Inactivation of Myosin Subfragment One by Cobalt(II)/Cobalt(III) Phenanthroline Complexes. 2. Cobalt Chelation of Two Critical SH Groups†

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ABSTRACT: Treatment of myosin chymotryptic subfragment one (SF_1) with $CoCl_2$, 1,10-phenanthroline (phen), and $[Co^{III}(phen)_2CO_3]^+$ in the presence of ADP leads to parallel inactivation of the NH_4^+-EDTA, Ca^{2+}, and Mg^{2+} ATPase activities. The incorporation of a single cobalt correlates with complete loss of enzyme activity. With prolonged treatment up to two cobalts per SF_1 can be incorporated, indicating the existence of noncritical cobalt site(s). The noncritical site(s) can be prelabeled with a mixture of cobalt(II)- and cobalt(III)-ethylenediamine-*N*,*N*′-diacetic acid complexes without affecting the ATPase activity. Subsequent labeling by cobalt phenanthroline complexes incorporates essentially one more cobalt with complete loss of enzyme activity. The loss of two SH groups by cobalt phenanthroline modification correlates linearly with ATPase inactivation. In addition, selective reduction with a variety of reducing agents and subsequent removal of slightly greater than one cobalt allows recovery of approximately two SH groups and almost all ATPase activity. The loss of SH groups is shown to result from direct cobalt chelation as opposed to coincident disulfide formation or indirect steric blockage. Cobalt incorporation and thiol modification with cobalt phenanthroline complexes can be blocked by prior treatment of SF_1 with low-fold excesses of *N*,*N*′-*p*-phenylenedimaleimide in the presence of MgADP (believed to cross-link two reactive cysteines called SH_1 and SH_2) or *N*-ethylmaleimide (believed to label SH_1). In addition, pretreatment with cobalt phenanthroline in the presence of MgADP provides significant protection against reaction with both maleimide reagents. These data suggest that cobalt phenanthroline inactivation in the presence of MgADP results from the incorporation of a single Co(III) into a dithiol site consisting of SH_1 and SH_2. Cobalt chelation of these SH groups means that they must move to within 3–5 Å of each other. These findings, combined with the earlier 12–14-Å thiol to thiol distance determined by Reisler et al. [Reisler, E., Burke, M., Himmelfarb, S., & Harrington, W. F. (1974) *Biochemistry 13*, 3837] with a rigid cross-linking agent, indicate the region of myosin containing SH_1 and SH_2 can undergo large conformational changes.

Myosin and its active proteolytic subfragments are known to undergo conformational changes as a function of magnesium nucleotide binding based on observed increases in intrinsic tryptophan fluorescence (Werber et al., 1972; Mandelkow & Mandelkow, 1973), changes in ultraviolet absorption (Morita, 1967; Malik & Martonosi, 1972), changes in circular dichroism (Murphy, 1974), increases in mobility of a nitroxide electron paramagnetic spin-label (Seidel et al., 1970; Tokiwa, 1971; Seidel & Gergely, 1973; Blumenfeld & Ignatêva, 1974), increases in the fluorescent lifetime of a coupled fluorescent probe (Mendelson et al., 1975), and changes in the reactivity of SH groups to alkylation by MalNEt[1] (Watterson & Schaub, 1973; Reisler et al., 1974a) and fluorodinitrobenzene (Reisler et al., 1977). Changes in intrinsic tryptophan fluorescence monitored by stopped-flow techniques (Bagshaw & Trentham, 1974) and alkylation of SH groups (Watterson & Schaub, 1973) have been correlated with intermediates during ATP hydrolysis. These and other studies have led to a kinetic model for myosin-catalyzed ATP hydrolysis [for reviews see Trentham et al. (1976) and Taylor (1979)].

In spite of the overwhelming evidence for conformational changes accompanying binding and hydrolysis of MgATP, little is known about the enzyme structures or conformational changes that accompany these kinetic intermediates. Recently, however, Reisler et al. (1974b) have reported that pPDM, a dimaleimide cross-linking reagent with a span of 12–14 Å, cross-links two activity essential SH groups, the so-called SH_1 and SH_2. These two SH groups appear to move toward each other upon binding of magnesium nucleotide because in the presence of MgADP they can be rapidly cross-linked by bifunctional reagents with spanner lengths as short as 7–10 Å (Burke & Reisler, 1977). The circular dichroic spectrum of myosin's proteolytic SF_1 cross-linked by pPDM is very similar to the spectrum of the kinetic intermediate following binding of MgATP to myosin, M**MgADP·P_i (Burke et al., 1976). These results suggest that pPDM traps an enzyme conformation which resembles that of M**MgADP·P_i. Although pPDM-labeled peptide(s) has not been isolated to show unequivocally the presence of an SH_1–SH_2 cross-link, a single cyanogen bromide peptide containing SH_1 and SH_2 from rabbit skeletal myosin has been isolated and sequenced, showing that these residues are only 10 amino acids apart in the primary sequence (Elzinga & Collins, 1977).

