. Since the crosslinker can be cleaved under mild conditions, the nativeness of the recovered protein can easily be assessed.

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2. MATERIALS AND METHODS

Ellman's Reagent (DTNB) was from Serva (Heidelberg), 1,9-nonane-dithiol from Aldrich (Steinheim), phalloidin and [^{3}H]dehydroxymethyl phalloidin (4 Ci/mmol) were our own preparations. Hexokinase C-302 was from Sigma.

Actin was prepared according to Spudich and Watt [7] with an additional gel filtration step on a Sephadex G-150 column equilibrated with 2 mM Tris, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.02% NaN_3 (pH 7.8) (Tris/ATP buffer).

2.1 *Preparation of n-nonylen-1,9-bis-[5-dithio-2-nitrobenzoic acid)] (NBDN)*

To 440 mg (1.11 mmol) Ellman's Reagent dissolved in 20 ml of 0.1 M $NaHCO_3$, 50 μl 1,9-nonane dithiol (0.25 mmol) were added dropwise while stirring vigorously. To protect the thiol from oxidation the reaction was performed in an argon atmosphere. After two hours, during which time the colour of the reaction mixture turned orange, 1 M HCl was added until the pH reached 1.0. The suspension was extracted twice with peroxide free ether, then the ether was removed in a rotary evaporator and the residue diluted in methanol. Separation of the reagent from accompanying Ellman's Reagent and NTB was achieved by HPLC on RP18 with $CHCl_3$/CH_3OH/2 N acetic acid (65:25:4) or, for larger scale preparations, on analytical thin-layer plates (Merck HF254) in the same solvent mixture. (R_f values: NBDN = 0.30, Ellman's Reagent = 0.13). Detection of the reagent and the starting material on the thin-layer plates was achieved under UV light or by the yellow colour developing after spraying of the plates with 1% DTT. The methanolic extract of the scraped-off silica was submitted to a further filtration step on a short Sephadex LH_{20} column equilibrated with methanol.

Yield: 17%

[^{1}H]-NMR data:

	δ [ppm]	coupling [Hz]
H^a	7.93 (d, 2H)	
		$^3J_{H,H}$ = 2.20
H^b	7.59 (dd, 2H)	
H^c	7.56 (dd, 2H)	
		$^3J_{H,H}$ = 8.60
$(CH_2)^\alpha$	2.78 (t, 4H)	
$(CH_2)^\beta$	1.68 (m, 4H)	
		$^3J_{H,H}$ = 7.00
$(CH_2)^\gamma$	1.38 (m, 4H)	
$(CH_2)_3$	1.28 (s, 6H)	

2.2. *Reaction of the crosslinker with cysteine-374 of monomeric actin. Preparation of (A–C)*

To a cuvette containing 2.9 ml of a G-actin solution (47.2 nmol) in Tris/ATP buffer (2 mM Tris-HCl, 0.2 mM ATP, 0.1 mM $CaCl_2$ 0.02% NaN_3, pH 7.8) was added 0.1 ml of methanolic NBDN (472 nmol, 10-fold excess). The increase of absorbance at 412 nm was followed relative to a control cuvette containing no actin. After the release of 1 equiv. of NTB (ca. 1 h) the actin derivative was separated from the reagent and low molecular weight reaction products by passing it through a Sephadex G-25 column equilibrated with Tris/ATP buffer. 1:1 stoichiometry of reacted reagent and actin was proved by cleaving the disulfide bridge with excess of mercaptoethanol and determining actin and NTB concentrations by UV spectrophotometry.

2.3. *Intramolecular crosslinking of monomeric actin between the cysteine residues 10 and 374. Preparation of (A=C)*

(A–C) was polymerized by the addition of 1 mM $MgCl_2$/0.2 mM EGTA, and, for removal of ATP, treated with 1 mM glucose and 5 U/ml hexokinase according to Pollard [8]. After 2 h at room temperature, the actin was pelleted (100 000 × *g*, 4°C, 1 h), rinsed with Tris/ADP buffer (2 mM Tris-HCl, 1 mM ADP, 0.02% NaN_3, pH 7.8) and kept under these conditions for 16 h at 0°C. After careful homogenizing the solution in a Teflon potter it was stored at 0°C. Spectrophotometry (412 nm) showed that after ca. 65 h the intrachain crosslinking reaction had reached completion. Yield was up to 82%.

