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Reaction of 5,5′-Dithiobis(2-nitrobenzoic acid) with Myosin Subfragment One: Evidence for Formation of a Single Protein Disulfide with Trapping of Metal Nucleotide at the Active Site†

James A. Wells and Ralph G. Yount*

ABSTRACT: Treatment of rabbit skeletal myosin chymotryptic subfragment one (SF_1) in the presence of MgADP with a twofold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) at 0 °C, pH 8.0, results in complete ATPase inactivation. Inactivation occurs in two phases that ultimately result in the modification of three SH groups. In the first phase there is a rapid reaction ($t_{1/2} \sim 10$ min) of DTNB with two SH groups which leads to activation of the Ca^{2+}-ATPase and inactivation of the K^+-EDTA-ATPase. Only one of these fast-reacting SH groups is believed essential for activity. In the second phase ($t_{1/2} \sim 2$ h), a thionitrobenzoic acid (TNB) group blocking one of the fast-reacting SH groups is displaced by a neighboring thiol believed to be the critical thiol called SH-2 to form a cystine disulfide bond. This latter reaction resulted in the loss of all Ca^{2+}-ATPase activity with concomitant trapping of MgADP at the active site ($t_{1/2,\text{off rate}} \sim 6$ days). Treatment of fully inactivated SF_1 with dithioerythritol reduced the disulfide, reduced the remaining TNB-SF_1 mixed disulfide, and released MgADP with the full recovery of all ATPase activity. In this reaction the remaining TNB was released more rapidly than ATPase activity was recovered, suggesting it plays a noncritical role in the original inactivation. Prior treatment of SF_1 with DTNB in the presence of MgADP was found to protect against subsequent modification by *N,N′*-(*p*-phenylene)dimaleimide or cobalt phenanthroline complexes. These reagents which have cross-linking spans of 12–13 and 3–5 Å, respectively, cross-link two critical thiol groups per active site in myosin [Reisler, E., Burke, M., Himmelfarb, S., & Harrington, W. F. (1974) *Biochemistry 13*, 3837; Wells, J. A., Werber, M. M., & Yount, R. G. (1979) *Biochemistry 18*, 4800]. The apparent formation of a disulfide bond between the cross-linked SH groups means they can move as close as 2.0 Å from each other. This demonstrates the large range of movement in the myosin molecule in response to binding of magnesium nucleotide. Furthermore, these studies extend prior observations [Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966] that metal nucleotide is trapped at the active site during magnesium nucleotide stimulated inactivation by thiol cross-linking reagents.

Myosin and its active proteolytic subfragments double-headed heavy meromyosin and single-headed SF_1[1] are well-known to undergo spectroscopically sensitive conformational changes during the MgATP hydrolytic cycle [Werber et al., 1972; Morita, 1967; Murphy, 1974; Bagshaw & Trentham,

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[1] Abbreviations used: SF_1, chymotryptic subfragment one; pPDM, *N,N′*-(*p*-phenylene)dimaleimide; phen, 1,10-phenanthroline; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; DTE, dithioerythritol; AMP-PNP, adenyl-5′-yl imidodiphosphate; TNB-SF_1, SF_1 inactivated in the presence of MgADP and a twofold excess of DTNB as described under Materials and Methods; MalNEt, *N*-ethylmaleimide.

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1974; for reviews, see Trentham et al. (1976) and Taylor (1979)]. Until recently very little evidence has been provided to structurally define these conformational states of the myosin molecule.

Reisler et al. (1974) reported that two critical thiol groups of myosin, called SH-1 and SH-2, could be cross-linked 12–13 Å apart using the reagent pPDM.[2] Burke & Reisler (1977) later reported that these SH groups could be cross-linked by a 10-Å dithiol cross-linking reagent, 4,4′-difluoro-3,3′-dinitrobenzene sulfone. Since the rate of cross-linking in both studies was greatly stimulated by addition of MgADP, they proposed that these SH groups move toward each other upon nucleotide binding. Furthermore, it has been proposed that these cross-linked SH groups can approach within 3–5 Å of each other based on a report of their simultaneous chelation by a single exchange-inert Co(III) (Wells et al., 1979b). Recently, it was discovered that magnesium nucleotide stimulated cross-linking of thiols by cobalt phenanthroline complexes or pPDM led to stable stoichiometric trapping of a 1:1 Mg·nucleotide complex at myosin's active site (Wells & Yount, 1979). To account for these observations, it has been proposed that myosin contains a jawlike active-site structure which closes on the Mg·nucleotide complex, bringing together two critical SH groups to allow cross-linking by Co(III) or pPDM (Wells & Yount, 1979). Here, the possibility was investigated that these SH groups can approach close enough to form a disulfide bond (2 Å) in the presence of MgADP.

The thiol reagent DTNB is known in a number of cases to promote the formation of intramolecular disulfides in proteins (Carlson et al., 1978; Flashner et al., 1972; Wassarman & Major, 1969). DTNB has been previously employed by Seidel (1969) to modify SH-1 in the absence of MgADP by formation of an SH-1-TNB mixed disulfide. Evidence is presented here to suggest that a twofold excess of DTNB reacts rapidly in a specific manner with SF_1 in the presence of MgADP to create two TNB-SF_1 mixed disulfides: one with a critical thiol and another with a nonessential thiol. Subsequently, the TNB group attached to the critical thiol is believed to be displaced by a second critical thiol, yielding a cystine disulfide with concomitant trapping of Mg·nucleotide and loss of Ca^{2+}-ATPase activity. The properties of this inactive form of SF_1 are investigated, and the relationship of this modification to the well-known effects of modifying SH-1 and SH-2 is discussed.

