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Regulatory light-chain Cys-55 sites on the two heads of myosin can come within 2Å of each other

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The di-thiol reagent, 5,5′-dithiobis (2-nitrobenzoic acid) is shown to induce disulfide bond formation between *Mercenaria* regulatory light-chain Cys-55 sites on either head of scallop hybrid myosin. This indicates that these two sites on opposite heads of myosin can come within 2Å of each other and this confirms a prediction based on earlier data [Chantler, Tao and Stafford (1991) Biophys. J. 59, 1242–1250]. Results demonstrate that myosin heads in solution show a considerable mutual freedom of movement which can be monitored by probes in the vicinity of regulatory light-chain residue 55. Implications for light-chain movement on the myosin head are discussed.

Myosin; Light-chains; Crosslinking; 5,5′-Dithiobis(2-nitrobenzoic acid); Disulfide

1. INTRODUCTION

The construction of hybrid myosins has been a particularly useful approach to obtaining information concerning myosin-linked regulation, head–head interactions and proximity relationships [1–7]. The natural location of sulfhydryl groups in the primary sequence of myosin regulatory light-chains is variable. Of those hybrids constructed to date possessing foreign regulatory light-chains, it is the *Mercenaria* regulatory light-chain that possesses a sulfhydryl group closest to the N-terminus, at position 55 [8]. Scallop hybrid myosins possessing *Mercenaria* regulatory light-chains modified specifically at Cys-55 have been used in cross-linking [3–5] and fluorescence resonance energy transfer (FRET) [2,16] studies. Steady-state FRET studies suggested that the mean distance between these Cys-55 sites on the two heads of myosin is >50 Å [2]. Nevertheless, 4,4′-dimaleimidylstilbene-2,2′-disulfonic acid (DMSDS) could cross-link this same pair of translationally equivalent sites with high efficiency [3] indicating that the sites can come within 18 Å of each other. The low efficiency of energy transfer between these sites, as observed by time-resolved fluorimetry [2], indicates that these probes spend a small but finite percentage of time in close proximity with each other.

All of these observations were successfully integrated into a single theory which demonstrated that there was no real contradiction between a low probability of energy transfer and a high efficiency of protein cross-linking between probes attached to Cys-55 of the *Mercenaria* regulatory light-chain on either myosin head [10]. The theory was a general one, but it further predicted that the two Cys-55 sites, on either myosin head, had a finite probability of coming within 2Å of each other. The thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) is known to promote disulfide bond formation in favorable cases [9,14]; when this occurs it indicates that the two cross-linked thiol groups can come within 2Å of each other. Here, we have investigated whether DTNB facilitates cross-linking between the two *Mercenaria* regulatory light-chain Cys-55 residues, one on each head of scallop hybrid myosin.

2. MATERIALS AND METHODS

Myosin regulatory light-chains, scallop myofibrils, myosin and hybrid myosins were all prepared by procedures described in earlier publications [1–3,11,12]. Western blots using polyclonal anti-light-chain antibodies were performed as described earlier [3]. The secondary antibody used in these studies was peroxidase-conjugated swine anti-rabbit IgG (Accurate Chemicals).

DTNB (Calbiochem) was made as a 0.1 M stock in 5 mM sodium phosphate, pH 7.0, taking all precautions as described by Lehrer [9] so as to ensure that the pH did not rise above 7.5 during dissolution. DTNB-induced cross-linking was performed on hybrid myosin (1 μM) for 4 h in 0.6 M NaCl, 10 mM phosphate, 2 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM $CaCl_2$, pH 7.0, with gentle agitation, prior to termination by the addition of iodoacetic acid (IAA) to 2 mM. Material was allowed to stand for 15 min prior to dialysis against the above buffer so as to remove excess IAA. Aliquots were lyophilyzed prior to SDS/

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Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DMSDS, 4,4′-dimaleimidylstilbene-2,2′-disulfonic acid; BPM, benzophenone-4′-maleimide; EGTA, ethylene glycol bis(aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; IAA, iodoacetic acid; FRET, fluorescence resonance energy transfer

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acrylamide (15%) gel electrophoresis (in the absence of reducing agent) and immunoblotting. *Mercenaria* regulatory light-chains (10 μM) were similarly modified in the same buffer as for myosin, both in the presence and absence of 2% SDS.

3. RESULTS AND DISCUSSION

In order to assess whether the two Cys-55 residues of the *Mercenaria* regulatory light-chain, on either head of hybrid myosin, can get within 2Å of each other, hybrid myosin was treated with DTNB under conditions described in Materials and Methods. Fig. 1 illustrates results from non-reducing SDS/acrylamide gels and shows that a four hour incubation of hybrid myosin with DTNB produces regulatory light-chain homodimers, approximately 10–15% of regulatory light-chains being converted into dimers during this period. DTNB promotes disulfide formation through a two-step sulfhydryl–disulfide exchange reaction via an intermediate possessing a single covalently bound 2-nitro-5-thiobenzoate (NbS) moiety [9]. The apparent decline in dimer formation with increasing concentrations of DTNB (Fig. 1) is due to the increased probability of bound NbS being found at *both* sulfhydryl sites, thereby blocking dimer formation.

