Inactivation of Myosin Subfragment One by Cobalt(II)/Cobalt(III) Phenanthroline Complexes. 1. Incorporation of Co(III) by in Situ Oxidation of Co(II)†

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ABSTRACT: Myosin's chymotryptic subfragment one (SF_1) ATPase activity is rapidly inactivated in a mixture containing $CoCl_2$, 1,10-phenanthroline (phen), and $[Co^{III}(phen)_2CO_3]^+$. The inactivation results from the incorporation of exchange-inert Co(III). The inactivation can be quenched but not reversed by addition of EDTA in quantities stoichiometric with Co^{2+} and can be completely reversed by addition of a variety of reducing agents such as $NaBH_4$, $Fe^{II}EDTA$, cobaltous cyanide, or $[Co(phen)_3]^{2+}$ which allow removal of cobalt(III) and restoration of ATPase activity. $^{57}Co(II)$ labeling studies indicate that greater than 93% of the cobalt incorporated into SF_1 is incorporated via a Co(II) intermediate. These studies and others support a mechanism whereby an exchange-labile $Co^{II}(phen)_x$ complex is rapidly and reversibly chelated by SF_1. The $Co^{II}(phen)_x$·enzyme complex is oxidized in situ by $[Co^{III}(phen)_2CO_3]^+$ to yield an exchange-inert $Co^{III}(phen)_x$·SF_1 complex with no ATPase activity. Inactivations of SF_1 which result from $Co^{II}(phen)_x$ in situ oxidation by either H_2O_2 or O_2 are not reversed by reducing agents, despite the removal of bound cobalt. These data indicate in situ oxidation of $Co^{II}(phen)_x$ by the single electron-accepting oxidant $[Co^{III}(phen)_2CO_3]^+$ results in a very mild incorporation of Co(III) into SF_1. This approach may have general application for the incorporation of Co(III) into proteins.

While there has been significant progress toward delineating the kinetic steps and rate constants of ATP hydrolysis by myosin (Bagshaw & Trentham, 1974; Trentham et al., 1976) and by actomyosin ATPase (Eisenberg & Moos, 1970; Lymn & Taylor, 1971), there has been much less progress made toward elucidation of the structure and location of the ATPase site in the myosin molecule. Location and structural determination of the ATPase site is a critical step in determining how the chemical energy from ATP hydrolysis is coupled to movement by the myosin molecule in contractile systems. Use of ATP analogues (Yount, 1975) which have the potential to label the active site of myosin have only recently begun to show promise. A nitrophenyl azide photoaffinity derivative of ATP (Jeng & Guillory, 1975) inactivates myosin in an irreversible light-dependent manner and appears to label a 25 000 peptide from the amino-terminal end of the heavy chains of myosin (Szilágyi et al., 1979).

An alternative approach to affinity labeling of myosin has been reported by Werber et al. (1974). These workers reported labeling the MgATPase site of myosin with $Co^{III}(phen)ATP(O_2^-)$[1] by a ligand substitution mechanism. They proposed that, by mimicking the substrate, $Co^{III}(phen)ATP(O_2^-)$ bound to the MgATP site where a more stable ligand from the enzyme replaced the less stable superoxide ligand. This was believed to result in a kinetically inert $Co^{III}(phen)$-ATP·myosin complex. The apparently facile ligand substitution in $Co^{III}(phen)ATP(O_2^-)$ offered a distinct advantage to incorporation of Co(III) by in situ oxidation of Co(II) since substitution avoided the use of potentially damaging peroxide oxidants. Affinity labeling with a Co(III) probe has the additional advantage that it can be readily reversed by reducing agents which convert the exchange-inert cobalt(III) back to exchange labile Co(II).

Further investigations of $Co^{III}(phen)ATP(O_2^-)$ (J. Wells, M. Werber, and R. Yount, unpublished experiments) indicated that standard preparations of $Co^{III}(phen)ATP(O_2^-)$ were impure and rapidly decomposed at pH 8.0 in the presence of Tris buffer to yield a mixture of cobalt complexes. Despite this decomposition the mixture remained effective with respect to its ability to inhibit myosin's ATPase activity. Chromatography of the decomposition mixture allowed isolation and characterization of purified cobalt complexes which caused the ATPase inhibition. We report here that two complexes, Co^{II}phen and $[Co^{III}(phen)_2CO_3]^+$, act together to rapidly inactivate myosin's ATPase activity. Inactivation results from the incorporation of Co(III) via an in situ oxidation of an enzyme-bound $Co^{II}(phen)_x$ complex by $[Co^{III}(phen)_2CO_3]^+$. The inactivation is markedly stimulated by addition of MgADP. This mechanism is in contrast to incorporation of a Co(III) complex by direct ligand substitution.

ATPase inhibition following cobalt incorporation is highly reversible by a variety of reducing agents unlike inhibition following in situ oxidation of Co^{II}phen using either H_2O_2 or dissolved oxygen as the oxidant. The use of $[Co^{III}(phen)_2CO_3]^+$ as a mild oxidant for incorporation of Co(III) into myosin chymotryptic SF_1 is a novel approach for in situ oxidation of a protein-bound Co(II) complex. Use of Co(III) complexes as mild oxidants to in situ oxidize protein-bound Co(II) complexes may be of general use.

Materials and Methods

Materials

Carrier-free $^{57}CoCl_2$ in 0.1 M HCl and $CoCl_2·6H_2O$ were obtained from Amersham and Mallinckrodt, respectively. Spectroscopically pure $CoSO_4·7H_2O$ (Johnson Matthey Chemicals, Ltd.) was used as a cobalt standard for atomic

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[1] Abbreviations used: phen, 1,10-phenanthroline; SF_1, chymotryptic subfragment one; Co-SF_1, subfragment one inactivated with Co^{II}/Co^{III}phen complexes; DTE, dithioerythritol; en, ethylenediamine; EDDA, ethylenediamine-*N*,*N*′-diacetic acid.

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absorption spectroscopy. 1,10-Phenanthroline (Aldrich) was used without further purification. Na_2ATP and Li_3ADP were obtained from P-L Biochemicals and Schwarz/Mann, respectively. α-Chymotrypsin was obtained from Sigma Chemical Co. All other chemicals were of reagent grade. Water was doubly deionized.

