Trapping of Transition Metal-Nucleotide Complexes in Myosin Subfragment 1 by Cross-Linking Thiols; Divalent Transition Metal Probes of the Active Site†

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ABSTRACT: Recent experiments [Wells, J., & Yount, R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966] have shown it is possible to trap MgADP and other nucleotides stably at the active site of myosin by cross-linking two thiol groups. A variety of cross-linking reagents including chelation of the two thiols by cobalt(III) phenanthroline or covalent reaction with *N,N'-p*-phenylenedimaleimide (pPDM) are effective trapping agents. No trapping of nucleotides occurs in the absence of divalent metals. Thus far Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Ca^{2+} but not Zn^{2+} all function to promote trapping of the 1:1 divalent metal-ADP complex and to enhance the rate of ATPase inactivation. Substitution-inert Cr(III) complexes of ADP, ATP, or pyrophosphate that bind very weakly or not at all to the active site are not trapped by cross-linking. While the stability of the trapped divalent metals varies, e.g., $t_{1/2}$ of 0.5-7 days at 0 °C, they are stable enough to permit accurate spectral measurements of the Mn^{2+} and Co^{2+} trapped complexes. Electron paramagnetic resonance (EPR) measurements of Mn^{2+} bound to 5'-adenylyl imidodiphosphate or complexed to myosin chymotryptic subfragment 1 indicate that the metal is bound at the active site. Circular dichroism (CD) and visible absorption studies of the Co^{2+}·ADP trapped complex indicate the metal ion is in an asymmetric octahedral environment. EPR and CD measurements show that the environment of the metal nucleotide is the same whether bound reversibly or stably trapped at the active site.

Metal ions play a critical role in the hydrolysis of ATP by myosin and actomyosin. The rate of divalent metal ion supported ATPase activity (Seidel, 1969), the catalysis of oxygen-18 exchange (Yount & Koshland, 1963), and the affinity of the M(II)·ADP complex for the enzyme (Malik et al., 1972) critically depend on the divalent metal ion present. Only recently have the structural aspects of the bound metal-nucleotide complex become better understood. A major approach has been the use of thiophosphoryl nucleotide analogues (Goody & Hofmann, 1980; Yee & Eckstein, 1980; Goody et al., 1981; Connolly & Eckstein, 1981) in which different stereoisomers can be shown to be favored for binding and for hydrolysis. The best evidence (Connolly & Eckstein, 1981) indicates that the Δ,β,γ-bidentate metal chelate form of ATP is favored for hydrolysis by myosin and that ADP is liganded to M^{2+} only via its β-phosphate when bound to the active site (Goody et al., 1981). This latter suggestion has been greatly strengthened by the elegant Mn^{2+} EPR[1] studies of Webb et al. (1982) using ^{17}O-labeled ADP-metal complexes bound to SF_1. These studies indicate that ^{17}O substituted on the β-phosphate of ADP but not on the α-phosphate affects the Mn^{2+} EPR spectrum, a result consistent with SF_1 binding only the β-monodentate form of MnADP. Further studies by Webb and co-workers indicate that at least three of the six ligands bound to Mn^{2+} in the MnADP·SF_1 system come directly from the amino acid side chains of SF_1.

Recent studies from our laboratory (Wells & Yount, 1979; Wells et al., 1980a) have shown it is possible to trap MgADP at the active site of myosin by use of a variety of bifunctional thiol cross-linking agents [for a review, see Wells & Yount (1982)]. These findings, in which MgADP was shown to be stably bound for days at 0 °C, suggested that it might be possible to trap a variety of spectroscopically interesting metals to be used as probes of the active site. Such an approach, if the complexes are stably trapped, would make it possible to study metals bound only at the active site without interference from unbound metals or metals bound at auxiliary sites. We report here that it is possible to trap a number of divalent metals including Co^{2+}, Mn^{2+}, Ni^{2+} and Ca^{2+} as a 1:1 complex with ADP following cross-linking by a variety of reagents. No trapping occurred with any of the chromium(III) exchange inert complexes studied. Stability studies indicated that the dissociation rates of trapped M(II)-nucleotide complexes varied widely ($t_{1/2} \simeq$ 0.5-7 days) but were, in general, small enough to allow accurate spectral measurements of the trapped M(II). Absorption and CD studies of trapped Co(II)·ADP indicate the Co(II) is in an asymmetric though nondistorted octahedral environment. EPR spectra of trapped Mn(II)·AMP-PNP or reversibly bound Mn(II)·AMP-PNP were essentially identical and indicate the metal is trapped at the active site. Active site trapping then provides a site-specific and site-exclusive method to probe the microenvironment of the trapped metal ion on a nonmetalloenzyme.

Materials and Methods

Materials. [U-^{14}C]ATP and [8-^{14}C]ADP were from Amersham, and [2,8-^{3}H]AMP-PNP was from ICN. ATP (sodium salt) was from P-L Biochemicals, and AMP-PNP was prepared according to Yount et al. (1971). Ultrapure $(NH_4)_2SO_4$ and Li_3ADP were purchased from Schwarz/Mann. pPDM and phen were obtained from Aldrich. Bis(1,10-phenanthroline)(carbonato)cobalt(III) was prepared according to Ablov & Palade (1961) as previously described (Wells et al., 1979a). The α,β,γ-tridentate and β,γ-bidentate