Results from the preceding paper (Wells et al., 1979) indicated a mixture of Co^{II}phen and $[Co^{III}(phen)_2CO_3]^+$ rapidly inactivated myosin chymotryptic SF_1. This inactivation resulted from the in situ oxidation of an enzyme-bound Co^{II}phen complex to an enzyme-bound exchange-inert Co^{III}phen

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[1] Abbreviations used: MalNEt, *N*-ethylmaleimide; pPDM, *N*,*N*′-*p*-phenylenedimaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDDA, ethylenediamine-*N*,*N*′-diacetic acid; DTE, dithioerythritol; $[Co^{II}EDDA(H_2O)_2]Cl$, diaquo(ethylene-*N*,*N*′-diaceto)cobalt(III); SF_1, chymotryptic subfragment one; Co·SF_1, subfragment one inactivated with cobalt(II)/cobalt(III) phenanthroline complexes in the presence of MgADP.

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complex. The cobalt incorporation and ATPase inactivation were reversed by a variety of reducing agents, making the cobalt enzyme derivative suitable for further studies to evaluate the activity critical site(s) of cobalt incorporation.

In this paper we provide evidence that the incorporation of only one cobalt was critical for ATPase inactivation. Incorporation of the activity critical cobalt resulted in blockage of two SH groups which appear to be SH_1 and SH_2 based on protection studies with pPDM and MalNEt. A model is proposed in which ATPase inactivation results from the simultaneous chelation by Co(III) of SH_1 and SH_2. If true, this fixes a maximal distance of approach for these SH groups of 3–5 Å, depending on whether the SH groups are chelated cis or trans to the cobalt. These results further substantiate the results of Burke & Reisler (1977) that SH_1 and SH_2 can move close together upon binding magnesium nucleotide. In addition, the possibility is discussed that cobalt phenanthroline complexes may be general modifiers of vicinal thiol sites on enzymes.

Materials and Methods

Materials

DTNB, DTE, and MalNEt were from Pierce Chemical Co. pPDM and 1,10-phenanthroline were from Aldrich. $[Co^{III}EDDA(H_2O)_2]Cl$ and EDDA were kindly donated by Dr. M. S. Urdea. $[Co^{III}(phen)_2CO_3]Cl{\cdot}4.5H_2O$ and SF_1 were prepared as described in the preceding paper (Wells et al., 1979). All other chemicals were of reagent grade.

Methods

ATPase Assays and Inactivations. NH_4^+-EDTA ATPase activity was measured by adding 25 μL of SF_1 (2 mg/mL) to 2.0 mL of assay mixture containing 0.56 M NH_4Cl, 0.23 M KCl, 36 mM EDTA, 6.1 mM ATP, and 60 mM Tris, pH 8.0, at 25 °C. Samples (0.5 mL) were removed at times 2 and 12 min and analyzed for inorganic phosphate by addition of 3 mL of molybdate reagent (0.75 N in H_2SO_4 and 0.66% in ammonium molybdate). The resulting phosphomolybdate complex was then reduced by addition of 0.5 mL of freshly prepared 10% $FeSO_4$ in 0.15 N H_2SO_4 (Rockstein & Herron, 1951), and after 2 min the absorbance at 700 nm was read by using a Gilford 300 N rapid sampling spectrophotometer.

The Ca^{2+} ATPase activity was measured by addition of 0.2 mL of SF_1 solution (2 mg/mL) to 2.0 mL of assay mixture containing 7.6 mM ATP, 15 mM $CaCl_2$, 150 mM KCl, and 180 mM Tris-HCl, pH 7.4, at 25 °C. At 2 and 22 min 1.0-mL samples were pipetted into centrifuge tubes containing 0.5 mL of 10% trichloroacetic acid, and precipitated protein was removed by centrifugation. One milliliter of the supernatant was analyzed for inorganic phosphate as described above. Mg ATPase activities were measured in a similar manner except 8 times as much SF_1 was used. Assay solutions contained 6 mM $MgCl_2$, 6 mM ATP, 0.1 M KCl, and 40 mM Tris, pH 8.0, at 25 °C.

ATPase inactivations were performed on 12–25 μM SF_1 by using a 10-fold excess of $CoCl_2$ and 1,10-phenanthroline and a 100-fold excess of $[Co^{III}(phen)_2CO_3]^+$, 0.1 mM nucleotide, 2–20 mM $MgCl_2$, 0.1 M KCl, and 50 mM Tris-HCl, pH 8.0, at 0 °C. Quenching the inactivation reactions and purifying partially inactivated SF_1 samples were performed as described in the preceding paper (Wells et al., 1979).