One must be careful to rule out the possibility that dimers are formed because of trivial reasons. For example, although IAA will quench unreacted sulfhydryl residues, bound NbS groups will survive and remain on a subpopulation of light-chains, potentially initiating dimer formation after dissociation from the heavy chain during the preparation and execution of non-reducing SDS/acrylamide gel electrophoresis. To negate this hypothesis, purified *Mercenaria* regulatory light-chains were subjected to DTNB modification, both in the absence and presence of 2% SDS, the latter to simulate conditions during sample preparation for electrophoresis. In either condition minimal dimer formation was observed, even at a five-fold higher concentration of light-chain than was possible in the myosin experiment (Fig. 2). This substantiates our interpretation that dimer production in the hybrid myosin experiment (Fig. 1) represents intramolecular events occurring in solution on the intact myosin molecule and is of non-trivial origin.

The myosin results seen in Fig. 1, together with the control light-chain data shown in Fig. 2, demonstrate that DTNB can promote disulfide bridging between *Mercenaria* regulatory light-chain Cys-55 residues, one on either head of the hybrid myosin molecule. Disulfide bond formation indicates that these residues can come within 2Å of each other. The two heads of myosin therefore show considerable mutual flexibility in this region where Cys-55 is located. Quantitative analysis of dimer production suggests that our practical results are in harmony with theory. A 10–15% yield of regulatory light-chain homodimers in a four hour period corresponds to a pseudo-first-order rate constant in the range $7.3–11.0\times10^{-6}$ s^{-1}. Using the differential probability distribution functions shown in Fig. 6 of Chantler et al.

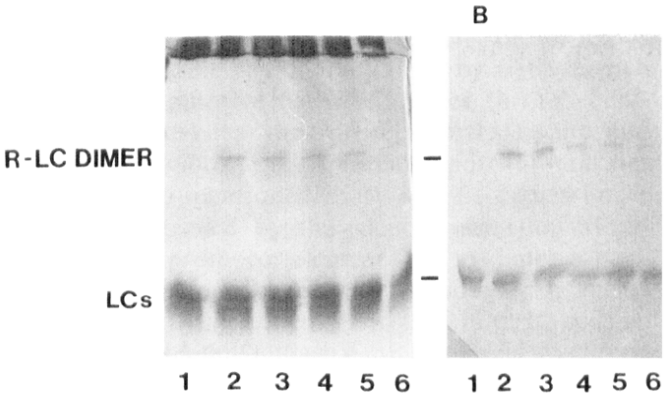

Fig. 1. Reaction of DTNB with scallop hybrid myosin possessing *Mercenaria* regulatory light-chains. Hybrid myosin was incubated with various concentrations of DTNB for 4 h in 0.6 M NaCl, 10 mM phosphate, 2 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM $CaCl_2$, pH 7.0 prior to termination of the reaction by addition of iodoacetic acid to 2 mM. [Myosin] = 1 μM. Aliquots were dialyzed against the above buffer then lyophilyzed prior to SDS/acrylamide (15%) gel electrophoresis (in the absence of reducing agent) and immunoblotting. Lanes 1 through 6 contain aliquots of myosin reacted with 0, 0.1, 0.2, 0.5, 1.0 and 2.0 mM DTNB, respectively. (A) Coomassie-blue stained gel. (B) Immunoblot of a sister gel of (A). Primary antibody is rabbit anti-*Mercenaria* regulatory light-chain IgG (1:250 dilution).

