Electrostatic Changes at the Actomyosin–Subfragment 1 Interface during Force-Generating Reactions†

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ABSTRACT: The ionic strength dependence of the binding of rabbit skeletal muscle myosin subfragment 1, S1, to F-actin in the presence of saturating concentrations of MgATP or MgADP was analyzed in order to determine the association constants at zero ionic strength [$K(0)$] and the products of the net effective electric charges ($|z_M z_A|$) at the binding interfaces. $K(0)$ and $|z_M z_A|$ were 1×10^6 M^{-1} and 17 esu^2 for S1–MgADP,P, and 5×10^7 M^{-1} and 7 esu^2 for S1–MgADP, respectively, for binding to F-actin at 25 °C. At ionic strengths near physiological, the increase in affinity is close to 10^4-fold for this transition that may correspond to force generation in muscle fibers. The large, from 17 to 7 esu^2, decrease in the electrostatic contribution to binding appears to be correlated with a much larger increase in nonelectrostatic interactions, unlike the simpler transition of actin-bound S1–MgADP to S1, which appears to be due entirely to electrostatic changes [Highsmith, S. (1990) *Biochemistry 29*, 10690–10694]. These results for acto–S1–MgADP,P to acto–S1–MgADP suggest that a substantial transformation of the actin binding site on S1 occurs even if there is a translocation to a new interface.

The actin–myosin–nucleotide complex generates force in muscle by means of actin–myosin structural rearrangements that depend on which of the MgATP hydrolysis products remain in the ATP binding site on myosin. It has been shown that an isolated domain of skeletal muscle myosin called subfragment 1 (S1)[1] is sufficient to interact with MgATP and actin to generate movement and presumably force (Toyoshima et al., 1987). S1, actin, and MgATP are water-soluble, and MgATPase kinetics indicate that for physiological conditions the force-generating steps of the contractile cycle probably start with S1–MgADP,P binding to actin, followed by force generation and hydrolysis product dissociation (Lymn & Taylor, 1971; Bagshaw & Trentham, 1974). The kinetic schemes have been made more realistic by the introduction of more intermediates and the correlation of intermediates and energetics (Eisenberg & Hill, 1985; Geeves et al., 1984). From the perspective of trying to understand the mechanical components of the mechanism of energy transduction, the key features of force generation are the acto–S1 structural manifestations of the communication between the ATP binding site on S1 and the acto–S1 interface. The proposed models for these force-generating protein structural changes remain hypothetical, low-resolution, and sometimes dependent on observations made on S1 in the absence of actin (Reedy et al., 1965; Huxley, 1969; Huxley, 1974; Shiver & Sykes, 1981; Thomas & Cooke, 1980; Botts et al., 1989; Highsmith & Eden, 1990; Geeves, 1991; Cheung et al., 1991) because tertiary structural information about the acto–S1–nucleotide complex is scarce and difficult to obtain.

The nucleotide and actin binding sites are about 5 nm apart (Tokunaga et al., 1987; Kasprzak et al., 1989). The binding of S1–N to actin to form a ternary complex, where N represents MgATP hydrolysis products or analogues, provides information about this chemomechanical process (Highsmith, 1976; Hoffman & Goody, 1978; Greene & Eisenberg, 1978). The actin affinity increases by a factor of about 1000 in progressing from N = MgADP,P to MgADP (Beinfeld & Martonosi, 1975; Highsmith, 1976, 1990; Greene & Eisenberg, 1978, 1980; Marston et al., 1979; Wagner & Giniger, 1981; Chalovich, et al., 1981; Chalovich & Eisenberg, 1982; Furukawa & Arata, 1984; Katoh et al., 1984; Geeves et al., 1986; Taylor, 1991) with significant variation in the specific association constants due to differences in conditions, methods, and protein preparations. The nature of the forces at the acto–S1 interface which contribute to this increase in affinity, and presumably to the contractile force that the complex generates, has been shown to be partially electrostatic (Tonomura et al., 1962; Highsmith, 1977, 1990; Gulati & Podolsky, 1978, 1981; Greene et al., 1983; Chalovich et al., 1984; Chaussepied & Morales, 1988; Yamamoto, 1989; Chaussepied, 1989; Kawai et al., 1990). We are interested here in quantitatively characterizing the electrostatic contribution to binding at the protein–protein interface of acto–S1–N as N is changed from MgADP,P to MgADP. A preliminary report on this work has appeared (Highsmith & Murphy, 1991).