To rule out that cobalt modification only shifted the ATPase pH optimum or ionic strength dependence, the NH_4^+-EDTA ATPase activities were measured at 0.5 pH intervals from pH 6 to 9.5 in 0.1, 0.6, and 0.9 M KCl. The results (data not shown) showed that the percentage of residual activity of

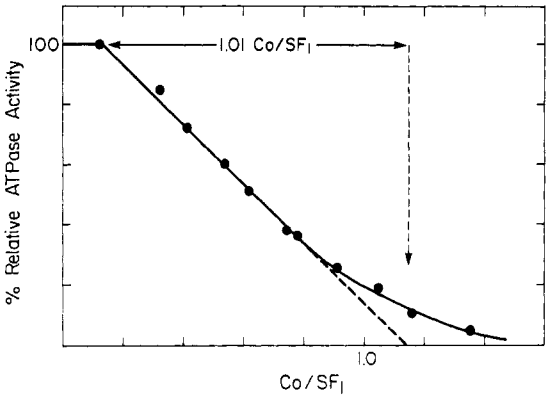

FIGURE 1: Percent relative NH_4^+-EDTA ATPase activity lost as a function of cobalt incorporation into SF_1. Subfragment one (90 mg, 14 μM) was inactivated in the presence of 20 mM $MgCl_2$, 0.1 mM ADP, 0.14 mM $CoCl_2$, 0.14 mM phen, 1.4 mM $[Co^{III}(phen)_2CO_3]^+$, 0.1 M KCl, and 50 mM Tris, pH 8.0, at 0 °C. At appropriate times small aliquots were taken for measuring NH_4^+-EDTA ATPase activities as described under Methods and Materials, and larger aliquots (8 mg of SF_1 each) were taken for measuring protein-bound cobalt. These latter samples were quenched by addition of EDTA to 10 mM and freed of extraneous cobalt by four successive $(NH_4)_2SO_4$ precipitations (see Materials and Methods). Cobalt was measured by atomic absorption, and protein was measured by the Bradford dye binding assay.

Co·SF_1 was the same under all of the above conditions.

Analytical Procedures. Determination of the thiol content was performed essentially according to Ellman (1959). SF_1 (0.6 mL of 12–25 μM) was added to 2 mL of freshly prepared 9 M urea, 10 mM EDTA, 50 mM Tris, and 0.1 M KCl, pH 8.0, and the absorbance at 412 nm was measured. After approximately 15 min 0.4 mL of freshly prepared 1 mM DTNB, 0.1 M KCl, and 10 mM phosphate, pH 7.0, was added, and the absorbance at 412 nm was followed until there was no further increase in absorbance (usually 10 min). The SH content was determined from the known $\epsilon_{412nm} = 13\,600$ (Ellman, 1959). Freshly prepared unmodified chymotryptic SF_1 contained 10.1 ± 0.2 SH groups (10 preparations). After 7 days, the SH content was found to decrease by 0.5–1.0 SH/SF_1. An average standard deviation of ±0.12 SH groups was compiled in over 30 duplicate trials.

Cobalt concentrations and protein concentrations were determined by atomic absorption and a dye binding assay (Bradford, 1976), respectively.

Results

Evidence for a Single Activity Critical Cobalt. The correlation of the loss of ATPase activity vs. Co/SF_1 (Figure 1) showed an initial phase of 0.15 Co/SF_1 which did not affect activity. A second phase showed a linear relationship between loss of ATPase activity and cobalt uptake which extrapolated to a net incorporation of one Co per SF_1 when all activity was lost. Exhaustive cobalt phenanthroline treatment incorporated up to two Co per SF_1 under these conditions, indicating the existence of nonessential cobalt site(s).

The nonessential cobalt site(s) could be prelabeled with 0.6–0.9 Co/SF_1 by prolonged incubation of SF_1 with $Co^{II}EDDA/Co^{III}EDDA$ without significantly affecting ATPase activity (Table I). Further labeling with cobalt phenanthroline complexes in the presence of MgADP led to an additional incorporation of 1.1 to 1.2 Co/SF_1 with concomitant ATPase inactivation.