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[1] Abbreviations: SF_1, myosin chymotryptic subfragment 1; SF_1 (A1) and SF_1 (A2), subfragment 1 isozymes containing the A1 and A2 alkali light chains, respectively; AMP-PNP, 5'-adenylyl imidodiphosphate; pPDM, *N,N'-p*-phenylenedimaleimide; phen, 1,10-phenanthroline; PP_i, pyrophosphate; ϵADP, 1,N^6-ethenoadenosine 5'-diphosphate; EPR, electron paramagnetic resonance; CD, circular dichroism; HMM, heavy meromyosin; LC_2, 18 000-dalton DTNB light chain that binds divalent metal ions; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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chromium complexes of ATP were prepared and purified according to Dunaway-Mariano & Cleland (1980a) and analyzed by paper electrophoresis (De Pamphilis & Cleland, 1973). CrADP (α,β-bidentate) and CrADP (β-monodentate) were prepared according to Bossard & Schuster (1981). The best yields of the β-monodentate CrADP complex were obtained by preheating 40 mM $CrCl_3$ and 40 mM NaADP (grade III, Sigma) to 80 °C separately and rapidly mixing, and the mixture was kept at 80 °C for exactly 1 min before rapidly cooling in an ice-cooled Erlenmeyer flask. The pH was adjusted to 2.0 with 0.1 N HCl, and the complex was purified by chromatography on Dowex 50 exactly as described by Bossard & Schuster (1981). The β-monodentate CrADP was shown to be free of ADP by paper electrophoresis (De Pamphilis & Cleland, 1973) and was shown to be essentially free of α,β-bidentate CrADP by hexokinase assays (Dunaway-Mariano & Cleland, 1980b). All other chemicals were of reagent grade, and water was twice deionized.

Rabbit skeletal myosin was prepared according to Wagner & Yount (1975) and was stored in 50% glycerol at −20 °C. Chymotryptic SF_1 was prepared as described by Weeds & Taylor (1975) and was assumed to have a molecular weight of 115 000 and an $\epsilon_{280}^{1\%} = 7.5\ cm^{-1}$ (Wagner & Weeds, 1977).

Methods. SF_1 (15–18 μM) in solutions containing 1.7 mM divalent metal ion, 0.1 mM nucleotide, 0.1 M KCl, and 50 mM Tris, pH 8.0 (KCl–Tris buffer), was inactivated at 0 °C by addition of cross-linking reagent as indicated. In general, inactivations were quenched by precipitation of the enzyme with 2.5 volumes of saturated $(NH_4)_2SO_4$–20 mM EDTA, pH 8.0. The enzyme derivatives were purified by a second $(NH_4)_2SO_4$ precipitation followed by Sephadex G-25 gel filtration (PD-10 columns, Pharmacia) equilibrated in KCl–Tris buffer at 4 °C. When necessary, protein samples were further concentrated by ultrafiltration (Amicon Model 12). Enzyme buffer solutions used in Ca^{2+} studies were normally passed over Chelex 100 resin columns (Bio-Rad) to reduce the concentration of contaminating calcium.

Analytical and Spectral Procedures. NH_4^+–EDTA ATPase and Ca^{2+}ATPase activities were measured as previously described (Wells et al., 1979b) except release of inorganic phosphate was measured at 2 and 8 min after addition of SF_1 to the assay mixture. Protein concentrations were measured by Coomassie blue dye binding (Bradford, 1976) with unmodified SF_1 as a standard as previously described (Wells et al., 1979a). Metal analysis was performed on 12–50 μM enzyme solutions by atomic absorption (Perkin-Elmer 360) with elemental magnesium (Mallinckrodt) dissolved in 1 N HCl, elemental manganese (Johnson Matthey Chemicals, Ltd.) dissolved in H_2SO_4, $NiCl_2$ (Baker), $ZnCl_2$ (Johnson Matthey Chemicals, Ltd.) or K_2CrO_4 (J. T. Baker Chemical Co.), or $CaCl_2 \cdot 2H_2O$ (Baker) as standards. Liquid scintillation counting was performed on a Beckman LS-230 in aqueous counting scintillant (Amersham).

Individual absorbance measurements were made on a modified Beckman DU, and absorption spectra were recorded on a Varian Superscan 3 spectrophotometer. CD measurements were made on a Jasco CD/ORD-5 instrument. EPR spectra were measured in high-purity quartz cells at 9.25 GHz (X-band) with a Varian E-9 spectrometer. The tryptophan fluorescence studies with CaADP or CaATP were performed on a Perkin-Elmer MPF-3L spectrofluorometer, and those with CrADP complexes were performed at 4 °C in an SLM 4800 spectrofluorometer.

Results

Trapping of Co(II)·ADP by Cobalt–Phen Complexes. It has been previously shown that inactivation of SF_1 by Co(II)/Co(III)·phen complexes in the presence of ADP and a large excess of Mg^{2+} leads to chelation of two thiols by a single Co(III)·$phen_x$ (Wells et al., 1979b) complex with concomitant trapping of MgADP (Wells et al., 1980a). Figure 1 shows that as the concentration of added $MgCl_2$ was decreased from 20 to 2 mM to zero, the cobalt incorporation increased from 1 to 1.3 to 1.8 as the enzyme activity went to zero in each case (see inset of Figure 1). This result is consistent with trapping a single CoADP complex coincident with the incorporation of 1 equiv of the Co(III)·$phen_x$ complex into SF_1. It further indicates the sites for trapping of Mg^{2+} and Co^{2+} are the same. The loss of ATPase activity during cobalt labeling shows an initial lag phase in which 0.15–0.3 cobalt per SF_1 is incorporated without loss of ATPase activity. This nonessential labeling phase likely results from carry-over of adventitious cobalt during purification and/or labeling of residual LC_2 fragments present in SF_1 (Wells et al., 1979b).