Separation from unreacted actin was achieved by the addition of 0.1 M KCl and 1 equiv. of phalloidin, followed by separation of the unreacted polymeric actin by ultracentrifugation (100 000 × *g*; 1 h). In order to remove KCl and excess of phalloidin the supernatant was run over a Sephadex G-25 column (25 × 1 cm) equilibrated with Tris/ ATP or Tris/ADP buffer, depending on the kind of nucleotide intended to be bound by the actin derivative. If necessary, the actin solution was concentrated by ultrafiltration.

2.4. *Intermolecular crosslinking of two monomeric actins at the cysteine-374 residues; (A–C–A)*

(A–C) was mixed with 1 equiv. of G-actin and frozen at −20°C. After thawing, the extent of dimerization was determined by measuring the extinction at 412 nm (ca. 60%). For separation of unreacted actin monomers the solution was passed through a Sephadex G-150 column (10 × 2 cm) equilibrated with Tris-ATP buffer at 4°C. The concentration of the actin dimer was raised by ultrafiltration through Amicon pm 30 membranes (N_2, 3 bar). For identification, the actin dimer was submitted to SDS-PAGE either without 2-mercaptoethanol or with 10-fold excess of 2-mercaptoethanol added, and detected with Coomassie blue. The molecular weight of the actin dimer was determined by HPLC in Tris-buffer (2 mM, pH 6.0) containing 0.1% SDS on a TSK 3000SW column (60 × 0.75 cm), using hexokinase, serum albumin and monomeric actin as marker proteins. Critical concentrations were determined by submitting samples of F-actin or actin-dimer (3×10^{-6} M to 2×10^{-5} M with equivalent amounts of phalloidin in 2 mM Tris, 0.2 mM ATP, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$) to ultracentrifugation at 4°C at 155 000 × *g* for 1 h. Protein concentration in the supernatant was determined according to Bradford [8].

Polymerizability was assayed by viscometry using a Canon viscosimeter as described previously [3].

2.5. *Phalloidin binding*

The equilibrium dissociation constant (K_d) of the actin dimer and [^{3}H]demethylphalloin was assayed by equilibrium dialysis.

One ml samples of the actin dimer (3×10^{-6} M, in Tris/ATP buffer, 0.1 M KCl) containing [^{3}H]demethylphalloin (3×10^{-8} M) and additional phalloidin up to total concentrations of phallotoxin of 3×10^{-7} M to 6×10^{-5} M, were dialyzed against 1 ml of the same buffer for 16 h at 4°C in Dianorm equipment. A control chamber contained no protein. Aliquots of 100 μl from each chamber were counted.

Phalloidin binding to the intramolecular crosslinked actin monomer (A=C) was assayed as described above with excess of phallotoxin up to 100-fold over protein concentration.

3. RESULTS AND DISCUSSION

3.1. *Preparation of the crosslinking reagent*

Alhough the yield was moderate, preparation of *n*-nonylene-1,9-bis[5-dithio-2-nitrobenzoic acid] (NBDN; Fig. 1) was easy and the reagent could be obtained with high purity.

Purity was proved by detection of a single peak, or spot, in HPLC, or TLC, respectively, as well as by ^{1}H-NMR (see Materials and Methods). In methanolic solution, NBDN was stable and could be stored for several months at 4°C. Reagent quantities were measured by UV absorption. The molar extinction coefficient in methanol, ε_{320} = 22.350 M^{-1}, was determined from the amount of NTB (ε_{412} = 14.150 M^{-1} [9]) released from the pure compound on the addition of excess mercaptoethanol. In the dry state, NBDN apparently undergoes partial decomposotion as indicated by the colour of the solid material turning slightly yellow.

3.2. *Attachment of the crosslinker to actin; preparation of (A–C)*

Monomeric muscle actin, when complexed to ATP, exposed only one thiol group, that of cysteine in position 374. Using a 10-fold excess of NBDN, derivatization of this thiol was found to be complete after ca. 1 h, as concluded from the spectrophotometric determination of the released NTB, which after that time leveled off, corresponding to 1.0 equiv. of the yellow anion (not shown). It is the particular advantage of this type of reagent that the endpoint of reaction can be easily determined, thus avoiding prolonged reaction times that may lead to unfolding and derivatization of thiols normally buried. Spectrophotometric control further helped in showing that the reaction of cysteine-374 was complete, so that a separation from unreacted material was unnecessary in most preparations. Actin bearing

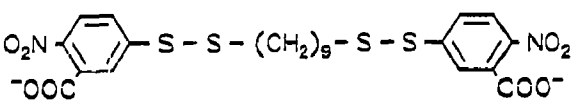

Fig. 1. Formula of 1,9-nonylenedithiol-bis[5-dithio-(2-nitrobenzoic acid)], NBDN.

