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THE REACTIVE SH_1 AND SH_2 CYSTEINES IN MYOSIN SUBFRAGMENT 1 ARE CROSS-LINKED AT SIMILAR RATES WITH REAGENTS OF DIFFERENT LENGTH

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SUMMARY: The rate of cross-linking the SH_1 and SH_2 residues in S-1 has been examined for several reagents which were recently shown to link these thiols with concomitant trapping of nucleotide at the protein's active site (Wells et al., (1980) J. Biol Chem. 255, 11140). Similar cross-linking rates were observed with naphthalene-1,5-dimaleimide, N,N'-p-phenylene dimaleimide, N,N'-o-phenylene dimaleimide, 4,4'-difluoro-3,3'-dinitrophenyl sulfone, and Co(II)/Co(III)-phenanthroline, both in the presence of MgADP or MgATP, at 0^{o} and 25^{o}C. These results suggest that the mobile region in S-1 coincides or communicates with the SH_1-SH_2 peptide.

INTRODUCTION

Most models for muscle contraction utilize the flexible properties of the myosin S-1/S-2 swivel and the HMM/LMM hinge in order to explain the mechanism of force generation. In addition to these regions, a significant portion of the myosin head, perhaps as much as 20%, was found in recent NMR studies to have a mobile character (1,2). These internal motions in S-1 are not affected by the binding of nucleotides, but are almost totally quenched by actin (1,2). Thus, it appears that the nucleotide binding site is located in a nonflexible area of the molecule, and the mobile region either interacts or coincides with the actin binding site. According to Prince et al. (2), the 41-residue segment of the A1 light chain in S-1 (A1) contributes significantly to the overall mobility in the myosin head.

Chemical studies on S-1, and in particular the bifunctional cross-linkings of the reactive SH_1 and SH_2 cysteine groups, indicate that the 21K fragment, which contains these residues, may be involved in the internal motions in the myosin head. Notable in this context is the ability of both short (3) and long (3,4) reagents to

The abbreviations used are: pPDM, N,N'-p-phenylene dimaleimide; oPDM, N,N'-o-phenylene dimaleimide; NDM, naphthalene-1,5-dimaleimide; F_2DPS, 4,4'-difluoro-3,3'-dinitrophenyl sulfone; phen, 1,10-phenanthroline; DTE, dithioerythritol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); S-1, subfragment 1

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bridge the SH_1 and SH_2 groups. Such bridging is facilitated in the presence of nucleotides, which in the process become non-covalently trapped at the protein's active site (5,6).

In the present study we have examined the rates of bridging the SH_1 and SH_2 groups with reagents of differing cross-linking span at 0° and 25°, and in the presence of MgADP and MgATP. In all cases, the cross-linking rates were similar, indicating segmental mobility of the SH_1-SH_2 region.

METHODS

α-chymotrypsin, ATP, ADP, and dithioerythritol were obtained from Sigma Chemical Co. [^{3}H]ADP was from New England Nuclear. o-PDM, p-PDM and 1,10-phenanthroline were from Aldrich, and NDM was from ICN. $[Co(III)(phen)_2CO_3]^+$ was a generous gift from Dr. R. G. Yount. F_2DPS was purchased from Pierce Chemical Co.

Myosin from rabbit skeletal muscle and S-1 were prepared as described previously (7).

Inactivations of S-1 were carried out in 30mM KCl, 25mM Tris, pH 8.0, at 0° and 25°. S-1 was present at a concentration of 1.5 to 2.0 mg/mL, MgADP or MgATP were present at either 5.0 or 6.8mM, and the molar excess of pPDM, oPDM, NDM, and F_2DPS over S-1 was 1.2:1. The reaction of S-1 with $[Co(III)(phen)_2CO_3]^+$ employed a 100-fold excess of this reagent over protein and a 10-fold excess of Co(II)phen. Inactivation reactions were started by addition of the reagents (in dimethyl formamide or in water) to a protein solution containing the Mg-nucleotide complex. The dimaleimide reactions were terminated by removing aliquots to receiver vessels containing 25mM EDTA, and 2mM DTE. The F_2DPS reaction was essentially stopped by dilution, and the $[Co(III)(phen)_2CO_3]^+$ inactivation was stopped with 25mM EDTA.

Reaction of S-1 with the bifunctional reagents was monitored by following the loss of EDTA (K^+) ATPase and CaATPase activities as a function of reaction time (up to 60 min). The loss of EDTA (K^+) ATPase activity reflects modification of SH_1 residues, whereas the loss of CaATPase is due to the intramolecular SH_1 to SH_2 bridging (3,8). $Ca^{2+}$and EDTA ATPase measurements were carried out at 37° as described previously (9). Trapping of Mg[^{3}H]ADP at the active site of S-1 was verified by the procedures of Wells et al., (5,6).