The nucleotide-induced electrostatic interactions at the acto–S1 interface cannot be calculated from the surface charge and location by using the Poisson–Boltzmann finite difference method (Warwicker & Watson, 1984; Sharp & Honig, 1989) at this time. Although the high-resolution tertiary structure of the crystalline heterodimer G-actin–DNase 1 is probably an adequate approximation of F-actin (Kabsch et al., 1990; Holmes et al., 1990), the tertiary structure of S1 is still only available at low resolution (Rayment & Winklemann, 1984). High-resolution structural information about the changes at the actin binding site on S1 which are caused by changes in the structure of the bound nucleotide is nonexistent. Estimates of the net effective electric charges at the actin binding site

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[1] Abbreviations: S1, myosin subfragment 1; AEDANS, 5-[[(acetylamino)ethyl]amino]naphthalene-1-sulfonate; TMA, tetramethylammonium; z_M, net effective electric charge of the actin binding site on myosin; z_A, net effective electric charge of the S1 binding site on actin; K_a, apparent association constant; $K(0)$, association constant in zero ionic strength; I, ionic strength; a, activity; m, molarity; c, concentration; γ, activity coefficient; b, α, $\beta(0)$, $\beta(1)$, and C are constants in the Pitzer (1979) treatment of ions in solution.

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of S1 (z_M) and the myosin binding site on actin (z_A) have been made from measurements of the product $z_M z_A$ for acto–S1 and acto–S1–MgADP by using electrolyte solution theory (Pitzer, 1973, 1979) to analyze the ionic strength dependence of acto–S1 binding (Highsmith, 1990). Knowing the product of the net effective electric charges does not reveal all the tertiary structural details at the interface, but the changes in the net effective electric charge were shown to be quantitatively consistent with the observed changes in affinity. Knowing the net effective electric charges of S1–nucleotide and actin should be useful for assigning surfaces on high-resolution tertiary structures of S1 and actin to binding sites when those structures are available. In addition, this electrolyte solution approach can provide data for some kinetic intermediates that may not be available for X-ray diffraction under any circumstances, but could be correlated to structural information obtained by nuclear magnetic resonance spectroscopy. In the present case, estimates are obtained of $|z_M z_A|$ for F-actin binding to S1–MgADP,P and S1–MgADP. The results indicate that for the transition from acto–S1–MgADP,P to acto–S1–MgADP in solution, $|z_M z_A|$ drops from 17 to 7 esu^2 as the affinity increases 100–10 000-fold, depending on the ionic strength.

Materials and Methods

Chemicals and Proteins. All chemicals were reagent grade or better. The stock solution concentration of TMAOAc was adjusted so that when diluted to 0.010 M in double-distilled water, it had a conductance equal to that of standard 0.010 M KCl (Ricca Chemical Co.). The ionic strength was calculated from $I = {}^1/_2\sum(m_i z_i^2)$ where z_i is the electric charge and m_i is the molar concentration of each of the i ionic species present.

Myosin was isolated from rabbit skeletal muscle (Nauss et al., 1969) and used to prepare S1 (Margossian & Lowey, 1978). S1 was also prepared from myofibrils (Cooke, 1972). In either case, papain was used for the proteolysis in the presence of 1–5 mM $MgCl_2$. S1 with both light chains present was purified by size-exclusion chromatography, using Sephacryl S-400; so the differences in the ionic strength dependencies observed for S1A1 and S1A2 (Chalovich et al., 1984) will not be resolved if they exist when all the light chains are present. Results for the binding of HMM to actin suggest that S1 moieties with all light chains present have the same ionic strength dependence (Greene, 1981). MgATPase activities at 25 °C were typically 0.044 s^{-1} for S1 and 1.05 s^{-1} for acto–S1. *N*-Acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine-labeled S1 (AEDANS-S1) was prepared (Duke et al., 1976), and unattached fluorescent label was removed by dialysis. AEDANS-S1 samples were centrifuged at 80000*g* for 1 h before use. F-Actin was prepared as described by Spudich and Watt (1971) from acetone-extracted muscle tissue (Feuer et al., 1948).