Materials and Methods

Materials. [U-^{14}C]Na_4ATP, [2,8-^{3}H]Na_4AMP-PNP, and [^{14}C]KCN were from NEN, ICN, and Amersham, respectively. DTNB and DTE were from Pierce. Na_2ATP, Li_3ADP, and pPDM were from P-L Biochemicals, Schwarz/Mann, and Aldrich, respectively. Bisphenanthroline(carbonato)cobalt(III) was prepared essentially according to Ablov & Palade (1961) as described previously (Wells et al., 1979a). Ultrapure $(NH_4)_2SO_4$ was from Schwarz/Mann.

Enzyme Preparations. Rabbit skeletal myosin was prepared according to Wagner & Yount (1975) and was stored in 50% glycerol at −20 °C. Chymotryptic SF_1 was prepared according to Weeds & Taylor (1975).

Enzyme Inactivations. SF_1 (13–18 μM) was treated with a twofold molar excess of freshly prepared DTNB solution (1 mM DTNB and 10 mM phosphate, pH 7.0, at 4 °C) in 0.1 mM ADP or ATP, 0.1–20 mM $MgCl_2$, and KCl–Tris buffer (0.1 M KCl and 50 mM Tris, pH 8.0) at 0 °C. Inactivations which proceeded 24 h or more usually reduced the Ca^{2+}-ATPase and NH_4^+-EDTA-ATPase activities to less than 10% of that of control. Inactivations were quenched, and modified SF_1 was purified by four successive $(NH_4)_2SO_4$ precipitations [to 70% saturation, pH 8.0, by using Chelex-treated $(NH_4)_2SO_4$ solutions] or a single $(NH_4)_2SO_4$ precipitation

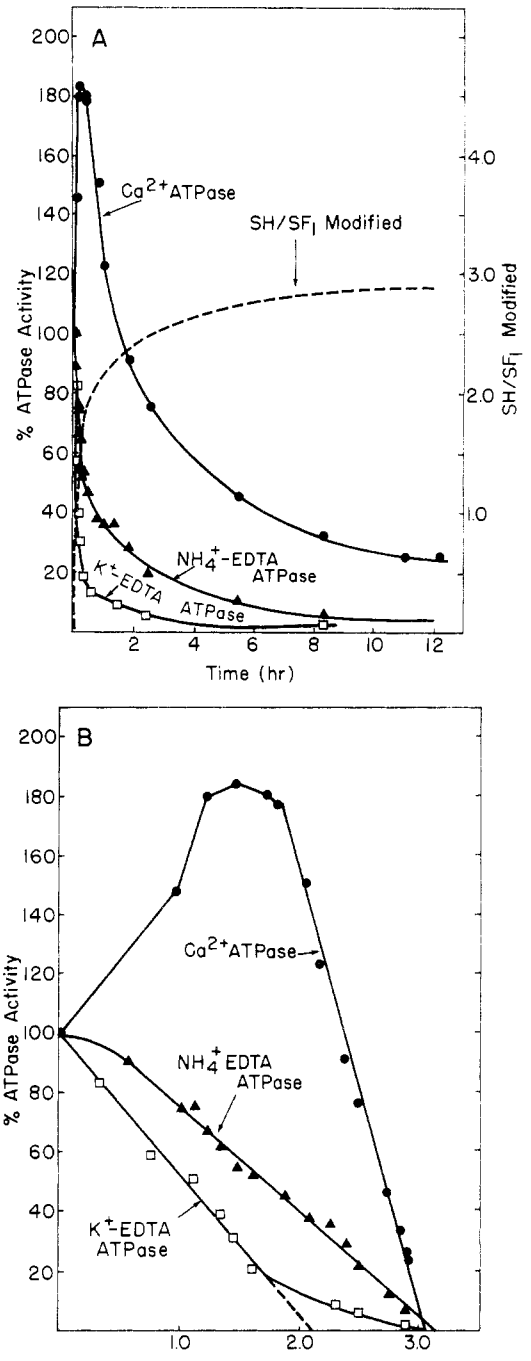


FIGURE 1: Panel A: Time course of the inactivation of the Ca^{2+}-ATPase (●), NH_4^+-EDTA-ATPase (▲), and K^+-EDTA-ATPase (□) activities as a function of SH groups modified per SF_1. SF_1 (16 μM) was inactivated at 0 °C in the presence of 0.1 mM ADP, 0.2 mM $MgCl_2$, and 32 μM DTNB. The absorbance at 412 nm was followed continuously in a Cary 14 recording spectrophotometer (see Materials and Methods) and was used to determine the SH/SF_1 modified (---). Panel B: The Ca^{2+}-ATPase (●), NH_4^+-EDTA-ATPase (▲), and K^+-EDTA-ATPase (□) activities are replotted as a function of SH/SF_1 modified from the data in panel A.