A Cary-14 spectrophotometer was used for all spectral determinations. Cobalt atomic absorption was performed on a Perkin-Elmer 360 atomic absorption spectrophotometer, and ^{57}Co was determined by using a Beckman γ-4000 counter.

Methods

Synthesis and Characterization of $[Co(phen)_2CO_3]Cl\cdot 4.5H_2O$. Bis(phenanthroline)(carbonato)cobalt(III) was synthesized and twice recrystallized as described by Ablov & Palade (1961) by heating a suspension of recrystallized $[Co(phen)_2Cl_2]Cl$ (Ablov, 1961) in the presence of sodium carbonate. The $[Co(phen)_2CO_3]\cdot Cl$ crystals were dried in a vacuum desiccator overnight and subsequently dried in vacuo at 25 °C for 1 h before elemental analyses (Galbraith Laboratories). Anal. Calcd for $[Co(phen)_2CO_3]Cl\cdot 4.5H_2O$; C, 50.22; H, 4.18; N, 9.37; Cl, 5.94. Found: C, 50.48; H, 4.41; N, 9.39; Cl, 6.08.

Qualitatively, the spectrum of the carbonato complex was the same as that reported by Ablov & Palade (1961) and was pH independent from pH 2 to pH 10. High-resolution proton NMR spectra of the carbonato complex were identical with those reported by Francis & Jordan (1972) and showed no apparent broadening, indicating the absence of large quantities of cobalt(II). However, the molar absorptivities based on cobalt (atomic absorption) were higher than those previously reported (Ablov & Palade, 1961; Francis & Jordan, 1972) at 509 nm (ϵ = 133), 350 nm (ϵ = 2300), 333 sh (ϵ = 3200), 317 sh (ϵ = 6900), 295 sh (ϵ = 22000), 272 nm (ϵ = 68000), and 222 nm (ϵ = 82000) and minima at 435 (ϵ = 25) and 242 (ϵ = 30000).

The purity of the $[Co(phen)_2CO_3]^+$ complex was evaluated in part by silica thin-layer chromatography (20 cm long × 0.25 mm thick; aluminum bonded), developing with pyridine–H_2O–glacial acetic acid (50:35:15 v/v/v) or 1-propanol–H_2O–acetic acid–2 M triethylamine bicarbonate (50:35:15:5). The carbonato complex ran as a single spot in both of these solvent systems with R_f values of 0.48 and 0.40, respectively. Cobalt-containing complexes were visualized as black spots when sprayed with 20% $(NH_4)_2S$. As little as 10 nmol of 1,10-phenanthroline could be visualized, either when free or when complexed to cobalt, as a pink spot when sprayed with 1.0% freshly prepared $FeSO_4$. The carbonato complex ran as a single spot on Whatman (31ET) paper electrophoresis at 60 mA, 500 V, for 3 h at 4 °C in 150 mM Tris-HCl, pH 8.0. The complex ran with a net charge of +1 as expected for $[Co(phen)_2CO_3]^+$ when compared with $Co(en)_2$ glutamate$^+$ and $Co(en)_2$ glutamine^{2+} standards (Keyes et al., 1976). The complex eluted as a single pink band from a Sephadex C-25–carboxymethyl column at a concentration of 0.4–0.5 M triethylammonium bicarbonate, pH 7.7.

Measurement of the Rate of Solution Exchange Reaction between $[^{57}Co^{II}(phen)_2(H_2O)_2]^{2+}$ *and* $[Co^{III}(phen)_2CO_3]^+$. To measure the rate of electron exchange between Co^{II}- and Co^{III}phen complexes, a reaction mixture containing 0.16 mM $^{57}CoCl_2$ (4550 cpm/nmol), 0.16 mM phen, 1.6 mM $[Co(phen)_2CO_3]^+$ in 0.1 M KCl, and 50 mM Tris, pH 8.0, at 0 °C was prepared. At various times 1.0-mL samples were agitated on a Vortex mixer for 15 s in 5-mL disposable tubes containing 100 mg of Chelex-100 resin (Bio-Rad) to preferentially remove cobalt(II) complexes from the mixture. The Chelex resin was sedimented for 5 s in a clinical centrifuge, 0.5 mL of the supernatant was immediately removed, and ^{57}Co was determined. In the absence of $[Co(phen)_2CO_3]^+$ under these conditions, 97.4 ± 0.5% of $^{57}Co(II)$ could be removed. Chelex treatment of 1.0-mL solutions containing only $[Co(phen)_2CO_3]^+$ removed 31.4 ± 0.6% of the Co(III) complex as measured by the loss of 350-nm absorbing material before and after Chelex treatment. The extent of extraction depended upon the amount of Chelex-100 used and depended critically upon the vortex mixing time. When performed carefully, this technique yielded extraction results reproducible to ±1% and allowed determination of electron exchange rates with improved precision over commonly used solvent extraction methods (Baker et al., 1959).

Enzyme Preparations. Rabbit skeletal myosin was prepared according to Wagner & Yount (1975). Chymotryptic SF_1 was prepared according to Weeds & Taylor (1975) except that myosin stored in 50% glycerol at −20 °C was dialyzed directly against 50 volumes of 10 mM phosphate, pH 7.0, and 0.1 M NaCl at 4 °C prior to digestion with α-chymotrypsin in the presence of 1 mM EDTA.

Protein Concentrations. Protein concentrations of SF_1 were determined by a Coomassie blue dye binding assay (Bradford, 1976). The diluted Bradford reagent was allowed to stand for at least 2 h and was filtered through a stack of Millipore filters (AP20 fiberglass) and two Millipore filters (1.2 and 0.45 μm) before being used. Unmodified chymotryptic SF_1 ($\epsilon_{280nm}^{1.0\%}$ = 7.5) was used as a protein standard (Wagner & Weeds, 1977) for cobalt phenanthroline modified SF_1. The M_r of SF_1 was taken as 120000 (Weeds & Taylor, 1975). Cobalt-modified SF_1 after purification on Sephadex G-25 columns (Pharmacia PD-10 columns) gave the same response in the dye binding protein assays as unmodified SF_1.

Enzyme Inactivations. ATPase activities were measured as described in the following paper (Wells et al., 1979). Normally, inactivations of chymotryptic SF_1 were carried out by using approximately 16 μM SF_1 at 0 °C in 0.1 M KCl, 50 mM Tris, pH 8.0, in 2 mM $MgCl_2$, 0.1 mM ADP, 0.16 mM $CoCl_2$, 0.16 mM phen, and 1.6 mM $[Co(phen)_2CO_3]Cl\cdot 4.5H_2O$ unless otherwise indicated. Zero time for the inactivation was taken with the addition of $[Co(phen)_2CO_3]Cl\cdot 4.5H_2O$.