Association Constants. In some experiments, the method of Marston and Weber (1975) was used to measure the fractions of bound and free S1 in the presence of F-actin except that AEDANS-S1 fluorescence was used to detect S1 instead of the radioactivity of [^{14}C]acetamide-labeled S1 (Highsmith, 1990). The AEDANS is attached to Cys-707, which does not appear to alter significantly the affinity of S1 for F-actin (Highsmith et al., 1976; Yoshimura & Mihashi, 1982). In other experiments, unmodified S1 binding to F-actin was determined by the method of Furukawa and Arata (1984; Katoh et al., 1984). The results from the different methods were combined. The primary purpose of the present study is to detect changes in the affinity caused by nucleotide binding to S1, so label effects that might change absolute values by small amounts will not affect the conclusions, which depend on ratios.

The conditions for F-actin binding to S1–MgADP were as described (Highsmith, 1990; Highsmith & Murphy, 1991) except that TMAOAc was the salt used to adjust the ionic strength in the present case. For F-actin binding to S1–MgADP,P, the ionic strength was adjusted with TMAOAc, KOAc, or KCl with no detectable difference between ionic species. The concentrations of F-actin and S1 were typically 0.5 and 30 μM, respectively, with [MgATP] in the 2 mM range. MgATPase activities in the buffers used for the measurements of K_a were measured to ensure that the [MgATP] remained high enough to saturate the S1 ATP binding site thoughout the binding measurements. K_a was determined from [bound S1]/[free S1][free actin] where conservation of mass equations were combined with [free S1] and [bound S1] determined as follows: For AEDANS-S1, the fraction of free S1 was determined from the fluorescence intensity of an acto–S1 solution and an S1-only solution after airfuge centrifugation to pellet actin and bound S1 followed by removal and dilution of aliquots of the supernates (Highsmith, 1990). For unmodified S1, the fraction bound was determined from the areas under densitometric tracings of the Coomassie blue stained S1 heavy-chain bands from the pellets of an acto–S1 plus MgATP solution and an acto–S1 without MgATP solution, similarly to an established method (Furukawa & Arata, 1984).

The ADP case is reversible equilibrium binding between F-actin and S1–MgADP. For ATP, the S1 species is the steady-state intermediate S1–MgADP,P, which reacts with F-actin, when bound. This makes the bound species that are isolated by sedimentation a mixture of acto–S1–MgADP,P and acto–S1–MgADP so that K_a is

$$K_a = K_{D,P} f_{D,P} + K_D f_D \quad (1)$$

where $K_{D,P}$ and K_D are the equilibrium binding constants for F-actin to S1–MgADP,P and S1–MgADP, respectively, and $f_{D,P}$ and f_D are the fractions of the total free S1 that are S1–MgADP,P and S1–MgADP, respectively. If the free S1 is nearly all S1–MgADP,P, as is thought to be the case (Geeves, 1991), then K_a approaches $K_{D,P}$. If f_D is significant, K_a will be larger than $K_{D,P}$, so K_a is an upper limit for the affinity of F-actin and S1–MgADP,P.

Data Analysis. The reversible equilibrium binding of the solutes M and A:

$$M + A \rightleftharpoons MA \quad (2)$$

can be described by the expression

$$K(0) = a_{MA}/a_M a_A = (c_{MA}/c_M c_A)(\gamma_{MA}/\gamma_M \gamma_A) \quad (3)$$

where a is chemical activity, c is concentration, and γ is the activity coefficient (Moore, 1962). The ratio of the concentrations is K_a, the apparent association constant, which usually is measured. For ions M and A binding in solution, $\gamma_{MA} = 1$ and $\gamma_M \gamma_A$ is represented as $\gamma_\pm$, which has values that decrease from 1 as the ionic strength increases. Thus, eq 3 can be written

$$\ln K(0) = \ln K_a - \ln \gamma_\pm \quad (4)$$

which describes the lowered K_a in terms of $K(0)$ at ionic strength = 0 and the activity coefficient of the ions. The greater the charges on M and A, the stronger is the dependence of K_a on the ionic strength of the solution.