[2] The center to center distance between the cross-linked thiols was estimated from careful consideration of CPK space-filling models. The values we determined have a somewhat more restricted range than those reported by Burke & Reisler (1977).

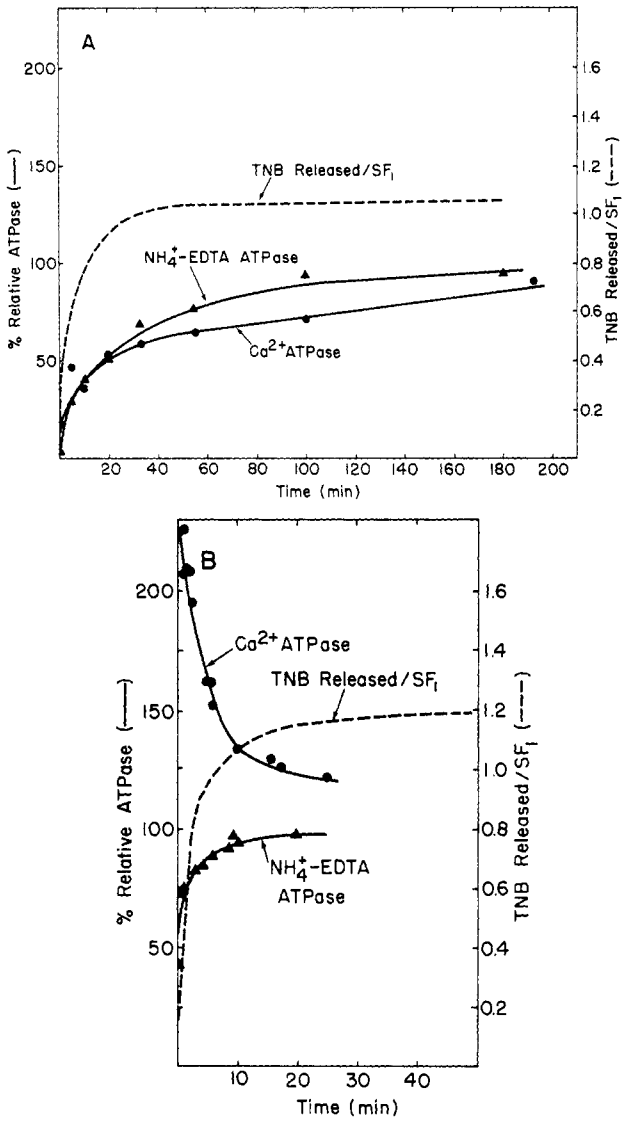


FIGURE 2: Dithioerythritol reactivation of DTNB-inactivated SF_1. Panel A: SF_1 (16 μM) was inactivated for 24 h in the presence of 0.1 mM MgADP as described in Figure 1. The enzyme was purified by $(NH_4)_2SO_4$ precipitation, followed by gel filtration as described under Materials and Methods. Purified DTNB-treated SF_1 (11 μM) was treated with 0.92 mM DTE at 0 °C while monitoring as a function of time the ATPase activities and TNB released per SF_1 (see Materials and Methods). Panel B: Same as (A) except SF_1 was treated with DTNB until the Ca^{2+}-ATPase was fully activated (~20 min). The inactivation was rapidly quenched by two sequential additions of an 80-fold weight excess over SF_1 of the chloride form of Dowex 1-X8 (100–200 mesh). Control experiments had shown the Dowex treatment was sufficient to remove greater than 98% of the unreacted DTNB or free TNB.

followed by passage over Sephadex G-25 (Pharmacia PD-10 columns) as described previously (Wells et al., 1979b). In some instances DTNB inactivations were rapidly quenched by batchwise treatment of inactivation mixtures with Dowex 1-X8 100–200 mesh (Bio-Rad; Cl^- form) which removed unreacted DTNB, TNB, and nucleotide.

Analytical Procedures. The change in absorbance at 412 nm, indicative of production of TNB (ϵ_{412nm} = 13600; Ellman, 1959), was followed with time of inactivation on a Cary-14 double-beamed spectrophotometer thermostated at 0.5 °C. Cobalt and magnesium were determined by atomic absorption using a Perkin-Elmer 360 atomic absorption spectrophotometer. Spectroscopically pure $CoSO_4 \cdot 7H_2O$ (Johnson Matthey Chemicals, Ltd.) and elemental magnesium (Mallinckrodt)