The results of activity measurements were analyzed as follows. Initial EDTA ATPase activity losses (in the first 5 to 7 min of the reaction), which are due to SH_1 modification at close to stoichiometric reagent-to-protein ratios, were analyzed in terms of second order process to yield the rate constants, k_E, and half-times, $t_{1/2}(E)$. Initial CaATPase activity loss was assumed to follow first order kinetics, and the linear semilogarithmic plots of activity vs. time were used to derive the first order rate constants, k_C, and half times, $t_{1/2}(C)$. In order to compare the rates of SH_1 to SH_2 bridging by different reagents, we employed the ratios of half-times, $t_{1/2}(C)/t_{1/2}(E)$. In this way, the rates of the secondary reactions, the cross-linking of SH_1 to SH_2, are corrected for the somewhat different reactivities of the various reagents towards the SH_1 residues.

RESULTS AND DISCUSSION

The recent peptide work of Burke and Knight (10) has shown that the bifunctional thiol reagent, pPDM, indeed cross-links the SH_1 and SH_2 residues on myosin. The previous circumstantial evidence for this mechanism included the characteristic changes in the Ca^{2+} and EDTA (K^+) activated ATPases of S-1 (3,4,8) and the trapping of MgADP on the protein. Other bifunctional thiol reagents were assumed to react in

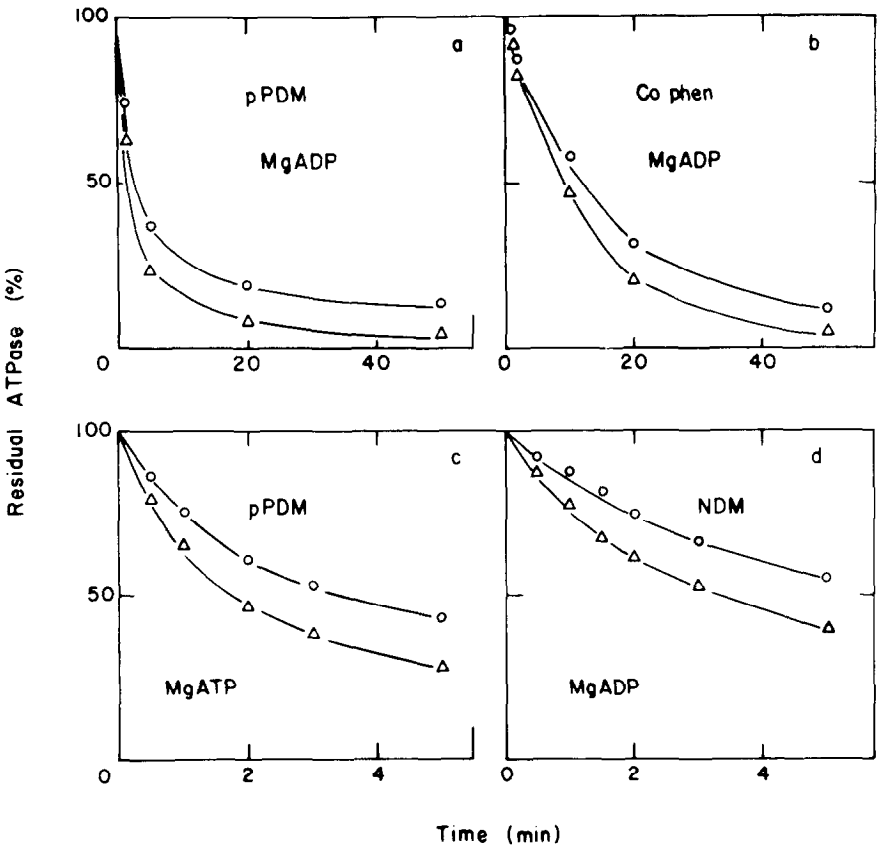

Fig. 1. Inactivation of EDTA ATPase (△) and CaATPase (○) activity of S-1 by pPDM, Co phen and NDM. The modifications were carried out in 30mM KCl, 25mM Tris, pH 8.0 in the presence of 5mM MgADP (a,b,d) or 5mM MgATP (c), at either 0° (a,b,c) or 25° (d). Protein and reagent concentrations were as described in the Methods. Rate constants for SH_1 modification and SH_1-SH_2 bridging were obtained from the initial inactivation rates (c,d) as described in the Methods.

the same manner with the SH_1 and SH_2 groups primarily on the basis of similar reaction stoichiometries, activity changes, and nucleotide trapping (3,7).

The thiol cross-linking on S-1 proceeds in two steps. The initial reaction, modification of the SH_1 groups, is characterized by the loss of the EDTA ATPase and elevation of the CaATPase activities. The subsequent bridging to the SH_2 groups is accompanied by inactivation of the CaATPase of S-1 (4,8). For fast bridging reactions, particularly those occurring at pH 8.0, the rates of the primary and secondary reactions are close enough to mask the kinetic evidence for the above sequence of events. Instead of transient elevation in the CaATPase activity, one observes in such reactions that the loss in this activity lags behind the loss in the EDTA ATPase of S-1. Typical time profiles of S-1 inactivation with thiol

Table 1. REDUCED BRIDGING RATES OF SH_1 AND SH_2 GROUPS IN S-1.