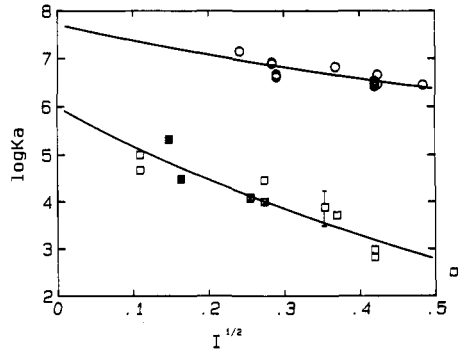

FIGURE 1: Ionic strength dependence of F-actin binding to S1–MgADP and S1–MgADP,P. The apparent association constant, K_a, was calculated from the fractions of free and actin-bound S1 in the presence of MgADP (○) or MgATP (□), as described in the text. The ionic strength was adjusted with TMAOAc (○, □), or KOAc (■), or KCl (⊠).

The theory developed by Pitzer (1973, 1979) has the expression relating $\gamma_\pm$ and I:

$$\ln \gamma_\pm = -0.392|z_M z_A|[I^{1/2}/(1 + bI) + (2/b) \ln (1 + bI^{1/2})] + m\{2\beta(0) + [2\beta(1)/\alpha^2 I] \times [1 - (1 + \alpha I^{1/2} - \alpha^2 I/2) \exp(-\alpha I^{1/2})]\} + m^2(3C/2) \quad (5)$$

where $b = 1.2$ and $\alpha = 2.0$ for all electrolytes and $\beta(0)$, $\beta(1)$, and C are parameters that are specific for particular electrolytes. Equation 5 can be used with eq 4 to fit data from measurements of K_a that are made over any range of I in order to obtain estimates of the values of $K(0)$ and $|z_M z_A|$. Accordingly, these parameters were obtained by fitting the data with a BASIC computer program which utilizes a nonlinear regression algorithm (Bevington, 1969; Marquardt, 1963). A more detailed description of the usefulness of the application of this approach to acto–S1 binding has been given (Highsmith, 1990).

Results and Discussion

The affinity of F-actin for S1–MgADP at 25 °C in solutions containing increasing amounts of tetramethylammonium acetate (TMAOAc) decreased by an order of magnitude in the ionic strength (I) range 0.06–0.25 M (Figure 1, shown as a function of $I^{1/2}$). When the data are fit to eq 4 by varying the values of $K(0)$ and $|z_M z_A|$, one obtains $(5 \pm 3) \times 10^7$ M^{-1} and 7 ± 2 esu^2, respectively. These values are close to those obtained for acto–S1–MgADP in solutions containing increasing amounts of LiOAc to adjust the ionic strength and confirm the conclusion that the results do not reflect specific ion effects (Highsmith, 1990).

The apparent affinity of F-actin for the steady-state intermediate S1–MgADP,P, produced from S1 and MgATP, is weaker and more sensitive to ionic strength (Figure 1) with values of $(1 \pm 0.6) \times 10^6$ M^{-1} and 17 ± 2 esu^2 for $K(0)$ and $|z_M z_A|$, respectively. The ionic strength dependence of F-actin binding to S1–MgADP,P has not previously been determined systematically, but combined measurements at isolated ionic strengths (Katoh et al., 1984; Furukawa & Arata, 1984) are consistent with the data in Figure 1. Slightly smaller values for K_a, but a similar ionic strength dependency, can be demonstrated by comparing values for regulated F-actin binding to S1–MgADP,P in the presence of Ca^{2+} at different ionic strengths (Chalovich et al., 1981; Wagner & Giniger, 1981; Chalovich & Eisenberg, 1982). The increase in F-actin affinity at zero ionic strength is 10^2-fold for going from S1–MgADP,P to S1–MgADP, and at physiological ionic strength is almost 10^4-fold (Figure 1) because of the much larger value of $|z_M z_A|$ for the Mg–ADP,P case.