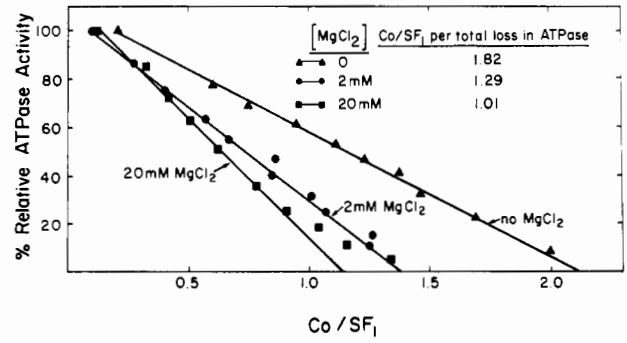

FIGURE 1: Percent relative NH_4^+–EDTA ATPase activity vs. Co/SF_1 incorporated. 14 μM SF_1 was incubated at 0 °C in the presence of 0.14 mM $CoCl_2$, 0.14 mM phen, 1.4 mM $Co^{III}(phen)_2CO_3^+$, 0.1 mM ADP, 0.1 M KCl, 50 mM Tris, pH 8.0, and $MgCl_2$ as indicated. At various times small aliquots were taken for measuring NH_4^+–EDTA ATPase activities as described under Materials and Methods. For measurement of protein-bound cobalt, 8 mg of SF_1 was quenched at various times by addition of EDTA. Samples were freed of extraneous cobalt complexes by two successive $(NH_4)_2SO_4$ precipitations followed by gel filtration (see Materials and Methods). Cobalt concentration was measured by atomic absorption, and protein concentration was measured by the Bradford dye binding assay.

Trapping of M(II)–Nucleotides by pPDM. The above studies with cobalt indicated that divalent metals other than Mg could be trapped at the active site of myosin. In order to study the trapping of other metals systematically, it was necessary to use the bifunctional cross-linking agent pPDM. pPDM has been shown previously to be equally effective to the Co(II)/Co(III)·phen system in trapping Mg–nucleotide complexes and it avoids the complication of having cobalt present with other metals of interest (Wells & Yount, 1979; Wells et al., 1980a). Figure 2 shows that in addition to MgADP a variety of M(II)·ADP complexes (e.g., Co^{2+}, Ni^{2+}, Mn^{2+}, Ca^{2+}) stimulated the rate of inactivation of SF_1 by pPDM in comparison to a M(II)·ADP-free control. The divalent metal ion dependence for inactivation was $Mg^{2+} \simeq Co^{2+} > Mn^{2+} > Ni^{2+} > Ca^{2+}$. Stimulation of inactivation of SF_1 by pPDM was not observed for Zn(II)·ADP (Figure 2A), β-monodentate Cr(III)·ADP, or β,γ-bidentate Cr(III)·ATP (Figure 2B).

The data in Table I show that those M(II)·ADP complexes that stimulated inactivation of SF_1 by pPDM were trapped as a 1:1 complex with [^{14}C]ADP immediately following inactivation. Initially no trapping of CaADP was observed even though it stimulated the rate of inactivation of SF_1 by pPDM (see Figure 2B). This anomaly was resolved when it was observed that the $(NH_4)_2SO_4$ precipitation step released the

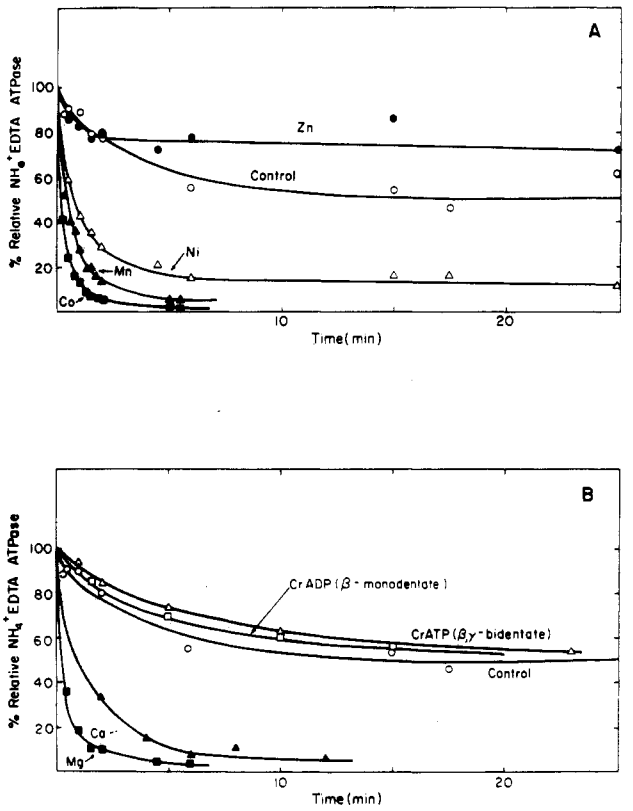

FIGURE 2: Inactivation of SF_1 NH_4^+-EDTA ATPase by pPDM cross-linking in the presence of various divalent metal ions and ADP. SF_1 (17 μM) at 0 °C in 0.1 M KCl, 50 mM Tris, pH 8.0, 1.6 mM divalent metal ion, and 0.14 mM ADP was treated with a 1.3-fold excess of pPDM. At indicated times, 25-μL aliquots were assayed for NH_4^+-EDTA ATPase activity as described under Materials and Methods. (Panel A) Inactivations in the presence of Zn^{2+}, Ni^{2+}, Mn^{2+}, and Co^{2+}. (Panel B) Inactivations in the presence of Mg^{2+}, Ca^{2+}, and 0.85 mM β-monodentate Cr(III)·ADP or 0.85 mM β,γ-bidentate Cr(III)·ATP.

Table I: Stoichiometry of Trapped Divalent Metal and ADP in pPDM-Inactivated SF_1 [a]

added metals	% remaining ATPase activities [b] NH_4^+	Ca^{2+}	metal/SF_1 [c]	ADP/SF_1 [c]
Mg^{2+}	2	3	0.71	0.70
Co^{2+}	6	24	0.74	0.75
Ni^{2+}	17	30	0.65	0.70
Mn^{2+}	1	9	0.60	0.64
Ca^{2+}	5	8	0.65	0.70
Zn^{2+}	70	85	0.07	0.10
none	59	71		0.05

[a] SF_1 (16–30 μM) was treated with 1.3-fold excess pPDM in 0.1 M KCl–50 mM Tris, pH 8.0, at 0 °C for 25 min in the presence of 1.6 mM divalent metal ion and 0.1 mM ADP. Enzyme derivatives except Ca^{2+} were purified by two $(NH_4)_2SO_4$ precipitations in the presence of 20 mM EDTA (pH 8.0) followed by Sephadex G-25 chromatography as described under Materials and Methods. The CaADP solutions were 56 μM SF_1 and were purified by Sephadex G-25 chromatography only (see text). [b] NH_4^+-EDTA ATPase and Ca^{2+}ATPase activities were determined following purification of cross-linked enzyme derivatives. Activities are expressed relative to appropriate non-cross-linked controls. [c] A control incubated with each M^{2+}ADP but without pPDM was used to correct for the background metal ion and ADP resulting from nontrapped binding and/or carry-over during purification. Such controls typically gave less than 0.1 metal or ADP/SF_1.