Reagent	Cross-linking span, A	Ligand	Temp.	Reduced bridging rate, $t_{1/2}^{(C)}/t_{1/2}^{(E)}$
NDM	13-14	MgADP	0°	1.6±0.3
naphthalene		MgADP	25°	1.3±0.3
dimaleimide		MgATP	0°	1.5±0.4
		MgATP	25°	1.0
pPDM	12-13	MgADP	0°	1.3±0.3
p-phenylene		MgADP	25°	1.8±0.4
dimaleimide		MgATP	0°	1.4±0.4
F_2DPS	10	MgADP	0°	1.4±0.4
oPDM	4-9	MgADP	0°	1.6±0.3
o-phenylene		MgADP	25°	1.5±0.4
dimaleimide		MgATP	0°	1.3±0.3
		MgATP	25°	1.0
Co phen	3-5	MgADP	0°	1.4±0.4
		MgADP	25°	1.0

All the modifications of S-1 with bifunctional reagents were carried out in 30mM KCl, 25mM Tris, pH 8.0 in the presence of 5mM Mg-nucleotide complexes. S-1 concentration was 1.5 to 2.0 mg/ml and the reagents were employed at 1.2:1 molar excess over the protein. $[Co(III)(phen)_2CO_3]^+$ and Co(II)phen were used at 100- and 10-fold molar excesses respectively. Half-times for the SH_1 modification $[(t_{1/2}^{(E)})]$ and the SH_1 to SH_2 bridging $[(t_{1/2}^{(C)})]$ were determined from the initial inactivation rates of the EDTA ATPase and CaATPase activities.

cross-linking reagents are shown in Fig. 1. The same activity decay curves were obtained for modifications carried out with all the reagents listed in Table I, in the presence of either MgADP (5mM) or MgATP (5mM), at 0° and 25°. The appropriate rate constants for SH_1 modification, bridging to SH_2, and the corresponding half-times of these reactions were calculated from the plots of initial activity loss [eq. Fig. 1 c,d] as described in the Methods. To correct for the different reactivities of various bifunctional reagents towards the reactive thiol groups, we present the bridging rates in a reduced form, i.e., as ratios of the half-times for

the loss of Ca^{2+} and EDTA ATPase activities (Table I). Although such procedure corrects mainly for the different reactivities towards the SH_1 groups, it appears to be adequate in view of the intramolecular nature of the secondary bridging reaction.

As shown in Table I, the reduced rates of bridging cluster around 1.4 for all the employed reagents with cross-linking spans between 3 and 14A. The same bridging rates are observed both at 0^{o} and $25^{o}C$, even though at the higher temperatures the primary modification at the SH_1 groups occurs at a faster rate. Similarly, in the presence of MgATP which reduces the rate of the SH_1 modification by a factor of two (at $25^{o}C$) the reduced bridging rate is close to 1.0.

At present, there are at least two examples for a very slow rate of SH_1-SH_2 bridging in the presence of MgADP. 1,5-difluoro-2,4-dinitrobenzene, which has a 5A cross-linking span, requires many hours to bridge to the SH_2 thiol, even though its monofunctional attachment to the SH_1 groups is quite rapid (3). However, this result is most likely due to the extremely low reactivity of the SH_2 residues towards the fluorodinitrobenzene moiety (11). In the case of a disulfide bond formation between the SH_1 and SH_2 groups in the reaction of DTNB with S-1, the loss of CaATPase is dramatically slower than the loss of EDTA ATPase activity (12). Yet, the interpretation of this result in terms of spatial limitations on the SH_1-SH_2 motions and proximity is unwarranted because of the complex mechanism of DTNB reaction with S-1 (12). This view is further supported by the fact that in a different disulfide yielding reaction, that of 5'-p-fluorosulfonylbenzolyadenosine with S-1, the rate of SH_1-SH_2 bridging is 1.4 (13), i.e., the same as for other reagents tested in this work.

To the extent that the ability of reagents of different cross-linking span to bridge the SH_1 and SH_2 groups is due to the internal motions in the myosin head, our results indicate that such motions are little affected by the particular kinetic state of S-1 (MADP or M**ADPP). This observation is consistent with the NMR studies (1,2), and further suggests that the mobile region in S-1 may coincide, overlap, or communicate with the SH_1-SH_2 peptide on the 21k fragment rather than with the nucleotide binding site. Although in principle the ability of different bifunctional thiol reagents to cross-link the SH_1 and SH_2 groups was established before

(3,4), the present kinetic analysis of the bridging reaction under various experimental conditions implicates more conclusively the 21k fragment in the internal motions in S-1. Our attempts to determine the effect of actin on the internal motions in this fragment by monitoring the rate of SH_1-SH_2 bridging in acto S-1 were hampered by the greater complexity of such a system, and in particular by the competitive reaction of actin with the bifunctional reagents (14).

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