Additional evidence that only one cobalt per SF_1 was necessary for inactivation is shown in Table II. Here, selective brief treatment of Co·SF_1 with several reducing agents allowed

Table I: Labeling of Nonessential Cobalt Site(s) by $Co^{II}EDDA/Co^{III}EDDA(H_2O)_2$ Pretreatment of SF_1

	expt 1		expt 2	
treatment	NH_4^+-EDTA ATPase	Co/SF_1	NH_4^+-EDTA ATPase	Co/SF_1
(A)[a] $Co^{II}EDDA/Co^{III}EDDA(H_2O)_2$	100	0.62	96	0.85
(B)[b] Co^{II}phen/$[Co^{III}(phen)_2CO_3]^+$	4	1.76		
(A) then (B)	4	1.85	6	1.95

[a] SF_1 (16 μM) was treated with 0.16 mM $CoCl_2$, 0.16 mM EDDA, 1.6 mM $[Co^{III}EDDA(H_2O)_2]$, 0.1 M KCl, and 50 mM Tris-HCl, pH 8.0, at 0 °C for 16 h, and excess reagents were removed by a $(NH_4)_2SO_4$ precipitation, followed by Sephadex G-25 gel filtration of the dissolved protein. [b] SF_1 (16 μM) was treated with 0.16 mM $CoCl_2$, 0.16 mM phen, 2 mM $MgCl_2$, 0.1 mM ADP, 0.1 M KCl, and 50 mM Tris-HCl, pH 8.0, at 0 °C for 9 min, quenched by addition of EDTA (to 10 mM), and purified as described in footnote *a*.

Table II: Selective Reduction of Activity Essential Co(III)

enzyme	reducing agent[a]	treatment time (min)	"A" fraction act.	Co/SF_1	ΔCo/"A"[b]	SH/SF_1	ΔSH/"A"[c]
SF_1			1.00			9.8	
Co·SF_1	none		0.04	1.7		7.0	
Co·SF_1	$Fe^{II}EDTA$	1	0.96	0.6	1.2	9.1	2.3
Co·SF_1	$Fe^{II}EDTA$	5	0.91	0.1		9.9	
Co·SF_1	$Co^{II}CN_5^{3-}$	12	0.85	0.9	1.0	8.9	2.3
Co·SF_1	$NaBH_4$	40	0.98	0.5	1.3	9.0	2.1

[a] Reaction conditions were essentially the same as described in the preceding paper (Wells et al., 1979). Excess reagents were removed as described under Materials and Methods prior to cobalt and thiol analysis. [b] ΔCo/"A" = (net loss of cobalt)/(net recovery of activity) = [1.7 − (cobalt remaining)]/("A"$_{final}$ − 0.04). [c] ΔSH/"A" = (net recovery of SH groups)/(net recovery of activity) = (SH_{final} − 7.0)/("A"$_{final}$ − 0.04).

nearly complete ATPase reactivation while removing approximately one Co per SF_1.

Evidence That Blockage of Two SH Groups Leads to ATPase Inactivation. Inactivation of SF_1 ATPase by cobalt phenanthroline complexes led to a net loss of 2.8 SH groups per SF_1 as shown in Table II. Brief exposure to a variety of reducing agents showed that approximately two SH groups returned along with all ATPase activity and the removal of the activity critical cobalt. Extended treatment with $Fe^{II}EDTA$ (5 min) allowed essentially full recovery of all SH groups and removal of all cobalt.

Direct addition of cobalt phenanthroline complexes to native SF_1 in the sulfhydryl assay solution caused only a small depression (4–6%) in the net absorbance change at 412 nm following addition of DTNB. This allowed the direct monitoring of SH groups as a function of ATPase activity as shown in Figure 2. The results were essentially the same whether the inactivation was performed in the presence of MgATP or MgADP (Figure 2A and 2B, respectively). The data show there was an initial loss of 0.3–0.5 SH groups which did not affect activity followed by a loss of essentially 2.0 SH groups per SF_1 which paralleled the complete loss of ATPase activity. The same values were obtained if cobalt complexes were removed prior to DTNB analysis, but the results were less precise because of the necessity to make separate protein determinations on each sample.

Other studies similar to those shown in Figure 2 were performed except the SH groups were assayed in the absence of urea. Again, the loss of 1.9 SH/SF_1 correlated with the loss of all ATPase activity, indicating the SH groups modified by cobalt were readily assessible. Our preparations of SF_1 exhibited 10 DTNB-detectable SH groups in the presence of 9 M urea but only about 6 SH groups in its absence.