CaADP. Subsequently, it was found that high ionic strengths, in general, destabilize trapped metal nucleotides. For example, [^{14}C]ADP trapped on SF_1 by pPDM in the presence of Ca^{2+} or Mn^{2+} and purified without $(NH_4)_2SO_4$ precipitation was found to be 6 times more stable in 0.1 M KCl than in 2.0 M KCl. Less than stoichiometric trapping of M(II)·ADP likely resulted from incomplete pPDM modification as well as from a small proportion of preexisting inactive enzyme. The approximate parallel loss of CaATPase and NH_4^+-EDTA ATPase activities after 25 min suggests cross-linking of SH 1 and SH 2 has occurred. The higher CaATPase activities in some cases probably result from some of the SF_1 being modified at SH 1 only. Zn(II), which did not stimulate inactivation by pPDM, was not trapped to a significant degree.

Table II: Inactivation of SF_1 with pPDM in the Presence of Cr(III) Complexes[a]

added metal nucleotides	% remaining ATPase activity NH_4^+	Ca^{2+}	metal/SF_1
none	59	71	
Cr(III)·ADP (α,β-bidentate)	60	73	0.07
Cr(III)·ADP (β-monodentate)	60	68	0.04
Cr(III)·ATP (α,β,γ-tridentate)	69	81	0.06
Cr(III)·ATP (β,γ-bidentate)	68	58	0.06
Cr(III)·PP_i	65	76	0.08
MgADP	2	3	0.77
$MgPP_i$	15		0.71

[a] SF_1 (16 μM) was treated with 1.3-fold excess pPDM in the presence of 0.8 mM Cr(III)–nucleotide and 0.1 mM KCl–50 mM Tris, pH 8.0 for 25 min. The MgADP and $MgPP_i$ reactions were 1.6 mM $MgCl_2$ and 160 μM ADP or PP_i. Enzyme derivatives were purified by two $(NH_4)_2SO_4$ precipitations in the presence of 20 mM EDTA, pH 8.0, followed by Sephadex G-25 chromatography. Cr and Mg concentrations were determined by atomic absorption. NH_4^+-EDTA ATPase and Ca^{2+}ATPase activities were determined as described in Table I. Controls as in Table I were used to correct for Mg or Cr contamination in the buffers and for nonspecific binding.

Trapping Experiments with Cr(III)–Nucleotides. It was of interest to try to trap Cr(III)–nucleotide complexes since Cr(III) is paramagnetic and could be used in conjunction with EPR or NMR techniques as probes of the active site of myosin. In addition, the trapping specificity could reveal which metal ion complex of ADP or ATP is necessary for binding. Previous studies (Wells et al., 1980a,b) have shown that a divalent metal is essential for stable trapping of nucleotides. The data in Table II show that none of the Cr(III)·ADP or Cr(III)·ATP complexes tested were trapped under conditions where Mg-ADP is trapped stoichiometrically. Cr(III)·PP_i was also not trapped even though Mg·PP_i is trapped stoichiometrically. It was possible that the β-monodentate Cr(III)·ADP had isomerized to α,β-bidentate Cr(III)·ADP or broken down to free ADP during the 25 min allowed for trapping to occur. However, the half-life of β-monodentate CrADP was found to be approximately 45 min under the reaction conditions used (0.1 M KCl, 50 mM Tris, pH 8.0 at 0 °C) by using the inhibition of hexokinase as an indication of the amount of β-monodentate isomer remaining. In addition, trapping studies performed at pH 7.0 where the β-monodentate CrADP was more stable ($t_{1/2} \simeq 5$ h) also show no significant trapping of Cr(III) and no promotion of inactivation of SF_1 by pPDM.

Stability of Trapped M(II)–Nucleotide Complexes. To evaluate the suitability of trapped transition metal–nucleotide complexes as stable spectral probes, it was necessary to measure their respective off-rates from SF_1 following pPDM cross-linking. The off-rate of the M(II)·ADP complex varied with the nature of metal ion trapped as shown in Figure 3. Panel A shows the retention of M(II) following gel filtration as a function of time after cross-linking. The order of in-