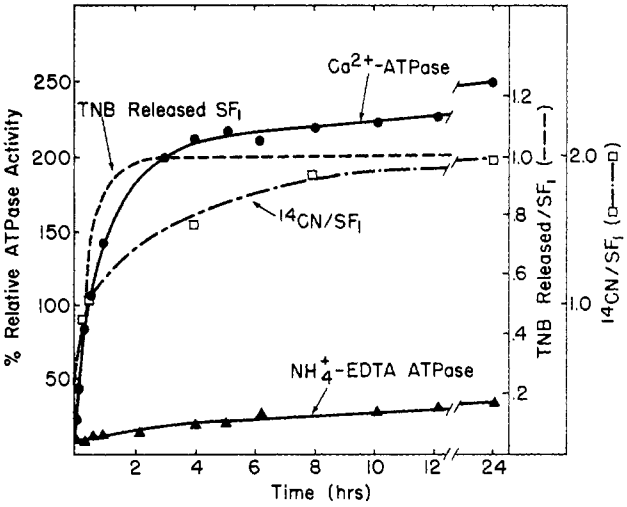


FIGURE 3: NaCN treatment of DTNB-inactivated SF_1. SF_1 (17 μM) was inactivated with 34 μM DTNB in the presence of 0.1 mM MgADP at 0 °C for 24 h, resulting in modification of 2.8 SH groups per SF_1. The enzyme was purified by $(NH_4)_2SO_4$ precipitation and gel filtration. Purified and inactivated SF_1 (12 μM) was treated with 12 mM freshly prepared NaCN at 0 °C and NH_4^+-EDTA-ATPase activity (▲), Ca^{2+}-ATPase activity (●), and production of TNB per SF_1 (---) was monitored with time. In a parallel experiment inactivated SF_1 was treated with $Na^{14}CN$ (6000 cpm/nmol), and at various times aliquots of the enzyme mixture were purified and ^{14}CN per SF_1 was determined (□).

dissolved in 1.0 N HCl were used as standards in atomic absorption measurements.

Ca^{2+}-ATPase and NH_4^+-EDTA-ATPase assays were performed as described previously (Wells et al., 1979b) except the release of inorganic phosphate was measured at 2 and 6 min after addition of SF_1 to the Ca^{2+}-ATPase mixture and 2 and 8 min after addition of SF_1 to the NH_4^+-EDTA-ATPase mixture. These times assured linearity of the ATPase assay over a more convenient range of enzyme specific activities. The K^+-EDTA-ATPase was measured in a manner similar to the NH_4^+-EDTA-ATPase except the assay mixture contained 0.6 M KCl, 5 mM ATP, 5 mM EDTA, and 50 mM Tris, pH 7.5, at 25 °C (Seidel, 1969). Protein concentrations were measured by a Coomassie blue dye binding assay (Bradford, 1976) as previously described (Wells et al., 1979a). Unmodified chymotryptic SF_1, used as the protein standard, was assumed to have a molecular weight of 120000 (Weeds & Taylor, 1975) and an $\epsilon_{280nm}^{1\%}$ = 7.5 cm^{-1} (Wagner & Weeds, 1977). Total thiol content was determined by DTNB analysis according to Ellman (1959) as described previously (Wells et al., 1979b).

Results

Preliminary determination of the reactivity of SF_1 thiol groups to titration with DTNB in the presence of MgADP indicated a twofold excess of DTNB was sufficient to inactivate SF_1-ATPase activity with subsequent modification of three SH groups. The kinetics of ATPase inactivation and SH-group modification by addition of a twofold molar excess of DTNB over SF_1 is shown in Figure 1A. Although the NH_4^+-EDTA- and the K^+-EDTA-ATPase activities were lost in a monotonic fashion, the K^+-EDTA-ATPase activity was lost more rapidly. This difference suggested the NH_4^+-EDTA-ATPase was less sensitive to the thiol modification than was the K^+-EDTA-ATPase. This suggestion was confirmed when SF_1 was treated with 1.4 excess *N*-ethylmaleimide at 0 °C in 0.1 M KCl and 50 mM Tris, pH 8.0, for 30 min and the K^+- and NH_4^+-

Table I: Protection of SF_1 against Cobalt Phenanthroline or pPDM Labeling by DTNB Pretreatment[a]

sample[b]	TNB[c] released	total[d] SH/SF_1	$\Delta(SH/SF_1)$[e]	Co/SF_1	% ATPase NH_4^+-EDTA	% ATPase Ca^{2+}
control		9.7 ± 0.5	0		100	100
TNB-SF_1	2.88 ± 0.11	7.2 ± 0.75	2.5 ± 0.4		3 ± 2	5 ± 3
TNB-SF_1 plus Co		6.4 ± 0.6	3.3 ± 0.4	0.20 ± 0.17	1 ± 0.6	9
TNB-SF_1 plus pPDM		6.4 ± 1.9	3.3 ± 1.5		0	0
TNB-SF_1 plus DTE	0.93 ± 0.08				105 ± 14	89 ± 3
(TNB-SF_1 plus Co) plus DTE	0.72				87 ± 14	80 ± 13
(TNB-SF_1 plus pPDM) plus DTE	0.73				93 ± 23	87 ± 24
Co·SF_1		7.3 ± 1.0	2.4 ± 0.7	1.65 ± 0.17	3 ± 1	2 ± 2
pPDM-SF_1		7.9 ± 0.1	1.8 ± 0.4		8 ± 6	9 ± 6