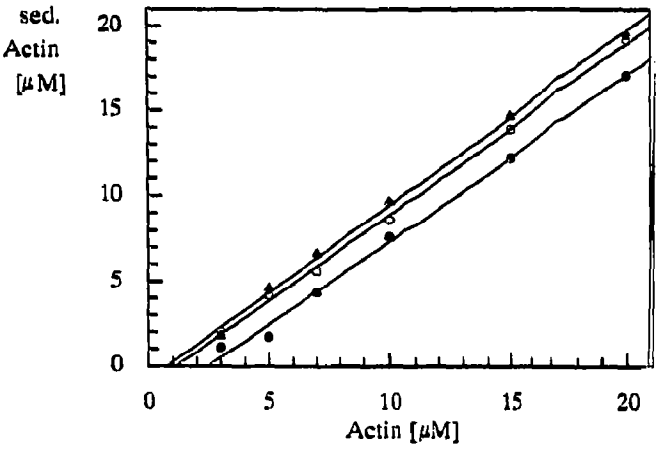

Fig. 2. Determination of the critical concentrations in the presence of 1 mM $MgCl_2$: actin (▲), (A–C) (●), (A–C) with equivalent amounts of phalloidin (○).

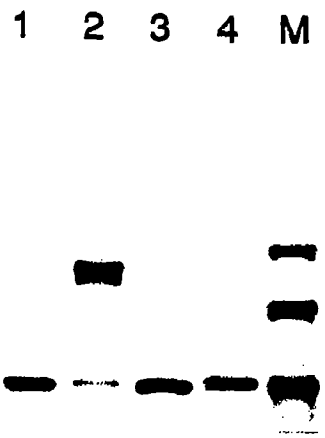

Fig. 3. SDS-PAGE (12.5%) of the actin derivatives. (Lane 1) G-actin; (lane 2) dimer after purification; (lane 3) dimer incubated with excess of 2-mercaptoethanol; (lane 4) intramolecular crosslinked actin; M = MW-marker: phosphorylase B (97.4 kDa); serum albumin (66.2 kDa); ovalbumin (45 kDa); from top.

the monovalently reacted crosslinker, (A–C), exhibits polymerizability similar to actin. Its critical concentrations in the absence and presence of phalloidin, C_{crit} = 2.7×10^{-6} M, and $C_{crit}^{PHD} = 1.0 \times 10^{-6}$ M, respectively were found to be very close to the corresponding values for normal actin, $C_{crit} = 1.0 \times 10^{-6}$ M, $C_{crit}^{PHD} = 5.0 \times 10^{-7}$ M (Fig. 2).

3.3. *Preparation of the actin dimer (A–C–A)*

When (A–C) was mixed with one equivalent of native G-actin, no reaction was observed, even after prolonged incubation times. The reaction occurred only when the mixture was frozen, and was then indicated by the appearance of yellow colour, which was monitored at 412 nm. After separation of the dimer from the monomer and NTB by gel filtration, the yield was ca. 60%. The dimer was identified by its molecular weight using SDS-PAGE (Fig. 3) and HPLC. A lane on SDS-PAGE, run in the presence of 2-mercaptoethanol, showed monomeric actin only.

3.4. *Polymerizability of the dimer*

The dimer was unable to polymerize on the addition of $MgCl_2$ (1 mM) or KCl (0.1 M) as proven by light scattering (not shown), or viscosity (Fig. 4). After 2 h of incubation with 2-mercaptoethanol, polymerizability was fully restored, with relative viscosity (η) values of up to 98% of the expected value for normal actin. This indicates that the dimerization process was not accompanied by denaturation. The recovered actin had a lower rate of polymerization and the onset of polymerization was delayed. Since it is unlikely that during mercaptoethanol reduction oligomers were formed, the delayed polymerization may be due to poor nucleation. Although not investigated in detail, monomers freshly prepared from the unpolymerizable dimers by this reaction may represent a model system for studying the kinetics of actin polymerization virtually in the absence of nuclei.