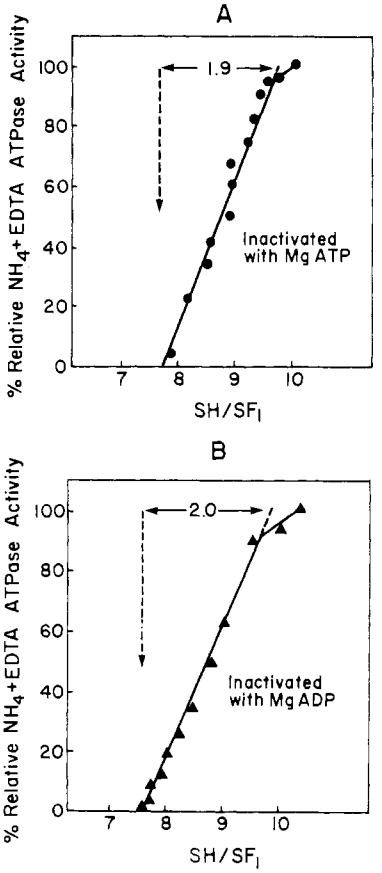

FIGURE 2: Percent relative NH_4^+-EDTA ATPase activity vs. SH/SF_1 remaining during cobalt phenanthroline inactivation. SF_1 (15 μM) was inactivated in the presence of 0.1 mM ADP (Δ) or 0.1 mM ATP (○), 2 mM $MgCl_2$, 0.15 mM phen, 1.5 mM $[Co^{III}(phen)_2CO_3]^+$, 0.1 M KCl, and 50 mM Tris, pH 8.0, at 0 °C. At various times 0.6-mL samples were added to 3.5-mL cuvettes containing 2 mL of 9 M urea and 10 mM EDTA. After 15 min at 25 °C the absorbance at 412 nm was measured and 0.4 mL of 2 mg/mL DTNB was added. The absorbance at 412 nm was monitored after 30 min, although no further increase occurred after 15 min. Total SH groups were corrected for a systematic 5% depression in the DTNB analysis caused by extraneous cobalt complexes. ATPase activities were measured on separate aliquots as described in Figure 1.

Evidence That the Critical SH Groups Modified Are SH_1 and SH_2. ATPase activity for SF_1 can be supported by $NH_4^+(K^+)$-EDTA, Ca^{2+}, or Mg^{2+}. Modification of SH_1 by MalNEt leads to a characteristic loss in the NH_4^+-EDTA ATPase activity and an increase in the Mg^{2+} and Ca^{2+} ATPase activity (Reisler et al., 1974a). Modification of both SH_1 and SH_2 by pPDM gives parallel loss of all ATPase activities (Reisler et al., 1976b). Inactivation of SF_1 by cobalt phenanthroline complexes resulted in a parallel loss of the NH_4^+-EDTA, Ca^{2+}, and Mg^{2+} ATPase activities (Figure 3), consistent with SH_1 and SH_2 modification.

Furthermore, we attempted to block cobalt incorporation into SF_1 by prior treatment with a low-fold excess of pPDM or MalNEt. Accordingly, SF_1 was titrated with pPDM in the presence of MgADP or MalNEt in the absence of MgADP until NH_4^+-EDTA ATPase activity was below 20% as shown in Table III. MalNEt and pPDM reacted with approximately 2.7 SH groups, although only 1 and 2 of these modified SH groups, respectively, are thought to cause NH_4^+-EDTA

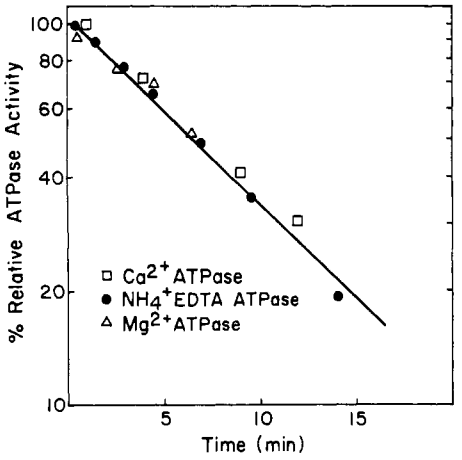

FIGURE 3: Inactivation of the Ca^{2+}, Mg^{2+}, and NH_4^+-EDTA ATPase activities by cobalt phenanthroline complexes. Inactivation conditions were 25 μM SF_1, 0.11 mM ATP, 0.1 mM $CoCl_2$, 0.1 mM phen, and 0.71 mM $[Co^{III}(phen)_2CO_3]^+$.

Table III: MalNEt and pPDM Protection against Cobalt Incorporation into SF_1

enzyme derivative	% NH_4^+-EDTA ATPase	SH/SF_1	loss of SH/SF_1[a]	Co/SF_1	ΔCo/SF_1[b]
SF_1	100	10.1			
MalNEt-SF_1[c]	16	7.4	2.7		
Co + MalNEt-SF_1[d]	3	7.0	3.1	0.75	−0.97
pPDM-SF_1[e]	18	7.4	2.7		
Co + pPDM-SF_1	2	6.8	3.3	0.43	−1.29
Co·SF_1[f]	11	7.4	2.7	1.72	

[a] Taken as the difference from control (10.1 SH groups). [b] Taken as the difference from Co·SF_1 (1.72 Co/SF_1). [c] Prepared by reacting 16 μM SF_1 with 2.3-fold excess of MalNEt for 50 min in 0.1 M KCl and 50 mM Tris, pH 8, 0 °C. [d] Prepared by reacting 17 μM MalNEt-SF_1 with cobalt phenanthroline complexes for 5 min as described in footnote *f*. [e] Prepared by reacting 16 μM SF_1 with 1.3-fold excess of pPDM for 50 min in the presence of 0.1 mM ADP, 2 mM $MgCl_2$, 0.1 M KCl, and 50 mM Tris, pH 8, 0 °C. [f] Prepared by reacting 16 μM SF_1 with cobalt phenanthroline complexes for 5 min as described under Materials and Methods in the presence of 2 mM $MgCl_2$ and 0.1 mM ADP.