[a] Values reported are averages and standard deviations of three separate experiments on three different SF_1 preparations. [b] TNB-SF_1 was prepared by inactivating 17 μM SF_1 in 0.1 mM MgADP with a twofold excess of DTNB at 0 °C for 24 h. Co·SF_1 was prepared as previously described (Wells & Yount, 1979) by reacting 17 μM SF_1 with 0.17 mM Co(II)phen, 1.7 mM $[Co(III)(phen)_2CO_3]^+$, 0.1 mM ADP, and 20 mM $MgCl_2$ in KCl–Tris buffer at 0 °C for 8 min. pPDM-SF_1 was prepared by reacting 17 μM SF_1 with a 1.3-fold excess of pPDM (1 mM in acetone), 0.1 mM ADP, and 10 mM $MgCl_2$ in KCl–Tris buffer at 0 °C for 20 min. (TNB-SF_1 plus Co) and (TNB-SF_1 plus pPDM) were prepared by reacting purified TNB-SF_1 with cobalt phenanthroline complexes or pPDM as described above. DTE treatments of purified enzyme derivatives were carried out by reacting the enzyme derivative (13–18 μM) with an 85-fold excess of DTE in KCl–Tris buffer at 0 °C for 4 h. All enzyme derivatives were purified by $(NH_4)_2SO_4$ precipitation in the presence of excess EDTA, followed by Sephadex G-25 (PD-10) gel filtration as described under Materials and Methods. [c] Moles of TNB per SF_1 produced by reaction of SF_1 with a twofold excess of DTNB or by reactivating TNB–enzyme derivatives with DTE. [d] Total SH content determined by DTNB titration (see Materials and Methods). [e] Difference in SH content of SF_1 control and SF_1 derivative.

EDTA-ATPase activities were found to be 31 and 49%, respectively. The Ca^{2+}-ATPase activity increased nearly twofold during early DTNB modification and subsequently lost activity, showing that the DTNB modification consisted of two phases. The height of the transient Ca^{2+}-ATPase activation varied from 175 to 250% of the control in several trials on different SF_1 preparations. A replot of the percent ATPase activity vs. the moles of SH groups modified per SF_1 (Figure 1B) shows modification of approximately two SH groups was linearly related to inactivation of the K^+-EDTA-ATPase activity while modification of essentially three SH groups correlated with the inactivation of the NH_4^+-EDTA-ATPase activity. Attainment of maximal Ca^{2+}-ATPase activation required reaction with 1.3–1.8 SH groups (first phase) while subsequent inactivation required the further loss of ~1.2 SH groups (second phase). Three SH groups were finally modified, one in excess of the moles of DTNB added, substantiating that there was formation of one cystine disulfide and one enzyme–TNB mixed disulfide.

The modification of SF_1 by DTNB was fully reversible by DTE either at the stage where all Ca^{2+}-ATPase was lost (Figure 2A) or at an earlier stage where the Ca^{2+}-ATPase was optimally activated (Figure 2B). SF_1 was inactivated with a twofold excess of DTNB until the NH_4^+-EDTA-ATPase and Ca^{2+}-ATPase were 3 and 14%, respectively, and had 2.9 SH groups modified. When this enzyme was purified and treated with DTE (Figure 2A), 1.06 mol of TNB was rapidly released as expected for the fully inactive form of SF_1 believed to contain a single cystine disulfide and a single SF_1-TNB mixed disulfide. The NH_4^+-EDTA-ATPase and Ca^{2+}-ATPase activities returned roughly in parallel, although more slowly than the reduction of the SF_1-TNB mixed disulfide, suggesting this enzyme-bound TNB does not affect the activity significantly. The slower return of ATPase activity is presumably related to the slower reduction of the cystine disulfide.

The SF_1 derivative which had nearly maximal Ca^{2+}-ATPase activity (~20-min reaction time; 1.4 SH groups modified) was purified and treated with DTE. As can be seen in Figure 2B, this derivative released 1.2 mol of TNB per SF_1 while giving rapid concomitant recovery of normal Ca^{2+}-ATPase and NH_4^+-EDTA-ATPase activities. The number of TNB groups released (1.2) when compared with the number of SH groups lost (1.4) means only 10–20% cystine disulfides have formed at the end of the first phase of modification. It further demonstrates that the early effects seen (Ca^{2+}-ATPase activated, NH_4^+- and K^+-EDTA-ATPases inactivated) result from mixed TNB-SF_1 disulfides. In addition, data in panels A and B show that the rate of TNB release by DTE treatment from both the early and late forms of DTNB-modified SF_1 was the same while the recovery of normal ATPase activity was more rapid from the early form (Figure 2B) than the late and completely inactive form of enzyme (Figure 2A). This is further evidence that the DTNB promoted inactivation and subsequent DTE reactivation of the Ca^{2+}-ATPase resulted from first the formation and then the reduction of a cystine disulfide.

Cyanide is known to cleave cystine disulfides (Catsimpoolus & Wood, 1966) and TNB mixed disulfides in proteins (Vanaman & Stark, 1970). Treatment of DTNB-modified SF_1 (Ca^{2+}-ATPase inactive) with NaCN, as shown in Figure 3, reactivates the Ca^{2+}-ATPase to about 230% activity, only slightly restores NH_4^+-EDTA-ATPase activity, and causes release of 1.0 mol of TNB per SF_1. In addition, 2 mol of $[^{14}C]CN$ was incorporated into SF_1 during this treatment concomitant with Ca^{2+}-ATPase reactivation and TNB release.[3] These data further substantiate that this form of SF_1 created after the second phase of DTNB modification contains a single SF_1-TNB mixed disulfide as well as a single cystine disulfide. The new cystine disulfide bond formed is intramolecular since control sedimentation velocity experiments did not detect any significant change in the sedimentation coefficient between native SF_1 and DTNB-modified SF_1 (data not shown).