Table I: F-Actin Binding to S1–MgADP and S1–MgADP,P[a]

ligand	$K(0)$ (M^{-1})	$\lvert z_M z_A \rvert$ (esu^2)	$\Delta G'^\circ$ (kcal/mol)	ΔE^{zz} (kcal/mol)
MgADP	$(5 \pm 3) \times 10^7$	7 ± 2	−10.4	−3.4
MgADP,P	$(1 \pm 0.6) \times 10^6$	17 ± 2	−8.1	−8[b]

[a] The values for $K(0)$, the apparent association constant at 0 M ionic strength, and $|z_M z_A|$, the absolute value of the product of the net effective electric charges of the actin binding site on S1, z_M, and the myosin binding site on actin, z_A, were calculated from the best fits of the data in Figure 1 to eqs 4 and 5. The standard free energy, $\Delta G'^\circ = RT \ln K(0)$, and the electrostatic contribution, $\Delta E^{zz} = |z_M z_A| e^2/\epsilon 4\pi r^2$, were determined from $K(0)$ and $|z_M z_A|$. [b] The distance, r, between z_M and z_A was set at 0.825 nm to make $\Delta E^{zz} = \Delta G'^\circ$ for the MgADP,P case and allow comparison to the MgADP case. The true value of r is unknown.

It is noteworthy that the electrostatic contribution to the free energy of binding decreases as the binding becomes stronger. At zero ionic strength as the free energy of binding, $\Delta G'^\circ$, increases from 8.1 to 10.4 kcal/mol, the electrostatic energy of binding, ΔE, decreases by more than a factor of 2, assuming that the separation, r, and the dielectric constant, ϵ, between z_M and z_A do not change (Table I). Unlike the increase in F-actin affinity for the S1–MgADP to S1 step, which can be accounted for quantitatively by the observed increase in $|z_M z_A|$ (Highsmith, 1990) and probably reflects modest structural changes at the interface, the larger increase in affinity for the S1–MgADP,P to S1–MgADP step appears to require substantial structural change. For the latter case, the net effective electric charges must decrease as the distance between them decreases and/or nonelectrostatic interactions increase.

If this MgADP,P to MgADP-induced transition consists of a transformation of a single interface, then z_M must be reduced by a structural rearrangement that removes positive or introduces negative charge, as z_A is unchanged. At the same time, to account for tighter binding, increased hydrogen bond formation and/or van der Waals contacts need to occur. There is evidence supporting the conclusion that a nucleotide-induced transformation of the actin binding involves electrostatic changes. Chemical cross-linking of acto–S1 indicates that the interface contains electric charge (Mornet et al., 1981; Sutoh, 1983). The sign for z_M is certainly positive, on the basis of amino acid analysis after cross-linking (Yamamoto, 1988) and the covalent attachment of a complementary anionic oligopeptide to S1 which blocks actin binding (Chaussepied & Morales, 1988). It has been shown that changes in the structure of the nucleotide change the actin binding site positive charge distribution (Bertrand et al., 1989; Highsmith, 1990) and that these changes may be localized to S1 heavy-chain residues 633–642 (Yamamoto, 1991). These results suggest that an actin binding site transformation is occurring. The increase in the number of actin-cross-linked positively charged S1 amino acid side chains when MgADP,P is replaced by MgADP (Yamamoto, 1989) suggests that the reduced charge (Table I) is due to the introduction of negative charge into the actin binding site, rather than the removal of positive charge.

On the other hand, instead of a transformation of the interface, it is possible that there is a translocation from a first acto–S1–MgADP,P interface to a second acto–S1–MgADP interface. Two interfaces have been suggested (Mornet et al., 1981; Katoh et al., 1984; Arata, 1986; Suzuki et al., 1987). It has been shown that oligopeptides or antibodies that are electrically and structurally complementary to the highly positively charged actin binding site on S1 (Chausspied &

Morales, 1988) or the highly negatively charged myosin binding site on actin (Mejean et al., 1986; Miller et al., 1987) interfere with F-actin binding to S1–ATP but not to S1, which supports a model of acto–S1 interaction with two interfaces. If there are two interfaces, the second stronger, less charged actin binding site on myosin needs to be inaccessible or nonexistent for S1–MgADP,P which suggests that even if translocation is occurring as acto–S1–MgADP,P progresses to acto–S1–MgADP, a substantial transformation of the stronger binding site occurs. It is possible that the weakly binding chemically cross-linked S1 (Reisler et al., 1974) and the hydrodynamically more compact S1–MgADP,V (Highsmith & Eden, 1990) are manifestations of the S1 structure that has the second actin binding site inaccessible.