ATPase inactivation. Further modification of MalNEt–SF_1 and pPDM–SF_1 by cobalt phenanthroline complexes allowed only 0.75 and 0.43 cobalts to be incorporated, respectively. A corresponding additional loss of 0.4 and 0.6 SH groups and of 13 and 16% ATPase activity also occurred. Table III shows that maleimide pretreatments blocked the incorporation of 1.0–1.3 Co/SF_1 and nearly completely prevented SH group modification by cobalt phenanthroline complexes.

Cobalt phenanthroline pretreatment of SF_1 reduced by greater than 50% subsequent maleimide modification of SH groups. It also allowed nearly two-thirds of the ATPase activity to be recovered by Fe^{II}EDTA reduction. Incomplete SH group protection by Co·SF_1 may result from reaction of other less reactive and exposed SH groups with MalNEt and pPDM. To test this, we reacted SF_1 with a 2.5-fold excess of pPDM for 20 min at 0 °C and quenched the reaction with a 500-fold excess of 2-mercaptoethanol. The purified pPDM-modified SF_1 had lost 3.5 SH groups, indicating more extensive reaction than when a 1.3-fold excess of pPDM was used.

Cobalt phenanthroline prelabeling followed by maleimide treatment allowed nearly two-thirds of the ATPase activity to be reactivated by Fe^{II}EDTA, indicative of cobalt protection. SF_1 treated with MalNEt or pPDM could not be reactivated by Fe^{II}EDTA. Incomplete reactivation and protection of SH

Table IV: Cobalt Protection against MalNEt and pPDM Labeling of SF_1

enzyme derivative	% NH_4^+-EDTA ATPase	SH/SF_1	loss of SH/SF_1	Co/SF_1	% reactivation[a]
SF_1	100	10.1			
Co·SF_1[b]	11	7.4	2.7	1.72	95
MalNEt + Co·SF_1[c]	1	6.0	4.1	1.40	65
pPDM + Co·SF_1[d]	1	6.0	4.1	1.40	65

[a] Reactivation performed by Fe^{II}EDTA reduction. Modified enzyme (13–16 μM) was treated with 1.0 mM $FeSO_4$, 5 mM EDTA, 0.1 M KCl, and 50 mM Tris, pH 8.0, 0 °C, and reactivation was monitored with time. Optimal reactivation occurred at 1 min. Percent reactivation values for MalNEt + Co·SF_1 and pPDM + Co·SF_1 have been corrected for the 10% additional loss of activity resulting from the maleimide modifications after Co(II)/Co(III) treatment. [b] See Table III. [c] Co·SF_1 (13 μM) was treated with a 2.3 molar excess of MalNEt as described in Table III. [d] Co·SF_1 (13 μM) was treated with 1.3 molar excess of pPDM as described in Table III.

groups after cobalt pretreatment may be explained by at least two factors. First, the maleimides modified SH groups other than SH_1 and SH_2 which in turn may have partially inactivated NH_4^+-EDTA ATPase activity. Second, the modification of Co·SF_1 by either MalNEt or pPDM resulted in a loss of 0.3 Co/SF_1 (Table IV). If this lost cobalt came from the critical dithiol binding site, then an additional 30% irreversible modification by MalNEt or pPDM could have resulted.

Discussion

Several lines of evidence strongly support the proposal that the incorporation of only one cobalt is critical for ATPase inactivation. These are as follows: (1) the stoichiometric incorporation of one cobalt per SF_1 led to complete inactivation, (2) the selective reduction and removal of one Co(III) per SF_1 led to complete recovery of ATPase aactivity, (3) prior treatment of SF_1 with Co^{III}EDDA/Co^{II}EDDA blocked nonessential cobalt phenanthroline sites, allowing only one activity critical cobalt site to be labeled, and (4) pPDM pretreatment blocked the incorporation of one cobalt per SF_1. These observations provide good evidence that ATPase inactivation results exclusively from the incorporation of cobalt into a specific site.

Experiments to characterize the activity critical cobalt site have indicated that this site probably consists of two thiolate ligands. Very close to two DTNB-detectable SH groups were lost concomitantly with the loss of all ATPase activity (Figure 2). Furthermore, selective reduction and removal of slightly more than one cobalt from Co·SF_1 resulted in the recovery of slightly over two SH groups and essentially all ATPase activity.