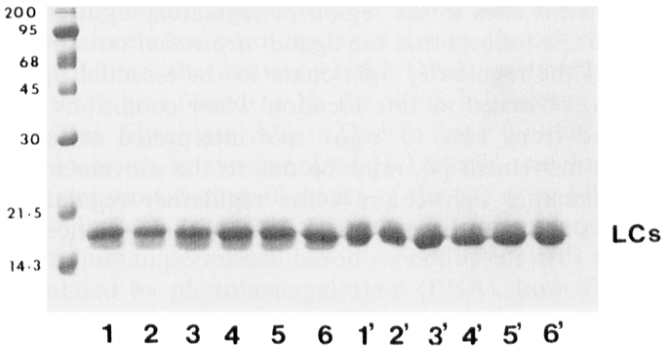

Fig. 2. Reaction of DTNB with *Mercenaria* regulatory light-chains, in the presence and absence of SDS. *Mercenaria* regulatory light-chains were incubated with various concentrations of DTNB for 4 h in 0.6 M NaCl, 10 mM phosphate, 2 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM $CaCl_2$, pH 7.0, ±2% SDS, prior to termination of the reaction by addition of iodoacetic acid to 2 mM. [Regulatory light-chain] = 10 μM. Aliquots were dialyzed against the above buffer then lyophilyzed, prior to SDS/acrylamide (15%) gel electrophoresis (in the absence of reducing agent) and staining with Coomassie blue. Lanes 1 through 6 contain aliquots of light-chain reacted with 0, 0.1, 0.2, 0.5, 1.0 and 2.0 mM DTNB, respectively, in the presence of 2% SDS; lanes 1' through 6' contain aliquots of light-chain reacted with 0, 0.1, 0.2, 0.5, 1.0 and 2.0 mM DTNB, respectively, in the absence of SDS. A lane of protein standards is seen on the LHS. One note of caution: these results were obtained on freshly purified material that had been fully reduced with 15 mM β-mercaptoethanol, prior to exhaustive dialysis to remove the reducing agent. When frozen stocks of light-chain were used, the same protocol did not work as well: non-reducing gels demonstrated the presence of dimer bands in *all* samples – including control samples that had never been exposed to DTNB.

1991 [10], together with an estimation of the rate of the second order reaction of DTNB with sulfhydryl groups (400 M^{-1} s^{-1} for DTNB and β-mercaptoethanol at pH 7.0 [15]), the calculated pseudo first order rate constant for disulphide formation between Cys-55 residues on either myosin head is in the range 2.9–47.0×10^{-6} s^{-1}. The measured rates are clearly within the theoretical range and emphasize the predictive value of our earlier treatment [10].

The photolabile cross-linker, BPM, linked at one end to the same light-chain Cys-55 residue in the hybrid myosin we have employed, has been shown to give rise to regulatory light-chain dimers in a nucleotide-independent manner [4,5]. Although the exact site of attachment at the photolabile end was never determined, our current results would suggest that attachment in the vicinity of the translationally equivalent site on the other head is plausible – for we have now bracketed the BPM cross-linking distance (9 Å span) by two other sulfhydryl-specific cross-linkers: DTNB (2Å span) (this paper) and DMSDS (18 Å span) [3]. Furthermore, two-dimensional SDS/ acrylamide gels of BPM and DMSDS cross-linked material, subjected to digestion by *S. aureus* V8 protease in the second dimension, appeared to give rise to identical cleavage products, when analysed by Western blotting using anti-*Mercenaria* regulatory light-chain IgG as primary antibody (Chantler, P.D. and Bower, S.M., unpublished observations). Given that BPM can cross-link translationally equivalent sites in the region of regulatory light-chain Cys-55, it follows that the ligand- *dependent* cross-linking of the regulatory light-chain to the essential light-chain, observed at this location when conditions are altered from 'rest' to 'rigor' and interpreted as light-chain movement [4], must be due to the movement of the essential light-chain – for regulatory–regulatory dimers are produced independent of the presence or absence of calcium and ATP [4]. This interpretation was suggested earlier by others [4,5] and is substantiated further here.

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REFERENCES

[1] Sellers, J.R., Chantler, P.D. and Szent-Gyorgyi, A.G. (1980) J. Mol. Biol. 144, 223–245.
[2] Chantler, P.D. and Tao, T. (1986) J. Mol. Biol. 192, 87–99.
[3] Chantler, P.D. and Bower, S.M. (1988) J. Biol. Chem. 263, 938–944.
[4] Hardwicke, P.M.D., Wallimann, T. and Szent-Gyorgyi, A.G. (1983) Nature 301, 478–482.
[5] Hardwicke, P.M.D. and Szent-Gyorgyi, A.G. (1985) J. Mol. Biol. 183, 203–211.
[6] Reinach, F.C., Nagai, K. and Kendrick-Jones, J. (1986) Nature 322, 80–83.
[7] Kendrick-Jones, J., Szentkiralyi, E.M. and Szent-Gyorgyi, A.G. (1976) J. Mol. Biol. 104, 747–775.
[8] Barouch, W.W., Breese, K.E., Davidoff, S., Leszyk, J., Szent-Gyorgyi, A.G., Theibert, J.L. and Collins, J.H. (1991) J. Musc. Res. Cell Motil. 12, 321–332.
[9] Lehrer, S.S. (1975) Proc. Natl. Acad. Sci. USA 72, 3377–3381.
[10] Chantler, P.D., Tao, T. and Stafford III, W.F. (1991) Biophys. J. 59, 1242–1250.
[11] Chantler, P.D. and Szent-Gyorgyi, A.G. (1978) Biochemistry 17, 5440–5448.
[12] Chantler, P.D. and Szent-Gyorgyi, A.G. (1980) J.Mol. Biol. 138, 473–492.
[13] Collins, J.H., Jakes, R., Kendrick-Jones, J., Leszyk, J., Barouch, W., Theibert, J.L., Spiegel, J. and Szent-Gyorgyi, A.G. (1986) Biochemistry 25, 7651–7656.
[14] Wells, J.A. and Yount, R.G. (1980) Biochemistry 19, 1711–1717.
[15] Means, G.E. and Feeney, R.E. (1971) in: Chemical Modification of Proteins, pp. 105–138, Holden-Day Inc., San Francisco, CA.
[16] Park, H-S., Tao, T. and Chantler, P.D. (1991) Biochemistry 30, 3189–3195.