The inactivation of SF_1·ATPase was quenched by the addition of 2.5 volumes of a saturated $(NH_4)_2SO_4$ solution, pH 8.0, and 5–20 mM EDTA, followed by centrifugation at 20000g for 15 min. The pelleted SF_1 was washed with EDTA-free saturated $(NH_4)_2SO_4$ solution (pH 8.0) and was dissolved in KCl–Tris buffer and passed over Sephadex PD-10 columns to remove extraneous cobalt. In cases where many samples were run, the SF_1 was purified by three additional EDTA-free $(NH_4)_2SO_4$ precipitations rather than gel filtration. Less than 0.02 extraneous cobalt/SF_1 remained after the above procedures when the final concentration of SF_1 was 2 mg/mL.

Results

The data in Figure 1 show that neither Co^{II}phen nor $[Co^{III}(phen)_2CO_3]^+$ caused appreciable inactivation of chymotryptic SF_1 ATPase activity in 1 h, but, when added together, there was a rapid inactivation with a half-life of 15 min. The inactivation was pseudo first order in enzyme for more than 4 half-lives, a result consistent with the modification of a single enzyme site. The addition of MgADP (or MgATP) increased the rate of inactivation some 10-fold. Neither $CoCl_2$ nor phen nor a mixture of phen and $[Co^{III}(phen)_2CO_3]^+$ caused appreciable loss in ATPase activity in a 60-min period (data not shown).

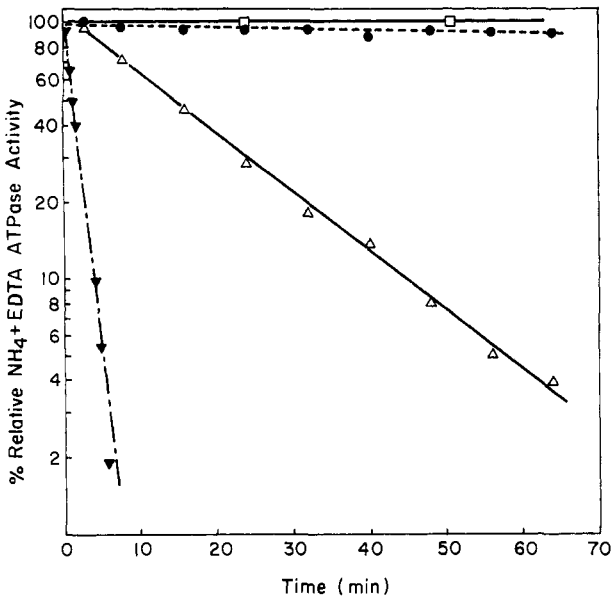

FIGURE 1: Inactivation of SF_1 ATPase activity by Co^{II}phen/$[Co^{III}(phen)_2CO_3]^+$ mixtures in the presence and absence of MgADP. All inactivations were performed in 0.1 M KCl and 50 mM Tris, pH 8.0, at 0 °C. (□) 19.5 μM SF_1 and 1.95 mM $Co^{III}(phen)_2CO_3^+$; (●) 18.5 μM SF_1, 0.185 mM $CoCl_2$, and 0.185 mM phen; (△) 18.0 μM SF_1, 0.18 mM $CoCl_2$, 0.18 mM phen, and 1.8 mM $[Co^{III}(phen)_2CO_3]^+$; (▼) 15.0 μM SF_1, 0.1 mM Li_3ADP, 2.0 mM $MgCl_2$, 0.16 mM $CoCl_2$, 0.16 mM phen, and 1.6 mM $[Co^{III}(phen)_2CO_3]^+$.

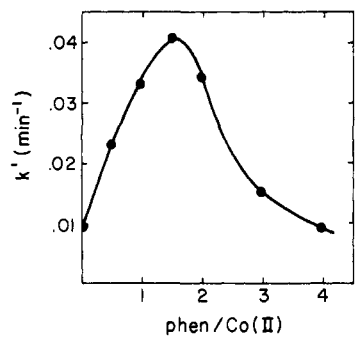

FIGURE 2: Dependence of the apparent first-order rate constant, k', of inactivation on the phen to Co(II) ratio. SF_1 (14 μM) was treated with 1.4 mM $[Co^{III}(phen)_2CO_3]^+$, 0.14 mM $CoCl_2$, variable phen, 0.1 M KCl, and 50 mM Tris, pH 8.0, at 0 °C.

Addition of varying amounts of phen to inactivation mixtures containing $CoCl_2$ and $[Co^{III}(phen)_2CO_3]^+$ showed that an optimal rate of inactivation occurred at a ratio of about 1.5 phen to 1 cobalt(II) (Figure 2). The nonchelating analogue, 4,7-phenanthroline, did not enhance the rate of $CoCl_2/[Co^{III}(phen)_2CO_3]^+$ inactivation (data not shown). These data suggest that the role of phen in promoting inactivation is in its ability to chelate Co(II) and not in its ability to act as an allosteric effector. The optimal phen/Co(II) ratio seen in Figure 2 suggests that the reactive Co(II) species is either a mono- or bisphenanthroline complex.

The addition of stoichiometric quantities of EDTA per Co(II) at several stages of the Co^{II}phen/$[Co^{III}(phen)_2CO_3]^+$ inactivation immediately blocked further ATPase inactivation (Figure 3). The ability to quench inactivations with EDTA was essential in correlating changes in enzymic properties with the extent of cobalt phenanthroline modification [see Wells et al. (1979)]. Furthermore, that complete chelation of Co(II) by EDTA or phen inhibits inactivation suggests that readily accessible ligand sites on Co(II) are essential for inactivation to occur.