The concomitant loss of two SH groups and ATPase activity with the incorporation of a single cobalt could result from at least two other causes other than direct cobalt chelation. The first alternative was that indirect steric blockage of SH groups resulted from cobalt modification. However, the fact that the DTNB analysis was performed in the presence of 9 M urea makes this alternative unlikely. For example, under these conditions all SH groups in SF_1 react with MalNEt, iodoacetamide or DTNB, suggesting the enzyme was completely denatured.

A second alternative was that the loss of SH groups and possibly ATPase activity resulted from the coincident formation of a disulfide bond during cobalt modification. In model experiments it was shown that Fe^{II}EDTA did not reduce disulfides (e.g., L-cystine) under conditions used for the Fe^{II}EDTA reactivation of Co·SF_1. Furthermore, Fe^{II}EDTA

did not reduce mixed disulfide preparations of SF_1 prepared by reaction with DTNB or 6,6′-dithiobis(inosinyl imidodiphosphate). In addition, reduction of disulfides by $Fe^{II}EDTA$ is thermodynamically unfavorable at pH 8.0 because the reduction potential for disulfides is typically between −0.2 and −0.3 V (Clark, 1960), while for $Fe^{III}EDTA$ it is +0.1 V (Belcher et al., 1955), predicting $\Delta E°$ at pH 8.0 of −0.3 to −0.4 V. In fact, prolonged treatment (24 h) with $Fe^{II}EDTA$ led to the net loss of SH groups in SF_1 (Wells et al., 1979). Thus, the rapid return of SH groups following $Fe^{II}EDTA$ treatment of Co·SF_1 is very unlikely due to coincident reduction of a disulfide. A similar argument can be made for the return of SH groups following $Co^{II}(CN)_5^{3-}$ treatment. Although cyanide alone is known to cleave disulfide bonds (Catsimpoolas & Wood, 1966), it generally does so at much slower rates (typical $t_{1/2}$ of 3 h at 37 °C) than the rate at which reactivation for Co·SF_1 occurs ($t_{1/2}$ of 2–5 min at 25 °C). This indicates reactivation and recovery of SH groups resulted only from cobalt removal from Co·SF_1.

More direct evidence for cobalt chelation of SH groups came from the protection against cobalt incorporation that resulted from pretreatment of SF_1 with maleimide reagents shown in Table III. In addition, pretreatment with cobalt phenanthroline complexes afforded protection against SH group modification by the same maleimide reagents as shown in Table IV. Direct evidence for cobalt chelation of SH groups may be available from difference spectra experiments comparing cobalt-modified and unmodified SF_1. Preliminary evidence shows the presence of a new intense absorption band at 365 nm following cobalt incorporation. The appearance of a new absorption band in this region would be expected as a result of cobalt-thiolate charge transfer; however, assignment is uncertain in the absence of spectra of model compounds.

The protection studies presented in Table III and Table IV strongly suggest the critical dithiol site modified by cobalt incorporation is the same as that modified by MalNEt or pPDM. Since the critical SH group modified by MalNEt is believed to be the so-called SH_1 and the critical thiols cross-linked by pPDM are thought to be SH_1 and SH_2 (Reisler et al., 1974b), it would seem reasonable to propose that the two critical SH groups lost during cobalt incorporation are SH_1 and SH_2. However, definitive localization of the two thiols modified awaits isolation and characterization of appropriately labeled peptides.

Kinetic evidence suggests that the pPDM and cobalt phenanthroline dithiol site is the same. For both inhibitors the rate of ATPase inactivation is greatly stimulated in the presence of MgADP (Reisler et al., 1974b; Wells et al., 1979). In addition, it is generally believed that modification of SH_1 and SH_2 leads to complete inactivation of all metal ion supported ATPase activities. Both pPDM (Reisler et al., 1974b) and cobalt phenanthroline treatment (Figure 3) of SF_1 in the presence of MgADP led to parallel inactivation of all ATPase activities.

The fact that nucleotide enhanced so markedly the rate of ATPase inactivation indicates the pPDM and cobalt phenanthroline dithiol site does not overlap the single nucleotide site of SF_1. The available evidence supports the concept that the binding of nucleotide favors an enzyme conformation which makes the dithiol site more reactive. Burke & Reisler (1977) have proposed that SH_1 and SH_2 move perhaps as close together as 7–10 Å when MgADP binds to SF_1 based on inactivation studies with varying spanner-length reagents which cross-link thiols. The data presented here are consistent with their proposal. Cobalt(III) chelation of these critical SH groups would fix a distance between them of 3.1 Å if cis chelated or 4.5 Å if trans chelated, based on known crystal structures of cobalt thiolate compounds (Helis et al., 1977; deMeester & Hodgson, 1976). It is known that two phenanthroline ligands can only assume a cis configuration about cobalt (Josephsen & Schäffer, 1969; Gibson et al., 1969), which would necessitate a bis(phenanthroline)cobalt(III) complex of SF_1 be cis chelated to the thiolate ligands.