It was of interest to determine if the thiols modified by DTNB were the same as those cross-linked by pPDM and cobalt phenanthroline complexes. Accordingly, SF_1 was treated with DTNB prior to modification with these other cross-linking reagents. Inactivation of SF_1 with DTNB largely prevented further thiol-group modification by pPDM or cobalt phenanthroline complexes (Table I). In view of the large standard deviation in the total thiol determination shown in Table I, it was difficult to determine if significant further SH-group modification by these cross-linking reagents oc-

[3] In three separate NaCN reversal experiments, the final Ca^{2+}-ATPase activity was seen to vary between 180 and 260%; the final NH_4^+-EDTA-ATPase activity varied between 22 and 48%; the total TNB released varied between 0.8 and 1.1, and the total $^{14}CN/SF_1$ varied between 1.8 and 2.5.

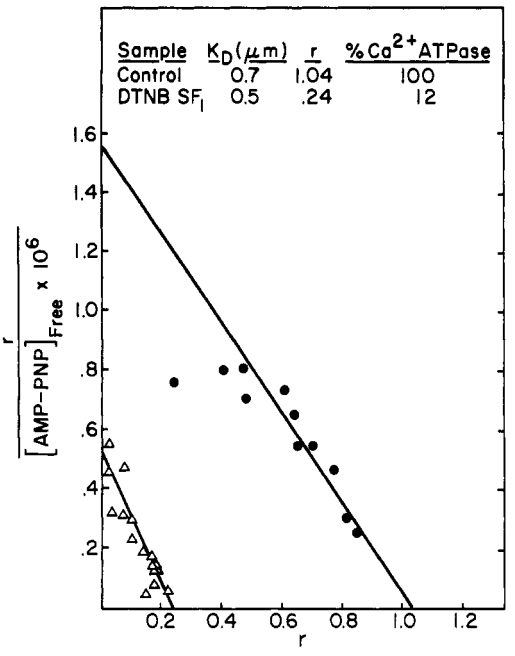


FIGURE 4: Scatchard plot for the binding of [^{3}H]AMP-PNP to DTNB-inactivated (△) and nontreated (●) SF_1. SF_1 was inactivated to 12% Ca^{2+}-ATPase activity by treatment with a twofold excess of DTNB in the presence of 0.1 mM MgADP at 0 °C for 24 h. The enzyme was purified and equilibrium dialysis with [^{3}H]AMP-PNP was performed as previously described (Wells & Yount, 1979). The control experiment (●) was performed on a different preparation of chymotryptic SF_1 than the DTNB-treated SF_1 (△).

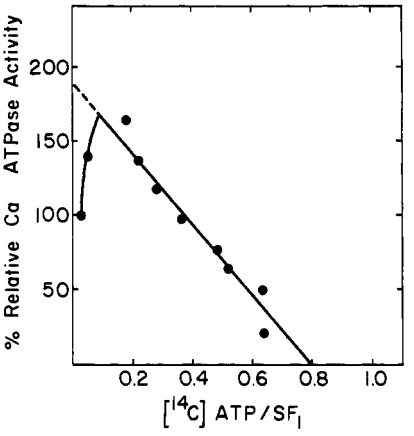


FIGURE 5: Percent Ca^{2+}-ATPase activity vs. [^{14}C]ATP trapped per SF_1 during DTNB inactivation. SF_1 (17 μM) was inactivated with 34 μM DTNB in the presence of 0.1 mM Mg[^{14}C]ATP (5500 cpm/nmol). At various times 1.0-mL aliquots of the inactivation mixture were purified by four successive $(NH_4)_2SO_4$ precipitations, and [^{14}C]ATP per SF_1 was determined. Ca^{2+}-ATPase activities were measured on separate aliquots in parallel during inactivation.

curred. However, DTNB-modified SF_1 (Ca^{2+}-ATPase inactive) which was subsequently reacted with pPDM or cobalt phenanthroline recovered almost all its ATPase activity when treated with DTE. pPDM-modified SF_1 was not reactivated by DTE (data not shown). These data suggest the activity-critical SH groups modified by pPDM and cobalt phenanthroline are the same as those modified by DTNB. In addition, DTNB pretreatment essentially prevented reaction with cobalt phenanthroline complexes, suggesting that the activity-critical site of both modifications is overlapping or the same.

Figure 4 shows a Scatchard plot for the binding of [^{3}H]-AMP-PNP to SF_1 inactivated in the presence of MgADP by DTNB. The plot shows only a small amount of residual