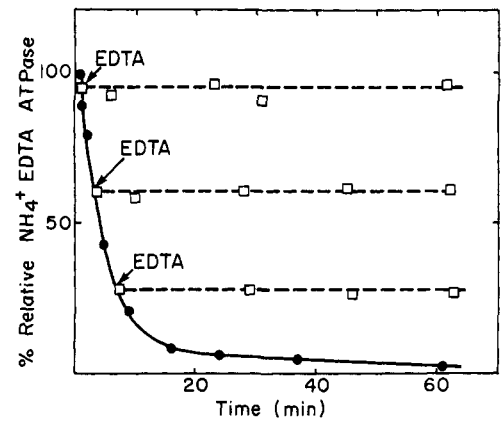

FIGURE 3: EDTA protection against cobalt phenanthroline inactivation. SF_1 (21 μM) was inactivated in the presence of 0.21 mM $CoCl_2$, 0.21 mM phen, 2.1 mM $[Co^{III}(phen)_2CO_3]^+$, 0.1 M KCl, and 50 mM Tris, pH 8.35, 0 °C. At indicated times aliquots of the inactivation mixture were made 0.21 mM in EDTA and assayed in parallel.

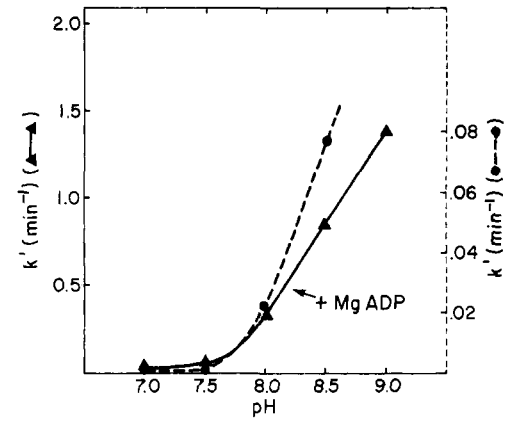

FIGURE 4: Dependence of the apparent first-order rate constant, k', on the pH of the inactivation mixture. (▲) 15.2 μM SF_1, 2 mM $MgCl_2$, 0.1 mM ADP, 0.072 mM $CoCl_2$, 0.072 mM phen, and 0.72 mM $[Co^{III}(phen)_2CO_3]^+$; (●) 9.8 μM SF_1, 0.25 mM $CoCl_2$, and 1.0 mM $[Co^{III}(phen)_2CO_3]^+$. All reactions were performed in 0.1 M KCl and 50 mM Tris, pH as indicated, 0 °C.

The pH dependence of inactivation in the presence or absence of MgADP is shown in Figure 4. Although the rate of inactivation is some 10-fold higher in the presence of MgADP, the overall shape of the curves is similar. This suggests that the ligands and the site of inactivation are similar, if not the same, in both cases.

The rate of inactivation of the ATPase by the mixture of cobalt phenanthroline complexes was markedly dependent on KCl concentrations. When no KCl was added and at 0.3 M KCl, the apparent rate constant for inactivation was 0.075 min^{-1} with a minimum of about 0.025 min^{-1} at 0.1 M KCl. The latter concentration was chosen to standardize reaction conditions and to minimize effects of the KCl concentrations on the rate of inactivation.

Cobalt Stoichiometry following Inactivation. To determine if cobalt was incorporated into SF_1 following loss of ATPase activity, we inactivated SF_1 in the presence of MgADP as described in Figure 1. Excess cobalt was removed from SF_1 by $(NH_4)_2SO_4$ precipitations following inactivation as described under Materials and Methods. Seven determinations of the Co/SF_1 after inactivation to less than 10% activity gave a ratio of 1.4–2.0, depending upon the length of the cobalt phenanthroline treatment time and the SF_1 preparation used. If care was taken to keep the treatment time constant, there was less than 0.1 Co/SF_1 difference between parallel inactivations using the same preparation of SF_1. The variability

Table I: Reversal of Cobalt Phenanthroline Inactivations[a]

reversal agent	treatment time	% EDTA ATPase act.[b]
$NaBH_4$ (4 mM) + EDTA (10 mM)	40 min	98
$Fe^{II}EDTA$[c]	2 min	93
$Co(CN)_5^{3-}$[d]	12 min	83
$CoCl_2$ (1.1 mM) + phen (4.5 mM)	40 h	93
DTE (8 mM) + EDTA (10 mM)	43 h	98
$CoCl_2$ (1.1 mM) + EDTA (2.2 mM)	24 h	4
$CoCl_2$ (1.1 mM)	24 h	4
phen (4.5 mM)	40 h	4
EDTA (2.2 mM)	24 h	4

[a] SF_1 (11–16 μM) was inactivated to ATPase activity in the presence of MgADP and purified as described under Materials and Methods. Reactivations were performed in 0.1 M KCl and 50 mM Tris, pH 8.0, at 0 °C. Reactivations were optimal when SF_1 was inactivated just long enough to reach less than 10% activity (6–8 min), quenched, purified, and reactivated within 1 h after being prepared. [b] NH_4^+-EDTA ATPase activity was followed with time of reactivation. The reported value at the given treatment time is the optimal activity recovered. [c] Co·SF_1 (14 μM) in 10 mM EDTA was treated with freshly prepared 1.4 mM $FeSO_4$. The average value for Fe^{II} EDTA reactivations of seven different Co·SF_1 preparations was 93 ± 4%. [d] SF_1 (30 μM) was treated with 6 μM $CoCl_2$ and 18 mM NaCN at 25 °C. The average value of reactivations of four different Co·SF_1 preparations was 83 ± 10%.

in Co/SF_1 in inactive enzyme suggests the existence of nonessential site(s) of labeling.

Reversal of Cobalt Phenanthroline Modification. The data in Table I show that exposure of purified Co·SF_1 to a variety of reducing agents returned essentially all the ATPase activity, whereas chelating agents alone had little effect. Reversal of the cobalt(III) modification with reducing agents is a critical control experiment to show that the inactivation results from cobalt incorporation and not from extraneous modifications to the enzyme. Attempts to incorporate Co^{II}phen or Co^{II}ATP into myosin, heavy meromyosin, or SF_1 using a 10-fold excess of Co(II) complex and in situ oxidation with H_2O_2 or perbenzoic acid led to irreversible inactivation of the enzyme, possibly because of coincident free-radical oxidative damage.