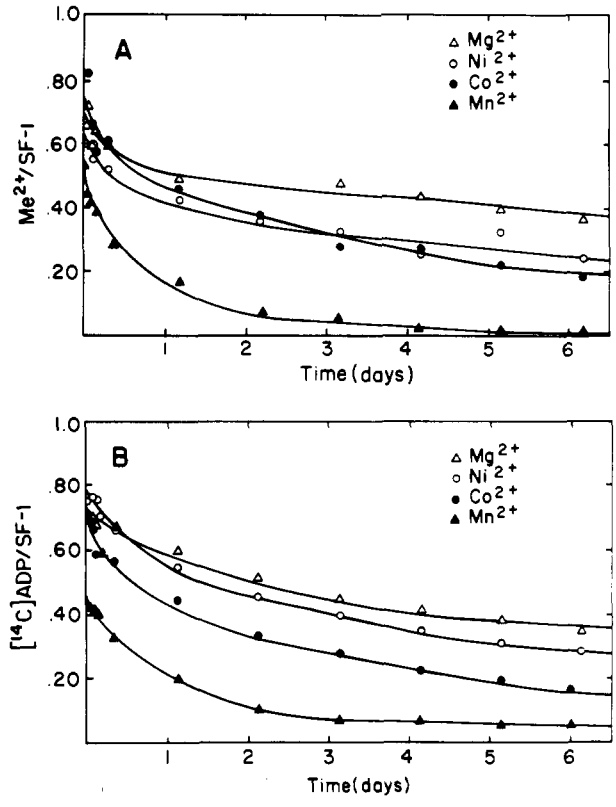

FIGURE 3: Retention of metal ions and ADP trapped on SF_1 by cross-linking with pPDM. SF_1 (16 μM) was treated with 1.3-fold excess pPDM in the presence of 1.6 mM metal ion and 0.1 mM ADP. Enzyme derivatives were then purified by two $(NH_4)_2SO_4$ precipitations in the presence of 20 mM EDTA followed by Sephadex G-25 chromatography. [^{14}C]ADP and metal ion determinations were analyzed on the same pPDM-modified SF_1. (Panel A) Retention of divalent metal ion from pPDM-modified SF_1 after gel filtration at the indicated time. The metal ion concentration was determined by atomic absorption as described under Materials and Methods. (Panel B) Retention of [^{14}C]ADP from pPDM-modified SF_1. The [^{14}C]ADP concentration was determined by scintillation counting.

creasing stability was Mn^{2+}, Co^{2+}, Ni^{2+}, and Mg^{2+} with apparent half-lives of 0.5, 2, 4, and 7 days, respectively, under these conditions. The release of trapped [^{14}C]ADP (panel B) was nearly coincident with the release of metal, yielding apparent half-lives of 0.8, 1.8, 3.5, and 7 days for the Mn^{2+}, Co^{2+}, Ni^{2+}, and Mg^{2+} complexes, respectively. The apparent half-life measured here is equal to the time it takes to release 50% of the initial trapped metal(II) or nucleotide.

The stability of trapped Mn^{2+} with other nucleotides was investigated. Figure 4 shows the order of off-rates for Mn(II) nucleotides was ϵADP ($t_{1/2} \simeq 3$ h), ADP ($t_{1/2} \simeq 8$ h), and AMP-PNP ($t_{1/2} > 50$ h). These studies (Figures 3 and 4) suggest the M(II)–nucleotide is released as a complex at a rate that depends on both the nature of the metal and nucleotide trapped.

Spectral Measurements with Trapped M(II)–Nucleotide Complexes. Binding of CaADP and CaATP to SF_1 is accompanied by characteristic increases in the intrinsic tryptophan fluorescence (Werber et al., 1972). This effect was used to compare the binding vs. trapping of Ca^{2+} nucleotides on the local environment about these sensitive tryptophan residues. Table III shows that CaATP (line 1) and CaADP (line 2) increase the tryptophan fluorescence of SF_1. CaADP was stably trapped by pPDM cross-linking (line 3) and gave tryptophan fluorescence values characteristic of that elicited by CaADP binding. This fluorescence value was unchanged by addition of CaATP, indicating that the CaADP could not

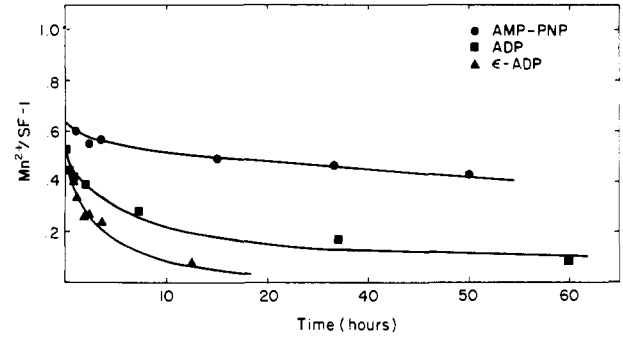

FIGURE 4: Retention of Mn^{2+} from SF_1 inactivated with pPDM in the presence of various Mn^{2+}–nucleotide complexes. SF_1 (17 μM) was incubated with a 1.3-fold excess of pPDM for 25 min in the presence of 1.7 mM $MnCl_2$ and 0.14 mM indicated nucleotide. Samples were purified and analyzed as described in Figure 3.

Table III: Enhancement of Tryptophan Fluorescence upon Binding or Trapping of CaADP and CaATP[a]

solution	% relative fluorescence: initial	% relative fluorescence: +0.25 mM ATP	% NH_4^+-EDTA ATPase
(1) SF_1	100	127	100
(2) CaADP·SF_1	105 ± 1	126 ± 3	100
(3) CaADP·SF_1 + pPDM[b]	110 ± 1	108 ± 2	1
(4) CaATP·SF_1 + pPDM[b]	110	110	1

[a] Enzyme solutions (16 μM) in 2 mM $CaCl_2$ and 0.1 mM nucleotide, 0.1 M KCl, and 50 mM Tris, pH 7.5 (0 °C), were diluted 1 to 12 by KCl-Tris buffer at 25 °C before fluorescence measurements were made. [b] Solutions were treated with 1.3 molar excess of pPDM over SF_1 for 30 min before fluorescence measurements were made.

be displaced. The trapping of CaATP (line 4) gave tryptophan fluorescence values appropriate for CaADP binding, a result consistent with earlier observations (Wells & Yount, 1979) that ATP is cleaved during the cross-linking step and trapped as ADP. Again, addition of excess CaATP after trapping (line 4) did not change the fluorescence observed. The lower relative fluorescence value of SF_1 with free CaADP (line 2) vs. trapped CaADP (lines 3 and 4) is the result of insufficient CaADP being present to saturate the active site after the 1 to 12 dilution required to make the fluorescence measurements. The dilutions did not affect the fluorescence values of the trapped CaADP or CaATP (ADP) SF_1 reactions since the nucleotides were stabilized at the active site by cross-linking.