The data in Figure 1 indicate there was a rapid incorporation of 0.15 cobalt/SF_1 which did not affect activity. This lag was present in all such studies performed to date and varied from 0.1 to 0.35 Co/SF_1, depending on the preparation of enzyme tested. Scatchard analysis of the reversible binding of $^{57}Co^{II}$phen to our SF_1 preparations has consistently shown the presence of 0.1 to 0.2 $^{57}Co(II)$ tight sites per SF_1 (K_D = 4 μM) and at least one weaker site (K_D = 200–500 μM). The dissociation constant for the residual tight site is comparable to the 3 μM dissociation constant reported by Bagshaw & Reed (1976) for Mn^{2+} binding to the DTNB light chain in papain SF_1. To evaluate the possibility of cobalt(III) labeling of this light chain, we measured ATPase activity as a function of cobalt incorporation into heavy meromyosin and myosin, both of which contain DTNB light chains. Between 1.3 and 1.8 Co/enzyme were incorporated into either heavy meromyosin or myosin without affecting activity, a result consistent with labeling of the DTNB light chains. Thus far, however, attempts to show ^{57}Co bound to light chains after separation by $NaDodSO_4$ gel electrophoresis have been unsuccessful.

In addition to the rapid nonactivity-related incorporation of Co into SF_1 shown in Figure 1, there was a corresponding rapid loss of 0.2–0.6 nonactivity-related SH groups (Figure 2). This SH loss may be related to cobalt binding to one or two of the SH groups of the DNTB light chain, but, in general, the loss of thiols exceeds the early burst of cobalt incorporation. Further studies are clearly needed to define this early phase of cobalt incorporation.

Transition metal phenanthroline complexes such as Cu^{II}phen show a strong affinity for SH groups. In fact, Cu^{II}phen is known to catalyze oxidation of SH groups to disulfide (Kobashi, 1968) and has been used for this purpose to cross-link oligomeric proteins (Steck, 1972; Murphy, 1976; Huang & Askari, 1978). We have found that Cu^{II}phen is a potent inactivator of SF_1 ATPase activity with a concomitant loss of two SH groups (unpublished results). However, Cu^{II}phen inactivations are not reversible by DTE or other reducing agents even with prolonged periods of treatment. The irreversible nature of the Cu^{II}phen inactivation may result from the generation of oxygen free radicals during thiol oxidation (Misra, 1974).

The sum of the data presented here and in the preceding paper (Wells et al., 1979) leads us to propose the mechanism for cobalt incorporation, SH modification, and ATPase inactivation shown in Figure 4. It is proposed that an enzyme conformation (depicted by the square), which is favored in the presence of nucleotide, chelates a Co^{II}phen complex via two specific proximal SH groups which are probably SH_1 and SH_2. This $Co^{II}(phen)_x$·enzyme complex is then in situ oxidized by $[Co^{III}(phen)_2CO_3]^+$ to yield an inactive exchange-inert enzyme complex.

Cobalt phenanthroline incorporation may prove to be generally useful for exchange-inert metal labeling of dithiol sites in other proteins. The cobalt modification is highly reversible, and one can selectively remove cobalt(III) dithiol labels by reduction with $Fe^{II}EDTA$ without reducing preexisting disulfides. Cobalt stoichiometry is facile to evaluate

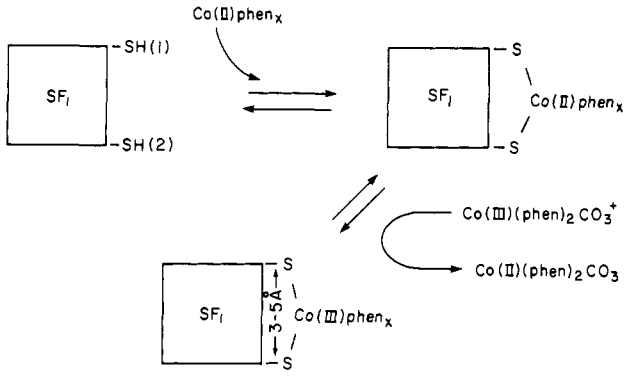

FIGURE 4: Proposed model for in situ oxidation of $Co^{II}(phen)_x$ chelated to critical SH groups SH_1 and SH_2 by $[Co^{III}(phen)_2CO_3]^+$.

by atomic absorption or by using the γ emitter ^{57}Co. The number of SH groups lost during cobalt incorporation can be conveniently followed without requiring the removal of extraneous cobalt complexes. A specific cobalt phenanthroline modification of a dithiol site may prove useful as a CD and absorption spectral probe of that site since phenanthroline ligands attached to the cobalt are highly chromophoric. Furthermore, such a chromophoric label may prove useful in energy transfer distance measurements.

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