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Specific Cross-Linking of the SH1 Thiol of Skeletal Myosin Subfragment 1 to F-Actin and G-Actin†

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ABSTRACT: Recently, we reported that (maleimidobenzoyl)-G-actin (MBS-G-actin), which was resistant to the salt and myosin subfragment 1 (S-1) induced polymerizations, reacts reversibly and covalently in solution with the S-1 heavy chain at or near the strong F-actin binding region [Bettache, N., Bertrand, R., & Kassab, R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6028–6032]. Here, we have readily converted the MBS-G-actin into MBS-F-actin in the presence of phalloidin and salts. The binding of S-1 to the two actin derivatives carrying on their surface free reactive maleimidobenzoyl groups was investigated comparatively in cross-linking experiments performed under various conditions to probe further the molecular structure of the actin–heavy chain complex before and after the polymerization process. Like MBS-G-actin, the isolated MBS-F-actin, which did not undergo any intersubunit cross-linking, bound stoichiometrically to S-1, generating two kinds of actin–heavy chain covalent complexes migrating on electrophoretic gels at 180 and 140 kDa. The relative extent of their production was essentially dependent on pH for both G-and F-actins. At pH 8.0, the 180-kDa species was predominant, and at pH 7.0, the amount of the 140-kDa adduct increased at the expense of the 180-kDa entity. The cross-linking of MBS-F-actin to S-1 led to the superactivation of the MgATPase substantiating the ability of this derivative to stimulate the S-1 ATPase as the native protein. The 140-kDa complex was suppressed by blocking Cys-707 (SH1) in S-1 but not at all by the specific modification of Cys-697 (SH2). The addition of Mg (1 mM) increased selectively the yield of the 140-kDa product with both native S-1 and SH2-blocked S-1. The cross-linking between the MBS-actins and SH1-modified S-1 in the presence of MgADP did not yield the 140-kDa species. The cross-linking of the MBS-actins to fluorescently labeled split S-1 showed the conjugation of actin to the 50-kDa fragment in the 180-kDa species and to the 20-kDa fragment in the 140-kDa derivative. The data suggest that in the G- and F-MBS-actin–S-1 complexes the cross-linkable lysine side chain on actin to which the maleimidobenzoyl arm was attached is within 0.9–1.0 nm from two different S-1 heavy-chain segments, one of which includes the SH1 thiol; these may be spatially related, forming together an unique actin recognition site in S-1.

Previously, we have described for the first time the production and some of the main structural and functional features of (maleimidobenzoyl)-G-actin (Bettache et al., 1989, 1990). This derivative, which results from the reaction of skeletal G-actin with (*m*-maleimidobenzoic acid) *N*-hydroxysuccinimide ester, contains few intramolecular cysteine–lysine cross-links which stabilize the monomeric form of actin against the salt and myosin S-1[1] induced polymerizations. This peculiarity has permitted the initiation of solution studies on the reversible complexes formed between G-actin and the two-skeletal S-1 isoenzymes (Bettache et al., 1990). The MBS-G-actin seemed to represent a useful tool to gain further insights into the mechanism of the actomyosin interaction. Most of our observations have been confirmed by a recent work of Arata (1991) conducted along similar lines of investigations. In addition, more recently, Miki and Hozumi (1991) described the ability of MBS-G-actin to polymerize in the presence of phalloidin and analyzed the activation of S-1 by the resulting MBS-F-actin as well as the regulation of the MBS-F-actin–S-1 complex by the tropomyosin–troponin system.

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[1] Abbreviations: S-1, myosin subfragment 1; acto-S-1, actomyosin subfragment 1; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; $NaDodSO_4$, sodium dodecyl sulfate; ATPase, adenosine-5'-triphosphatase; DTE, dithioerythritol; MBS, (*m*-maleimidobenzoic acid) *N*-hydroxysuccinimide ester; MBS-actin, (maleimidobenzoyl)actin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid MOPS, 3-(*N*-morpholino)propanesulfonic acid; AN-S-1, anthroyl-S-1; ANN, 9-anthroylnitrile; EDANS, *N*-(5-sulfo-1-naphthyl)ethylenediamine; MTMR, tetramethylrhodamine-5-(and -6)-maleimide; pPDM, *N,N'*-(1,4-phenylene)dimaleimide.

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