The observation that MnAMP-PNP was stably trapped by pPDM cross-linking (Figure 4) indicated Mn EPR should be useful as a means to compare the microenvironment about MnAMP-PNP trapped or reversibly bound to SF_1. Figure 5 shows that addition of an excess of SF_1 over MnAMP-PNP results in a dramatic broadening of the Mn EPR spectrum similar to that previously reported for the binding of MnADP to SF_1 (Bagshaw & Reed, 1976). The EPR spectrum of MnAMP-PNP trapped by pPDM cross-linking (spectrum c) is nearly the same as that for MnAMP-PNP bound reversibly. The additional fine structure seen in the trapped spectrum (spectrum c) not seen in the reversibly bound spectrum (spectrum b) results from the presence of a small amount of free MnAMP-PNP. With time (spectra d and e), the trapped spectrum gradually changes to that of the free spectrum of Mn^{2+}. The kinetics of this spectral change were similar to the off-rate of MnAMP-PNP as measured directly by atomic absorption (Figure 4).

The ability to trap CoADP (Table I) and the stability of this complex once trapped (Figure 3) allowed the microen-

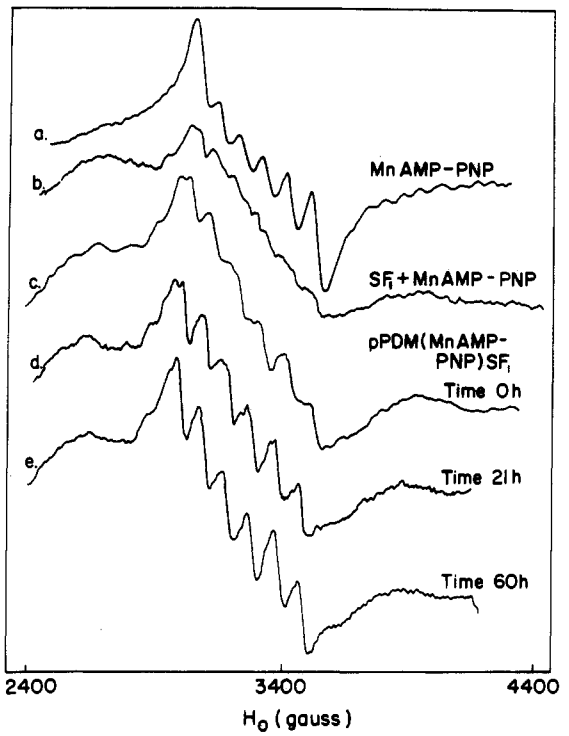

FIGURE 5: Mn^{2+} EPR spectra of free Mn·AMP-PNP and Mn·AMP-PNP bound reversibly or trapped on SF_1. All solutions contained 0.1 M KCl–50 mM Tris, pH 8.0 at 0 °C. EPR measurements were taken on a Varian E-9 instrument at 1–2 °C with a modulation amplitude of 32 G and gain setting of 8000. (Spectrum a) 0.48 mM $MnCl_2$ and 10.5 mM AMP-PNP; (spectrum b) 0.29 mM $MnCl_2$, 10.0 mM AMP-PNP, and 0.6 mM SF_1; (spectra c–e) 0.23 mM Mn·AMP-PNP trapped on SF_1 (0.48 mM). Spectra taken at 0, 21, and 60 h, respectively. This latter solution was prepared by inactivating 40 mL of 16 μM SF_1 with pPDM in the presence of 1.6 mM $MnCl_2$ and 160 μM AMP-PNP. After purification by $(NH_4)_2SO_4$ precipitations followed by Sephadex G-25 chromatography, this sample was concentrated at 2 °C by use of ultrafiltration (Amicon PM-10).

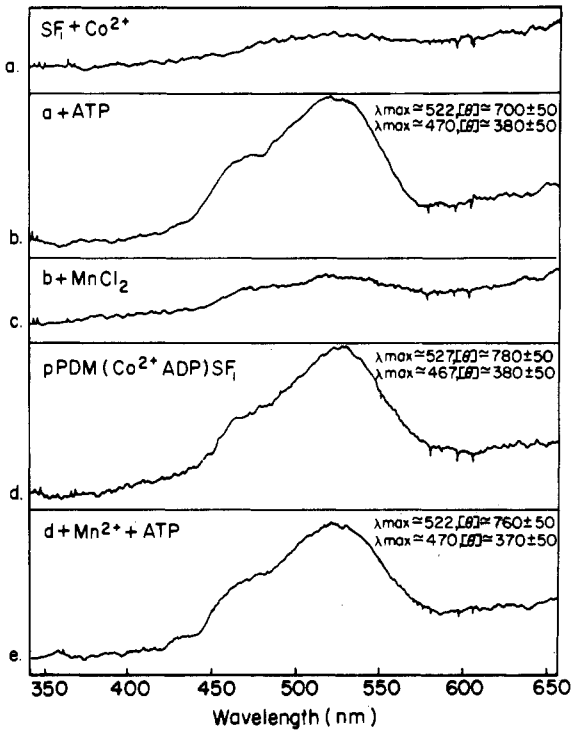

FIGURE 6: CD spectra of cobalt SF_1. Conditions: (spectrum a) 86 μM $CoCl_2$ and 43 μM SF_1 in 0.1 M KCl–50 mM Tris, pH 8.0 at 4 °C; (spectrum b) same as sample in spectrum a plus 86 μM ATP; (spectrum c) addition of a final concentration of $MnCl_2$ to 0.86 mM to sample in spectrum b; (spectrum d) 47 μM CoADP trapped on 71 μM SF_1 by pPDM cross-linking. The solution was prepared as described under Materials and Methods; (spectrum e) same as sample in spectrum d plus addition of $MnCl_2$ and ATP to 1.4 and 0.14 mM, respectively. The CD measurements were made on a Jasco CD/ORD 5 instrument at 1 °C in a 10-cm path-length cell. All molar ellipticities given in spectra b, d, and e were corrected for background absorbance.

vironment of the trapped vs. reversibly bound CoADP complex to be compared by CD spectroscopy. Spectrum a in Figure 6 shows there was no detectable CD spectrum produced between 350 and 650 nm upon mixing Co^{2+} and SF_1. Addition of ADP results in two CD active transitions: one at approximately 520 nm and a second of approximately half the intensity at 470 nm (spectrum b). Addition of CD-silent Mn^{2+} at 20-fold excess over Co^{2+} (spectrum c) eliminated the CoADP-bound spectrum as Mn^{2+} displaced the Co^{2+}. Trapping of CoADP by pPDM cross-linking results in a CD spectrum essentially identical with that for CoADP bound reversibly to SF_1 (compare spectra b and d). However, addition of Mn^{2+} to the pPDM cross-linked CoADP enzyme derivative (spectrum e) had no effect on the CD spectrum, indicating trapped Co^{2+} was not displaced. The visible spectrum of CoADP trapped in SF_1 with pPDM showed a maximum absorption at approximately 520 nm ($\epsilon \leq 15\ M^{-1}\ cm^{-1}$), coincident with the most prominent CD transition (data not shown).