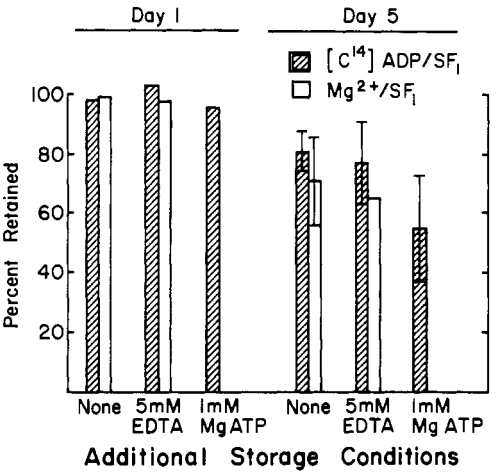


FIGURE 6: Retention of magnesium and [^{14}C]ADP in DTNB-treated SF_1 under various storage conditions at 0 °C. SF_1 (17 μM) was inactivated with a twofold molar excess of DTNB in the presence of 20 mM $MgCl_2$ and 0.1 mM ADP and purified by $(NH_4)_2SO_4$–EDTA precipitation, followed by gel filtration as described under Materials and Methods. Two derivatives were prepared in parallel: one for magnesium analysis employing nonradioactive ADP and a second for nucleotide analysis using [^{14}C]ADP (5300 cpm/nmol). At time zero, SF_1 contained an average in two trials of 0.75 magnesium and 0.73 ADP with 10% residual Ca^{2+}-ATPase activity. Enzyme derivatives were stored in KCl–Tris buffer at 0 °C with additional conditions as indicated. At various times, derivatives were subjected to Sephadex G-25 gel filtration (PD-10 columns), and the protein peak was collected and analyzed for magnesium or nucleotide. Error bars represent the standard deviation in two trials in the percent magnesium or nucleotide retained.

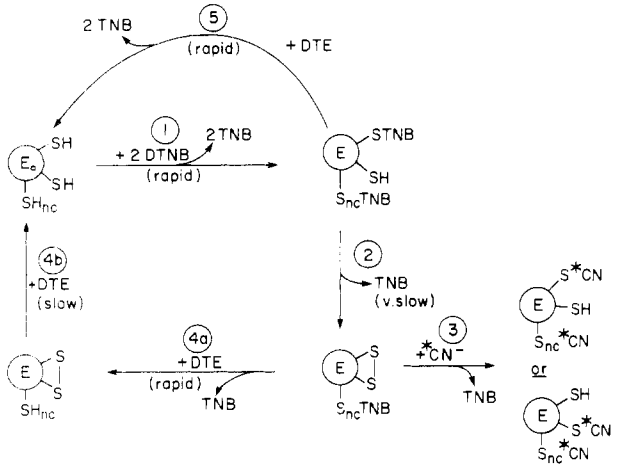
binding of [^{3}H]AMP-PNP to DTNB-inactivated SF_1 which could largely be accounted for as incompletely modified SF_1 (see table insert in Figure 4). It is worth noting that in AMP-PNP binding studies employing several different chymotryptic SF_1 preparations there was variation in the dissociation constant calculated for control SF_1 from Scatchard plots of between 0.7 and 1.2 μM. The basis for these discrepancies is presumably the result of variations in the enzyme preparations. There was much less variation in the stoichiometry of binding with values ranging between 0.9 and 1.1 AMP-PNP per SF_1.

The fact that SF_1 inactivated with DTNB in the presence of nucleotide did not bind AMP-PNP and that MgADP greatly stimulated DTNB inactivation (data not shown) suggested that nucleotide was trapped during nucleotide-stimulated inactivation. Such an effect has been seen previously for other dithiol cross-linking reagents (Wells & Yount, 1979). Figure 5 shows that nearly stoichiometric quantities of [^{14}C]ATP were trapped during nucleotide-stimulated DTNB inactivation.[4] After an initial activation phase, the extent of trapping correlated linearly with the extent of Ca^{2+}-ATPase inactivation. The amount of [^{14}C]ATP trapped during the early activation phase correlates well with the 10–20% cystine disulfide formation predicted from the reactivation studies shown in Figure 2B.

Magnesium was also trapped in a 1:1 ratio with nucleotide as shown in Figure 6. Furthermore, greater than 50% of the trapped Mg·nucleotide complex was retained following gel filtration after 5 days even when the enzyme derivative was stored with large amounts of EDTA or MgATP. In a separate

[4] Although [^{14}C]ATP is added initially, during cross-linking with other reagents it is hydrolyzed with release of inorganic phosphate and remains trapped as ADP (Wells & Yount, 1979). Although not studied directly, the same reaction presumably occurs with DTNB-promoted cross-linking.

Scheme I



study, after 7 days in KCl–Tris buffer at 0 °C, 43% of the initial magnesium and nucleotide were retained following gel filtration. This stability is comparable to that seen when cross-linking was performed with pPDM or cobalt phenanthroline complexes (Wells & Yount, 1979). The dissociation rate of magnesium was generally slightly greater than that for nucleotide although the standard errors in the measurement do not support a significant difference in the dissociation rates. The addition of EDTA or MgATP to the stored SF_1 derivative only marginally increased the dissociation rate of trapped Mg nucleotide. Variability in these data may be expected from the extended time scale of the experiment and the different enzyme preparations used.

Discussion

The data presented strongly support our conclusion that inactivation of SF_1 by DTNB results in the creation of a single activity-critical intramolecular cystine disulfide and a single noncritical TNB-SF_1 mixed disulfide. A scheme consistent with all our observations is shown in Scheme I.