3.5. *Phalloidin binding of the dimer*

While the addition of salt did not induce polymerization of the dimer, the addition of phalloidin caused a retarded but pronounced increase of viscosity as shown in Fig. 4. Since it is unlikely that this kind of crosslinking (bottom-to-bottom) produces a dimer that would fit into the filament model as proposed by Holmes et al. [10], we believe that under the influence of phalloidin the actin dimer aggregates in a manner different from actin filaments. On ultracentrifugation the aggregated dimer could be pelleted and thus allowed the estimation of the critical concentration from the supernatant (C_{crit} = 5.5×10^{-6} M).

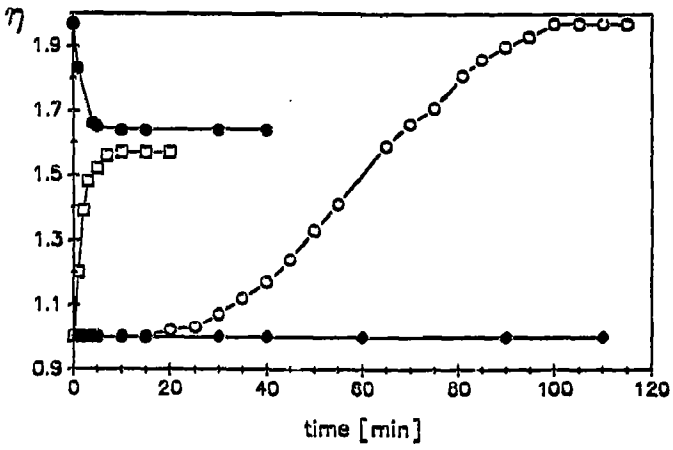

Fig. 4. Polymerizability of crosslinked actin dimer (A–C–A) as assayed by viscometry. No polymerization in the presence of 1 mM $MgCl_2$ or 0.1 M KCl (♦), polymerization of control G-actin (□), aggregation of crosslinked dimer in the presence of 1 mM $MgCl_2$ and phalloidin (○), change in viscosity of the aggregated dimer on the addition of 2-mercaptoethanol (●).

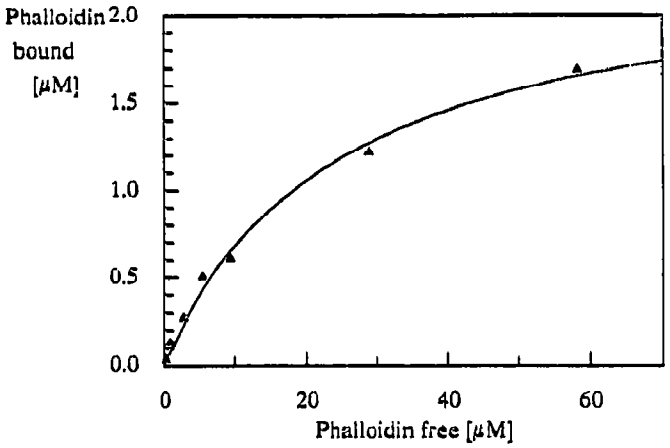

Fig. 5. Complexation of the crosslinked dimer (A–C–A) with phalloidin as measured by equilibrium dialysis. Fitting of the curve provides $K_d = 2.4 \pm 0.4 \times 10^{-5}$ M.

As further shown in Fig. 4, addition of excess of 2-mercaptoethanol reduced viscosity to a level that is similar to that of the corresponding concentration of native F-actin suggesting that the aggregated dimer undergoes a rearrangement to normal filaments. The structure of the polymeric actin dimer is under investigation at present.

Binding of phalloidin to the dimeric actin was established by equilibrium dialysis experiments using [^{3}H]demethylphalloidin as a tracer. Evaluation of the plot shown in Fig. 5 resulted in a K_d-value of $2.4 \pm 0.4 \times 10^{-5}$ M, a value indicating a roughly 10^3 times lower affinity of phalloidin for the aggregated dimer than for normal F-actin [11].