Discussion

The results presented show that the M(II)·ADP complexes (e.g., with Mg^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, and Ca^{2+}) previously shown to bind with high affinity to the active site (Malik et al., 1972) promote cross-linking and concomitant inactivation of SF_1 by pPDM (Figure 2). Furthermore, those divalent metals that promote inactivation also become trapped on SF_1 (Table I). Zn(II) that did not promote pPDM inactivation was not trapped to a significant extent. Zn(II) actually appeared to protect against inactivation (Figure 1). This is not surprising considering Zn likely binds to the same thiols that are cross-linked by pPDM. These M(II)·ADP-stimulated modifications with pPDM and subsequent trapping studies complement previous experiments, which demonstrated a variety of Mg–nucleotide complexes that stimulate thiol cross-linking become trapped (Wells et al., 1980b).

The Cr(III) exchange-inert nucleotide complexes investigated neither promoted inactivation by pPDM (Figure 2B) nor become trapped following treatment with pPDM (Table II). These results are consistent with the fluorescence studies of Eccleston & Trentham (1978) that demonstrated β,γ-bidentate Cr(III)·ATP and α,β-bidentate Cr(III)·ADP bound very weakly if at all to SF_1. We initially attempted trapping β-monodentate Cr(III)·ADP since the report by Yee & Eckstein (1980) suggested it was a potent competitive inhibitor (K_i = 3 μM) of myosin's MgATPase activity. It is shown here that β-monodentate Cr(III)·ADP did not promote inactivation by pPDM nor was it trapped. Furthermore, no increase in the intrinsic tryptophan fluorescence was observed (data not shown) when this complex was added to SF_1, suggesting it was not binding. Other more recent studies (Connolly & Eckstein, 1981; Goody et al., 1981) have been unable to repeat the earlier β-monodentate Cr(III)·ADP inhibition findings. These results suggest that either impurities in the initial Cr(III)·ADP preparation or breakdown to ADP were responsible for the inhibition observed. In our hands, β-monodentate Cr(III)·ADP at pH 7–8 primarily rearranges to α,β-bidentate Cr(III)·ADP, a result consistent with the studies of Bossard et al. (1982).

In any case the β-monodentate Cr(III)·ADP was stable enough to have been trapped had it bound in a manner analogous to MgADP. The data presented here indicating the absence of tight binding by any Cr(III)–nucleotide complex may reflect the requirement for a change in the hydration sphere of MgADP for tight binding to myosin (i.e., exchange of metal-bound H_2O for a ligand from the enzyme). The exchange-inert nature of the Cr(III)–nucleotide complexes would prevent such rearrangement or exchange as suggested by Eccleston & Trentham (1978).

The dissociation rate of trapped M(II)·ADP complex varied greatly depending on the metal ion and the nucleotide present. For instance, MgAMP-PNP was found to be the most stable complex whereas Mnε-ADP is the least stable of the complexes studied to date. The rates of dissociation for a given M(II) and nucleotide were comparable, suggesting they are released as a complex. In agreement with earlier studies (Wells et al., 1980b), trapping of a metal ion requires the presence of either pyrophosphate or a nucleotide diphosphate. In addition, it was found the rate of release of trapped metal nucleotide is increased in high salt. For example, when $(NH_4)_2SO_4$ precipitations were used to purify pPDM CaADP·SF_1 derivatives, less than 0.2 Ca[^{14}C]ADP/SF_1 was found after two precipitations. Thus exposure to high salt should be avoided to prevent loss of the less stable M(II)–nucleotide complexes.

The kinetics of release of trapped M(II)–nucleotide did not follow a single exponential rate process. Replots of the data in Figure 3 for CoADP and NiADP on semilog scales indicate the rates are biphasic. This result may reflect different rates of release of trapped M(II)–nucleotide by the isozymes SF_1 (A1) and SF_1 (A2) or it may indicate a heterogeneity in the thiol groups cross-linked. Experiments are in progress to establish the exact cysteines that are modified.

The spectroscopic evidence strongly suggests that the divalent metal nucleotide is trapped at the active site. Simple binding or trapping of CaADP, MnAMP-PNP, and CoADP led to nearly the same intrinsic tryptophan fluorescence enhancement (Table III), EPR spectra (Figure 5), and CD spectra (Figure 6), respectively. In each case the environment about the metal nucleotide was not changed by pPDM cross-linking. However, spectral changes induced by simple binding of M(II) nucleotides could be distinguished from those induced by trapping by the fact that they were reversible whereas changes induced by trapping were irreversible.

The broadened EPR spectra of MnAMP-PNP bound or trapped on SF_1 are characteristic of an immobilized Mn^{2+} relative to MnAMP-PNP free in solution (Figure 5). The increased fine structure of the EPR spectrum observed as a function of time suggested that MnAMP-PNP was leaking out irreversibly. Hence, it appears once metal nucleotide leaks out, the trapping site remains relatively inaccessible to metal nucleotide. An alternative explanation is that the enzyme isomerizes following release so as not to rebind MnAMP-PNP.