Optimal recovery of NH_4^+-EDTA ATPase activity by Fe^{II}EDTA reduction (Table I) was found to occur between 1- and 10-min incubation time. After this time there was a slow decrease in ATPase activity such that at 100 min recovered activity had decreased 10–15%. Prolonged exposure to Fe^{II}EDTA also led to extensive loss of SH groups. Treatment of 4.8 μM Co·SF_1 with a 70-fold excess of Fe^{II}EDTA for 24 h resulted in a 23% decrease in reactivated activity and a loss of 4 to 5 SH groups per SF_1. These observations are consistent with known Fe(II)-catalyzed oxidation of thiols by dissolved oxygen (McAuliffe & Murray, 1972). In addition, oxygen free radicals generated in this process (Misra, 1974) could cause further oxidative damage to the protein (McCord & Day, 1978).

Evidence for Incorporation of Co(III) into SF_1 by in Situ Oxidation of an Enzyme-Bound $Co^{II}(phen)_x$ Complex. Of the possible mechanisms for rapid incorporation of Co(III) into proteins under our reaction conditions, only the one (eq 1a and 1b) involving a rapid binding of a $Co^{II}(phen)_x$ complex

$$E + Co^{II}(phen)_x \rightleftarrows E{\cdot}Co^{II}(phen)_x \quad (1a)$$

$$E{\cdot}Co^{II}(phen)_x + [Co^{III}(phen)_2CO_3]^+ \rightleftarrows E{\cdot}Co^{III}(phen)_x + Co^{II}(phen)_2CO_3 \quad (1b)$$

to the enzyme followed by in situ oxidation by $[Co^{III}(phen)_2CO_3]^+$ seemed reasonable. The known exchange lability of Co(II) complexes and the well-documented studies of electron transfer between Co(II) and Co(III) complexes

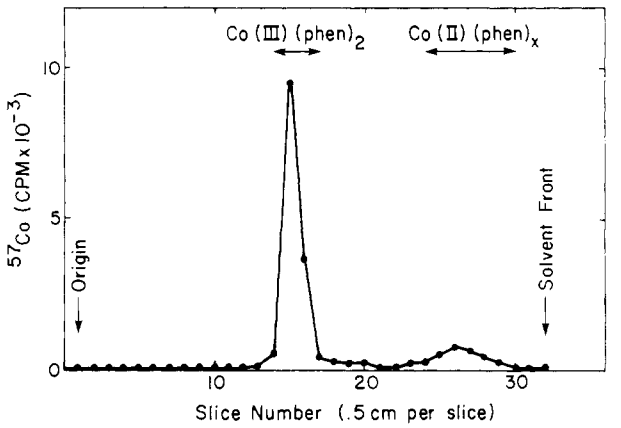

FIGURE 5: Silica TLC separation of a mixture containing initially 20 mM $[Co^{III}(phen)_2CO_3]^+$, 2 mM $^{57}CoCl_2$ (9000 cpm/nmol), and 2 mM phen incubated 60 min in 50 mM Tris, pH 8.0, and 0.1 M KCl at 25 °C. The TLC plate was developed with 50% pyridine–35% H_2O–15% acetic acid and then sliced into 0.5-cm strips and counted for ^{57}Co. The location of $[Co^{III}(phen)_2CO_3]^+$ and Co^{II}phen standards run in parallel are indicated by arrows. This TLC system did not resolve $[Co^{III}(phen)_2CO_3]^+$ from $[Co^{III}(phen)_2(H_2O)_2]^{3+}$.

(Davies & Warnqvist, 1970) lend support to this mechanism. However, it was important to determine that this mechanism was operative in light of our interests to employ this approach to modify other proteins and hormones. Accordingly, SF_1 was inactivated (>96%; 8–10-min treatment time) as described under Materials and Methods except that $^{57}Co(II)$ and unlabeled $[Co^{III}(phen)_2CO_3]^+$ were used. In three separate experiments, some 60–70% of the total cobalt incorporated was ^{57}Co. Furthermore, if the cobalt complexes were incubated together in enzyme buffer for 40 min before addition to SF_1 solutions, only 10% of the total cobalt incorporated was ^{57}Co. These anomalous findings could be explained if electron transfer occurred rapidly between $^{57}Co^{II}(phen)_x$ complexes and $[Co^{III}(phen)_2CO_3]^+$ as shown by eq 2. Since $[Co^{III}\text{-}{}^{57}Co^{II}(phen)_x + [Co^{III}(phen)_2CO_3]^+ \rightleftarrows {}^{57}Co(III)(phen)_x + Co(II)(phen)_2CO_3$ (2)

$(phen)_2CO_3]^+$ was present in a 10-fold excess over Co^{II}phen, complete scrambling of ^{57}Co would result in an 11-fold dilution of the $^{57}Co(II)$. Electron transfer reactions of this type are known to occur between $Co(phen)_3$ complexes (Baker et al., 1959; Ellis et al., 1957).

To test this hypothesis, a mixture of Co^{II}phen and $[Co^{III}(phen)_2CO_3]^+$ was incubated for 60 min at 25 °C and subsequently separated by TLC. Figure 5 shows that the ^{57}Co was uniformly distributed among the cobalt complexes. The fact that approximately 90% of the ^{57}Co ran coincident with $[Co^{III}(phen)_2CO_3]^+$ (R_f = 0.48) suggested that electron exchange had reached equilibrium and had occurred between $^{57}Co^{II}(phen)_2(H_2O)_2$ and $[Co^{III}(phen)_2CO_3]^+$.

To measure the rate of the reaction in eq 2, we developed a method for rapid preferential removal of cobalt(II) from solutions containing $^{57}Co^{II}$phen and $[Co^{III}(phen)_2CO_3]^+$ using Chelex-100. Figure 6 shows that there was a rapid increase with time ($t_{1/2} \simeq 6$ min) in the amount of ^{57}Co present in the Chelex supernatant following addition of $[Co^{III}(phen)_2CO_3]^+$ due to the production of $^{57}Co^{III}(phen)_2(H_2O)_2$. There was no increase in unbound ^{57}Co in the absence of added $[Co^{III}\text{-}(phen)_2CO_3]^+$ even in a 4-h period. The experiment shown in Figure 6 was also performed in the presence of MgADP to mimic the conditions for modifying SF_1 (Figure 7). MgADP and/or SF_1 had no effect on the measured rate of electron exchange between Co(II) and Co(III)phen complexes within experimental error.