3.6. *Crosslinking of monomeric actin (A=C)*

For the intrachain crosslinking reaction we made use of a conformational change of G-actin recently discovered in our laboratory [4]. In the absence of an excess of divalent cations, monomeric actin complexed to ADP slowly undergoes a conformational change that uncovers the thiol group of cysteine-10. We submitted (A–C) to these conditions expecting that the second activated disulfide moiety of the crosslinker would react with the thiol group of cysteine 10 that becomes more and more exposed under these conditions. A prerequisite for the expected reaction is of course that the crosslinker is long enough to span the distance between the two cysteine residues in position 10 and 374. By following the reaction with spectrophotometry we found that the crosslinker indeed reacted in the expected way and that after 65 h the reation was complete, yielding up to 82% of the intrachain crosslinked actin (A=C).

The structure of (A=C) as a monomer with a crosslink between the cysteine residues of 374 and 10 is mainly based on two kinds of evidence. One is that the only cysteine to be uncovered to any extent under these conditions (aside from cysteine-374) is cysteine 10. This was shown recently by labelling the exposed cysteine residues with [^{14}C]cysteine and identifying the position of the labels after degradation of the protein. Beside the well-known labelling of cysteine-374, the major part of the radioactivity was found in the N-terminal peptide 1–44, which contains only the cysteine residue in position 10. Only a small amount of the label was found in a third cysteine residue (cysteine-257), which under these conditions reacts to about 10% [4]. More evidence for the proposed structure comes from the X-ray model of actin [12] which shows that only cysteine-10 is located in the same domain as cysteine-374, and thus close enough to react with the crosslinker.

As discussed in more detail below, actin crosslinked in the intrachain manner had completely lost its ability to form polymers, even in the presence of phalloidin. This property facilitated its purification, because unreacted (A–C) (see above) could be separated by polymerization in the presence of phalloidin. As calculated from the critical concentration of the starting material (A–C), this purification procedure would leave less than 1% of the unreacted percursor as impurity in the solution of the crosslinked actin monomer. Indeed, after this purification step the crosslinked actin species was pure enough for most investigations. The crosslinked monomer was stable, particularly after the bound nucleotide, which after the crosslinking reaction was ADP, was exchanged for ATP. While after 10 days storage at 0°C (A=C)·ADP was found to be 50% denatured, (A=C)·ATP was only 5% denaturated after 20 days at 0°C, as determined from the yield of pelleted actin recovered after the addition of 2-mercaptoethanol and 0.1 M KCl. The stability of (A=C)·ATP seems high enough for investigating the conditions of its crystallization, which for example, would provide the possibility for studying the structure of an actin monomer which is not part of a complex with an actin-binding protein.

3.7. *Polymerizability and phalloidin binding capacity of the crosslinked actin monomer*

As already mentioned, (A=C) has completely lost its ability for polymerization. No increase in viscosity was observed when phalloidin was added to solutions of (A=C) containing either 1 mM $MgCl_2$ or 0.1 M KCl, or both (data not shown). Various actin derivatives have in the past been claimed to be unpolymerizable, although in several cases this conclusion had to be revised because at least partial polymerization was observed on the addition of phalloidin [13]; (A=C), however, showed no signs of filament formation even at protein concentrations of 20 mg/ml and in the presence of high excess of phalloidin. In agreement with this, no binding of [^{3}H]demethylphalloin was detected when (A=C) was assayed in equilibrium dialysis experiments. Experiments are in progress which will show whether the crosslinked actin monomer still possesses its binding capacity for actin binding proteins like DNase I or profilin.

Like the crosslink in the actin dimer the crosslink in mononeric actin represents a modification that can be reversed under mild conditions, for example, by the addition of 2-mercaptoethanol at pH 7.8. In this respect (A=C) differs from many other modified actins, for which it was difficult to decide, whether loss of polymerizability was due to the modification made, or to denaturation accompanying the modification reaction. When (A=C) was treated with 2-mercaptoethanol and subsequently polymerized, viscosity reached ca. 85% of the η-value expected for a given actin concentration, indicating that up to 15% of the actin had become denatured during the crosslinking and cleavage reactions. This reversibility of the crosslinking reaction may be of particular importance for biological studies, because it allows actin to be introduced into in vitro systems as an unpolymerizable, i.e. biological inactive species, and its biological activity to be restored under controlled conditions by the addition of a reducing agent. Since reactivation of actin from (A=C) occurs via a slow conformational change [4], it is tempting to look for means to study the refolding process in the crystal, e.g. by soaking them in 2-mercaptoethanol.

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