The metal nucleotide binding pocket on SF_1 is in an asymmetric environment since Co(II) d–d transitions were observed by CD measurements of CoADP·SF_1. The energies at which these transitions occur and the ε value of less than 15 M^{-1} cm^{-1} at 525 nm strongly suggest that the cobalt is in a nondistorted octahedral environment. Typically, tetrahedral and octahedral cobalt(II) complexes can be distinguished from each other since tetrahedral Co(II) d–d transitions occur at much lower energies (higher wavelengths) and have molar absorptivities about 100 times larger than those of octahedral complexes (Carlin, 1965). In fact, the visible spectrum of octahedral CoADP (ϵ_{517nm} = 10 M^{-1} cm^{-1}) is essentially identical with that of CoADP trapped on SF_1.

Recently, Webb et al. (1982) have proposed on the basis of Mn(II) EPR studies of MnADP·SF_1 systems that Mn(II) is in an octahedral environment and is chelated directly by three protein ligands. Our CD and visible measurements of CoADP·SF_1 support their suggestion of an octahedral environment about the metal ion. In addition, our inability to trap any of the chromium(III) exchange-inert nucleotide complexes is also consistent with the requirement that protein ligands must displace waters bound to Cr(III) for effective binding to occur. Finally, since we did not observe charge transfer bands for Co(II) on CoADP-trapped SF_1, it is unlikely that one of the protein ligands is sulfur (Cotton & Wilkinson, 1980).

In conclusion, trapping of spectroscopically interesting metal ions by thiol cross-linking can offer some distinct advantages over a system that allows the metal nucleotide to bind reversibly. Problems associated with a heterogeneous population of bound and free probes when approaching active site saturation are almost completely eliminated by trapping. There is only one site of stable trapping of M(II) nucleotides, and this eliminates binding of the M^{2+} to other metal binding sites in myosin, e.g., the metal binding site of LC_2 (Bagshaw & Reed, 1976). This has not been a major problem in studies of chymotryptic SF_1 since it does not contain significant amounts of LC_2, but it would be expected to be a significant problem for studies of HMM or myosin. Finally, trapped transition metal–nucleotide complexes may prove useful in topographic mapping of the active site by NMR (Mildvan, 1977), EPR, or fluorescence energy transfer techniques (Villafranca et al., 1978).

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Registry No. MgADP, 7384-99-8; Co(II)·ADP, 78969-59-2; Mn(II)·ADP, 69828-68-8; Ni(II)·ADP, 83862-74-2; Ca(II)·ADP, 79189-47-2; Ca·ATP, 15866-84-9; Mn(II)·AMP-PNP, 82050-25-7; Mn(II)·εADP, 83862-75-3; Co(III)·phen, 75559-30-7; pPDM, 3278-31-7.

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Antithrombin III Binding to SC5b-9 Attack Complexes of Human Complement: Dissociation of a Modified Antithrombin III Derivative Subsequent to Complex Formation†

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ABSTRACT: Antithrombin III (ATIII) was firmly bound to fluid-phase complement attack complexes assembled upon complement activation of normal human serum (SC5b-9 complexes). Analysis of gel filtration column profiles of SC5b-9 complex preparations by double immunodiffusion and immunoreplication procedures employing goat anti-human ATIII antibodies clearly indicated the presence of ATIII antigenic determinants in the 1×10^6 molecular weight, SC5b-9 complex containing fractions. Inclusion of ethylenediaminetetraacetic acid in normal human serum prior to the addition of complement activators inhibited SC5b-9 complex formation as well as the appearance of ATIII in gel filtration column fractions containing 1×10^6 molecular weight proteins or protein complexes. Although ATIII was bound firmly to SC5b-9 complexes, as demonstrated by gel filtration column chromatography in the presence of 0.5 M NaCl and 0.1% Triton X-100 and by rocket immunoelectrophoresis, the majority of SC5b-9 complex associated ATIII was dissociated upon incubation with 4 M guanidine hydrochloride or 10% deoxycholate for 16 h at 37% C (the SC5b-9 complex was not dissociated under these conditions). Anti-ATIII immunoreplication procedures further demonstrated that SC5b-9 complex associated ATIII comigrated with C9 as a 70000 molecular weight protein when subjected to sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis under nonreducing conditions. In addition, it was demonstrated that functionally active complement attack complexes assembled on biological membranes (MC5b-9 complexes) were completely devoid of ATIII. Therefore, it appears the protease inhibitor antithrombin III has the potential to react with and thereby modulate complement attack complex assembly and functional activity. The biochemical basis and the biological significance of this interaction are discussed.

Complement (C)[1] is a sequential, multicomponent system of plasma proteins which can be activated by a variety of immunological as well as nonimmunological stimuli (Müller-Eberhard, 1975). C activation can proceed via either the classical (Müller-Eberhard, 1969) or the alternative (Müller-Eberhard & Schreiber, 1980) pathway through a series of cascading reaction steps which are dependent upon the conversion of serum zymogens to serine active site, trypsin-like esterase enzymes (Porter & Reid, 1979; Götze, 1975). Activation of either C pathway results in the expression of

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[1] Abbreviations: Nomenclature for complement proteins follows that set forth by the World Health Organization (1982); C, complement; EDTA, ethylenediaminetetraacetate; TEMED, *N,N,N′,N′*-tetramethylenediamine; NHS, normal human serum; NHSE, normal human serum containing 10 mM EDTA; PBS, phosphate-buffered saline containing 5 mM potassium phosphate and 145 mM NaCl, pH 7.4; PBSE, PBS containing 10 mM EDTA; $NaDodSO_4$, sodium dodecyl sulfate; DOC, deoxycholate; ATIII, antithrombin III protease inhibitor; protein A, *Staphylococcus aureus* protein A; HSA, human serum albumin; SC5b-9, C5b-9 complexes containing S protein assembled in NHS upon complement activation; MC5b-9, C5b-9 complexes assembled on biological membranes upon complement activation; Cl_3CCOOH, trichloroacetic acid; Gdn·HCl, guanidine hydrochloride.

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