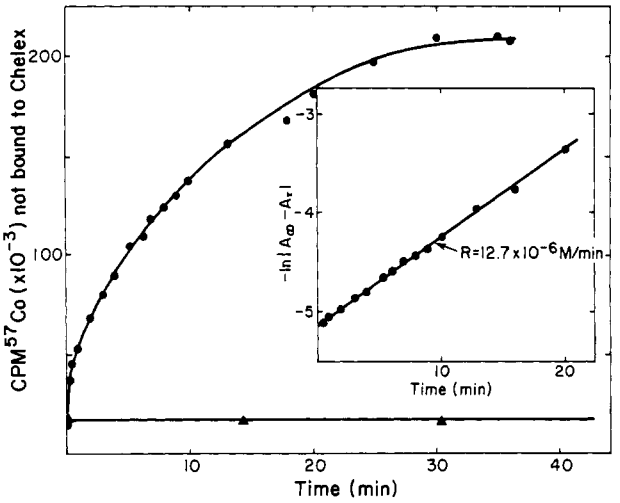

FIGURE 6: Measurement of the rate of electron exchange between $^{57}Co^{II}$- and Co^{III}phen complexes. See Materials and Methods for details. (▲) 0.16 mM $^{57}CoCl_2$ (4500 cpm/nmol), 0.16 mM phen, 2 mM $MgCl_2$, 0.1 mM ADP, 50 mM Tris, pH 8.0, and 0.1 M KCl, 0 °C; (●) same as above plus 1.6 mM $[Co^{III}(phen)_2CO_3]^+$. The insert shows a replot of the top curve (●) according to the McKay equation (see text for details).

The insert in Figure 6 shows a replot of the data according to the McKay equation (McKay, 1938) as treated by Baker et al. (1959). The data reploted according to eq 3 gave a straight line expected for electron exchange reactions of the type shown by eq 2

$$-\ln \frac{A_\infty - A_t}{A_\infty - A_0} = Rt\frac{a+b}{ab} \qquad (3)$$

where A_∞, A_t, and A_0 refer to radioactivity in the Chelex supernatant at equilibrium, time t, and time zero, respectively. The concentrations of Co(II) and Co(III) species correspond to a and b, respectively, and R is the gross exchange rate. R was calculated from the slope of the line in Figure 6 and in four trials gave an average value of $(12.5 \pm 1.1) \times 10^{-6}$ M/min, indicating the technique was highly reproducible.[2] The average R value was of comparable magnitude to those reported under similar conditions for Co(II)/Co(III) trisphen electron exchange (Baker et al., 1959).

Figure 7 shows the time course of incorporation of ^{57}Co into SF_1 compared to the total cobalt incorporation as monitored by cobalt atomic absorption in a parallel experiment. The ^{57}Co uptake curve underestimated the amount of cobalt incorporated as a cobalt(II) intermediate since there was a significant dilution of the cobalt(II) specific activity by electron exchange with Co(III). The initial specific activity of ^{57}Co(II) (4500 cpm/nmol) was changed to 409 cpm/nmol at equilibrium by reaction with a 10-fold excess of $[Co^{III}(phen)_2CO_3]^+$. Substituting this range of specific activities for the ordinate in Figure 6 (4500 at zero time and 409 at 35 min) allowed the

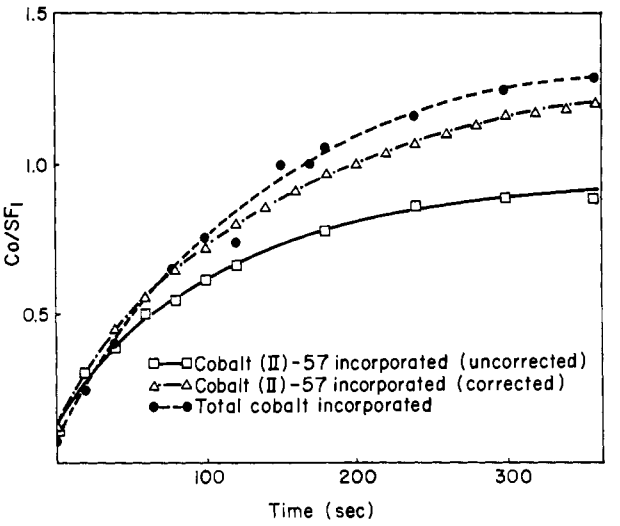

FIGURE 7: Evidence that SF_1 was labeled via a Co(II) to Co(III) in situ oxidation mechanism. At indicated times aliquots of SF_1-Co(phen) reaction mixtures were quenched by addition of 5 mM EDTA. Samples were subsequently precipitated 4 successive times by addition of 2.5 volumes of saturated $(NH_4)_2SO_4$ solution to remove nonbound cobalt. (□) Labeling of 16 μM SF_1 with ^{57}Co(II) in a mixture containing 2 mM $MgCl_2$, 0.1 mM ADP, 0.16 mM $^{57}CoCl_2$ (4500 cpm/nmol), 0.16 mM phen, and 1.6 mM $[Co^{III}(phen)_2CO_3]^+$; (▲) cobalt incorporated as a cobalt(II) intermediate. This curve was obtained by correcting (□) for the decrease in the specific activity of ^{57}Co(II) due to solution electron exchange with $[Co^{III}(phen)C_2O_3]^+$ as measured in Figure 6 (see text for details). (●) Same mixture as (□) except nonradioactive $CoCl_2$ was used and total cobalt incorporation was monitored by atomic absorption.

determination of ^{57}Co(II) specific activity at any time. These decreasing ^{57}Co(II) specific activities were used to correct the apparent uptake of ^{57}Co(II) by SF_1 in each succeeding 20-s interval. When these corrections were made, the middle curve in Figure 7 was obtained which shows that >93% of the ^{57}Co incorporated was via Co(II).

Further evidence in support of the in situ oxidation mechanism was obtained by measuring the rate of inactivation by using eight different concentrations of Co^{II}phen essentially under the conditions described in Figure 4 at pH 8.0 in the presence of MgADP. The rate of inactivation saturated with increasing concentrations of Co(II)phen and an inhibition constant (K_i) of 270 μM was calculated from the x intercept of the resulting linear plot of $1/k'$ vs. $1/[Co^{II}phen]$. This suggests a rapid and reversible binding of $Co^{II}(phen)_x$ to the enzyme prior to inactivation.

Evidence for Enzyme-Bound Cobalt Being Co(III). Copurification of cobalt bound to SF_1 following cobalt phenanthroline inactivation and brief exposure to EDTA suggests that the enzyme-bound cobalt is Co(III) but does not exclude the possibility of a tightly bound Co(II). The following data provide evidence that >85% of the bound cobalt is cobalt(III).