This scheme is based on the following observations. Reaction of SF_1 with 2 mol of DTNB (step 1) resulted in the rapid release of 2 mol of TNB with concomitant activation of the Ca^{2+}-ATPase and loss of the K^+-ATPase activity (Figure 1). Such a reaction is consistent with modification of a critical SH group, SH, and a second noncritical thiol, labeled SH_{nc}. With longer reaction times (step 2), an additional mole of TNB was lost with a parallel loss of Ca^{2+}-ATPase activity. This reaction suggests that the modification of a second critical SH group occurs by the formation of a disulfide bond. The displacement of TNB from enzyme thiols by neighboring thiols to form a cystine disulfide is a well-documented reaction [see Carlson et al. (1978) and references cited therein]. Moreover, the internal displacement of TNB was dependent on the presence of MgADP since in its absence only two SH groups were modified (J. A. Wells and R. G. Yount, unpublished observations). This observation is consistent with the second critical SH group being called SH-2 because of the well-known enhanced reactivity of SH-2 in the presence of MgADP (Yamaguchi & Sekine, 1966).

The reaction of $^{14}CN^-$ with inactive SF_1 (step 3) released 1 mol of TNB with the subsequent incorporation of 2 mol of ^{14}CN into the enzyme (Figure 3). Such a reaction is consistent with cyanide displacing TNB and cleaving the critical cystine disulfide. This latter reaction returns the Ca^{2+}-ATPase to its activated state presumably by cyanylation of one of the critical SH groups.

The reactivation of totally inactivate SF_1 by DTE (steps 4a and 4b) occurs in two stages. The more reactive enzyme-bound TNB mixed disulfide is first reduced (step 4a) with little effect on activity (Figure 2A). More slowly, the Ca^{2+}- and NH_4^+-ATPase activities return (step 4b), indicating that the slower reduction of the cystine disulfide is the key step to reactivation. The rapid reduction of SF_1 containing 1.2 bound TNB groups was also seen in Figure 2B as illustrated by step 5 in Scheme I. Here, all TNB groups are released rapidly ($t_{1/2}$ ~ 1–2 min) with concomitant recovery of normal Ca^{2+}- and NH_4^+-ATPase activities. These results are consistent with the early modification of a single critical SH group by mixed disulfide formation with one of the TNB groups and the absence of formation of significant amounts of cystine disulfides.

The kinetic pattern of ATPase inactivation by DTNB in the presence of MgADP and the kinetic pattern of ATPase reactivation by DTE and cyanide provide suggestive evidence that the critical thiols modified by DTNB are the so-called SH-1 and SH-2 thiols. Furthermore, prior reaction of SF_1 with DTNB prevented reaction with pPDM or cobalt phenanthroline complexes, cross-linking reagents believed to cross-link SH-1 and SH-2 (Reisler et al., 1974; Wells et al., 1979b). In addition, as with cross-linking by pPDM or cobalt phenanthroline complexes (Wells & Yount, 1979), magnesium nucleotide stimulated cross-linking with DTNB resulted in blockage of the active site by stable trapping of Mg·nucleotide. This adds further support that the thiols cross-linked are the same for all three reagents. However, it should be *emphasized* that the designation of the cross-linked SH groups as SH-1 and SH-2 is based on effects on ATPase activities and is largely circumstantial. Definitive localization of the two critical thiols modified by DTNB, pPDM, and cobalt phenanthroline complexes awaits the isolation and identification of appropriately labeled peptides. If the thiols modified are SH-1 and SH-2, they should be found on the same 92 amino acid CNBr peptide isolated and sequenced by Elzinga & Collins (1977). Finally, the designation of these thiols as "critical" is related to how their modification affects activity and is not related to their presumed location at the active site. In fact, the observation that these thiols can be cross-linked with trapping of MgADP at the active site means they are unlikely to be involved in either binding or hydrolysis of the substrate. This agrees with the recent work of Wiedner et al. (1978), who found that complete modification of the thiols of myosin with small blocking groups did not destroy enzyme activity.

We have recently proposed that myosin contains a jawlike active-site structure which closes upon binding of a Mg·nucleotide complex. The active-site closure is believed to be coupled to the juxtaposition of two SH groups which may be cross-linked. Burke & Reisler (1977) suggested the cross-linked thiols could not approach within the cross-linking span of 2,4 dinitro-1,5-difluorobenzene (3–5 Å) based on its inability to inactivate SF_1-Ca^{2+}-ATPase with approximately stoichiometric quantities of this reagent. Evidence has recently indicated, however, that a single Co(III) can simultaneously chelate these SH groups, placing a distance between the SH groups of 3–5 Å (Wells et al., 1979b). The formation of a disulfide bond, consistent with data presented here, would indicate that these SH groups can come within 2.0 Å of each other in the presence of Mg·nucleotide. These combined studies suggest that these thiols in the myosin globular head move in response to nucleotide binding distances >12 Å

(cross-linking span of pPDM) to 2 Å (length of a disulfide bond).

Trapping of spectroscopically conversant metal nucleotide analogues is currently under investigation in our laboratory. The trapping of metal nucleotides by DTNB modification offers distinct advantages over modification by pPDM or cobalt phenanthroline. The DTNB-promoted disulfide formation is a reversible modification unlike that of pPDM. In addition, the disulfide cross-link introduced is only weakly chromophoric, unlike that of the cobalt phenanthroline modification. Furthermore, DTNB treatment in the presence of Mg·nucleotide generates the most tightly thiol cross-linked enzyme conformation possible. The fact that DTNB is thiol specific and is a readily available reagent makes it particularly useful for trapping spectroscopic analogues and photoaffinity analogues to be used for probing the microenvironment and structure of the active site of myosin.

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