^{57}Co-labeled SF_1 was prepared as described in Figure 1 except additional $MgCl_2$ (20 mM) was added. Upon addition of 0.1 mL of 60% $HClO_4$ to 1.0 mL of freshly prepared ^{57}Co-SF_1 (2 mg/mL), 85% of the radioactivity precipitated with the protein. Control experiments showed that ^{57}Co(II) and $[Co^{III}(phen)_2CO_3]^+$ did not precipitate with SF_1 under the above conditions. These data argue that minimally 85% of the enzyme-bound cobalt is cobalt(III). This value may be higher since it is not known how stable Co(III)·peptide complexes are in $HClO_4$. These data also indicate that the bulk of cobalt(III) was directly chelated to the enzyme and not simply tightly adsorbed through the second sphere as is the case for the binding of $Co^{III}(NH_3)_4$ADP(glucose 6-

[2] These values of R assume a random exchange between all Co(II) species present such that a = total concentration of Co(II). Our exchange data in Figure 5, however, indicate there was preferential electron exchange between the bisphen complexes. From the known sequential association constants of Co(II) with phenanthroline (Schilt, 1969), the proportion of $Co^{II}(phen)_2(H_2O)_2$ to total cobalt(II) was calculated to be 0.23 under the conditions used for measuring electron exchange. If exchange occurs exclusively between bisphen complexes, the above R value should be a factor of 4 lower. It is interesting that the R values reported for electron exchange between the trisphen complexes (Baker et al., 1959) are of the same order of magnitude as we report here for the weaker ligand field strength bisphen complexes. In addition, we observe dramatic reductions in the rate of electron exchange in H_2O vs. 0.1 M KCl ($t_{1/2} \simeq 4$ h vs. 6 min).

phosphate) to hexokinase (Cornelius & Cleland, 1978).

When Co·SF_1 (30 μM) was stored in the presence of 5 mM EDTA, 0.1 M KCl, and 50 mM Tris, pH 8.0, at 0 °C for 24 h, less than 5% of the cobalt was removed following gel filtration. Only 15% was lost after 3 days under these conditions.

Confirmation of the absence of enzyme-bound cobalt(II) in Co·SF_1 prepared as described above came from cobalt low-temperature EPR.[3] After 3 days in 10 mM EDTA at 0 °C less than 15% of the cobalt present in 30 mg/mL Co·SF_1 was cobalt(II). This was measured with reference to a Co^{2+} and Co^{2+}·SF_1 standard.

Finally, as shown previously in Table I, brief treatment with a variety of reducing agents, followed by gel filtration in the presence of chelating agents, allowed the removal of essentially all of the bound cobalt. Without reducing agents the above treatment removed little, if any, of the enzyme bound cobalt.

Discussion

Through a reinvestigation of the reported affinity reagent, $Co^{III}(phen)ATP(O_2^-)$ (Werber et al., 1974), a mixture of $Co^{II}phen$ and $[Co^{III}(phen)_2CO_3]^+$ has been found to act together to inactivate myosin chymotryptic SF_1 ATPase activity. In the following paper, it is shown that ATPase inactivation results from the incorporation of a single cobalt per SF_1 even though uptake values of 1.4–2.0 cobalts per SF_1 were found. The data suggest that the Co binding site critical for activity loss does not overlap with the ATPase site since MgATP does not protect against inactivation.

The data presented strongly support the proposal that the mechanism for cobalt incorporation is by an in situ oxidation mechanism (eq 1a and 1b). It was observed that while essentially all (>85%) of the enzyme-bound cobalt was Co(III), essentially all cobalt was incorporated through a Co(II) intermediate. The fact that chelating agents such as EDTA or large excesses of phen protected against inactivation and the observation that the rate of inactivation saturated with increasing $Co^{II}phen$ are consistent with the reaction in eq 1a. The data support the role of $[Co^{III}(phen)_2CO_3]^+$ as the oxidant for $Co^{II}(phen)_x$ bound to SF_1 as shown in eq 1b. First $[Co^{III}(phen)_2CO_3]^+$ can rapidly oxidize $^{57}Co^{II}(phen)_2(H_2O)_2$ as shown by the data in Figures 5 and 6. Oxidation of Co(II) complexes by Co(III) complexes is well documented in the inorganic literature (Davies & Warnqvist, 1970). Second, if dissolved O_2 was lowered to less than 30 μM (monitored by an oxygen electrode) in the inactivation mixture, there was no significant effect on the rate of inactivation. Oxygen exclusion was performed on separate solutions of MgADP·SF_1 and cobalt complexes in a Thunberg tube by cycles of exhaustive evacuation and argon purge.

The in situ oxidation mechanism (eq 1a and 1b) predicts only catalytic quantities of $Co^{II}phen$ should be necessary to potentiate inactivation since $Co^{II}(phen)_2$ is regenerated in eq 1b. When SF_1 (16 μM) was incubated with a 1.6 μM $Co^{II}phen$ and 1.6 mM $[Co^{III}(phen)_2CO_3]^+$ in the presence of 2 mM $MgCl_2$ and 0.1 mM ADP, the ATPase activity was inactivated with a half-life of 8 min. In fact, small impurities of cobalt(II) complexes present in $[Co^{III}(phen)_2CO_3]^+$ preparations were sufficient to potentiate inactivation. When 14 μM SF_1 was incubated with 1.4 mM $[Co^{III}(phen)_2CO_3]^+$, the ATPase was inactivated with a half-life of 35 h or 22 min when 0.1 mM MgADP was added. Either inactivation was blocked by addition of EDTA or phen in amounts equivalent to 1–3% of the Co(III) complex added. These results argue for the presence of small quantities of Co(II) potentiating the slow ATPase inactivation as opposed to direct incorporation of $[Co^{III}(phen)_2CO_3]^+$ by ligand substitution. Previous investigators (Ellis et al., 1957) have found small quantities of cobaltous complexes in $[Co^{III}(phen)_3]^{3+}$ preparations.

1,10-Phenanthroline plays a major role in promoting ATPase inhibition. The observation that the nonchelating analogue, 4,7-phenanthroline, was ineffective suggests that the chelating properties of 1,10-phenanthroline are important for potentiating ATPase inhibition. Although slow ATPase inactivation occurred in the absence of added phen (Figure 2), this can be explained by the presence of small impurities of cobaltous phen present in $[Co^{III}(phen)_2CO_3]^+$. The optimal ratio for inactivation of 1.5 phen to 1.0 Co(II) (Figure 2) suggests the value of x in E·$Co^{II}(phen)_x$ as shown in eq 1a is either 1 or 2. Calculations based on the known sequential association constants for Co^{2+} and phen (Schilt, 1969) indicate mole fractions of 0.09, 0.37, 0.44, and 0.09 for free Co^{2+}, mono-, bis-, and trisphen cobaltous complexes, respectively, at a phen to Co^{2+} ratio of 1.5. The fact that electron transfer occurs preferentially between cobaltous bisphen and cobaltic bisphen in free solution (Figure 5) suggests the reactive E·$Co^{II}(phen)_x$ species in eq 1b is a bisphen complex. Preliminary difference spectra comparing cobalt phenanthroline modified and unmodified SF_1 indicate a large increase in the molar absorptivity of the modified protein between 267 and 274 nm consistent with the presence of at least one and probably two phenanthrolines per enzyme-bound cobalt. The exact stoichiometry will require use of radioactive phen since the difference spectra are difficult to quantify due to unknown microenvironmental effects of the protein and to changes in the molar absorptivity of phen upon coordination to cobalt.

Preliminary use of other cobalt complexes such as $[Co^{III}(en)_2CO_3]^+/Co^{II}en$ or $[Co^{III}(EDDA)CO_3]^-/Co^{II}EDDA$ indicates these complexes do not inactivate SF_1 ATPase activity in a 24-h period under conditions where cobalt phenanthroline complexes gave >96% inactivation after 8 min. The CoEDDA mixture does allow incorporation of cobalt into nonessential cobalt phenanthroline binding sites (Wells et al., 1979).

The role of 1,10-phenanthroline with respect to cobalt incorporation could be at least threefold. Stable cobalt(III) complexes require six ligands arranged in an octahedral configuration. Generally, ligands higher in the spectrochemical series are preferred in cobalt(III) complexes because they stabilize the complex with respect to reduction back to Co(II). If the enzyme-incorporated cobalt(III) is chelated by only two ligands from the enzyme, then four other ligands must be made available either from the solvent (H_2O) or from added phen. Since phen is a much stronger field ligand than H_2O (Josephsen & Schäffer, 1977), the expected order of stability assuming the absence of steric problems would be

$$[E\cdot Co^{III}(phen)_2] > [E\cdot Co^{III}phen(H_2O)_2] > [E\cdot Co^{III}(H_2O)_4]$$

A second role for phen might be to facilitate electron transfer as shown in eq 1b. For instance, while 1,10-phenanthroline and ethylenediamine are very close in the spectrochemical series (Josephsen & Schäffer, 1977), the relative rate of electron exchange is $>10^5$ faster between the trisphenanthroline complexes than between the tris(ethylenediamine) complexes of cobalt (Wilkens, 1974). It has been suggested that the π system of the phen ring serves as a channel for electron transfer (Przystas & Sutin, 1973).

A third role of phen might be to direct the cobalt(II) complex to a hydrophobic site where it may be chelated by

[3] Measurements were made at 4 K by Dr. George Reed at the University of Pennsylvania, Philadelphia, PA.

the enzyme. Our present results do not allow us to determine the relative importance of these roles.

Reaction 1b suggests that other oxidants such as O_2 or H_2O_2 may serve in place of $[Co^{III}(phen)_2CO_3]^+$ to oxidize enzyme-bound $Co^{II}(phen)_x$. Addition of as little as 10-fold excess of Co^{II}phen and H_2O_2 or *m*-chloroperbenzoic acid over enzyme in the presence of MgADP led to significant ATPase inactivation. The use of peroxide oxidants presented at least two problems: first, H_2O_2 alone significantly inactivated SF_1 and, second, Co^{II}phen/peroxide inactivations were not reversible over a 24-h period by either DTE or Fe^{II}EDTA even though all cobalt was removed. These data would indicate that extraneous modifications to the protein beyond cobalt incorporation resulted from Co^{II}phen/peroxide inactivation. The presence of free-radical scavengers, such as 40 mM phenol or 40 mM indoleacetic acid, during the oxidation did not significantly improve subsequent reactivations of Co^{II}phen/peroxide inactivated SF_1. These concentrations of free-radical scavengers might not be high enough to protect against local generation of oxygen free radicals during cobalt oxidation which could cause irreversible damage to the protein.

Dissolved oxygen supported ATPase inactivation when high concentrations of Co^{II}phen were used. A 200-fold excess of Co^{II}phen inactivates 10 μM SF_1 in 0.1 M KCl and 50 mM Tris, pH 8.0, at 0 °C with a half-life of 32 min. This inactivation was enhanced by MgADP and could be stopped by EDTA. The inactivation with Co^{II}phen showed a pH dependence similar to that seen for Co(II)/Co(III) inactivations (Figure 3) and could be blocked by prior degassing and argon purging to exclude O_2. However, even when dissolved O_2 was used as the oxidant for Co(II)phen, the ATPase activity was only marginally reactivated by Fe^{II}EDTA or DTE, despite removal of all bound cobalt, suggestive again that oxidative damage to the protein had occurred. A full report of Co^{II}phen inactivations in comparison with those observed with Co^{III}-(phen)ATP(O_2^-) and Co^{II}phen/$[Co^{III}(phen)_2CO_3]^+$ is in preparation.

The incorporation of exchange-inert Co(III) as a probe of metal binding sites in proteins has assumed increasing use [for a review, see Legg (1978)]. This is normally accomplished by in situ oxidation of exchange-labile Co(II) by use of H_2O_2 or O_2. These two electron oxidants, H_2O_2 or O_2, might be expected to generate potentially damaging free radicals such as ·OH or O_2^- during the single-electron oxidation of Co(II) to Co(III). However, the oxidant employed here, $[Co^{III}(phen)_2CO_3]^+$, can readily accept a single electron to yield $[Co^{II}(phen)_2CO_3]$ without generating reactive free-radical intermediates. These findings suggest the use of Co(III) complexes as mild oxidants to in situ oxidize Co(II) to Co(III) in other proteins; for instance, this may be of use in radiolabeling hormones with ^{57}Co(III) for use in radioimmunoassay.

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