The activation of yeast enolase by cobaltous ion in 0.1 M KCl is characterized by an activation constant of 1 μM and an inhibition constant of 18 μM. Measurements of binding of Co²⁺ to the apoenzyme show that a maximum of four Co²⁺ ions are bound per dimer in the presence or absence of substrate although binding is far tighter in the presence of substrate. Ultraviolet spectral titrations show evidence for a conformational change due exclusively to the binding of the first two ions of Co²⁺. Both visible and EPR spectra confirm that the environment of the first pair of cobalt ions ("conformational sites") is markedly different from that of the second pair in the "catalytic" sites. Cobalt at the conformational site appears to be a tetragonally distorted octahedral complex while the second pair of metal ions appears to be in a more regular tetrahedral symmetry. Addition of either Mg²⁺ or substrate to the enzyme with only one pair of cobalt ions per dimer causes striking changes in the metal ion environment. The conformational metal sites appear sufficiently shielded from solvent to be inaccessible to oxidation by H₂O₂, in contrast to the second pair of cobaltous ions whose ready oxidation by H₂O₂ inactivates the enzyme. Comparison of kinetic and binding data suggests that only one site of the dimeric enzyme can be active, since activity requires more than two metals bound per dimer and inactivation results from the binding of the fourth ion per dimer.

YEAST ENOLASE (EC 4.2.1.11) demonstrates an absolute divalent cation requirement for catalytic activity (Wold, 1971). Removal of activating metals and loss of activity is fully reversible. Two Mg²⁺ ions bind tightly to the apoenzyme in the absence of substrate, while two additional Mg²⁺ ions bind in the presence of substrate (Hanlon and Westhead, 1969a; Faller et al., 1977). Several other cations, including Zn²⁺, Mn²⁺, Fe²⁺, Cd²⁺, and Co²⁺, have been substituted for Mg²⁺ to yield enzyme with varying degrees of restored activity (Wold and Ballou, 1957; Hanlon and Westhead, 1969a, 1969b; Elliott and Brewer, 1980). The chemical or geometric nature of the binding sites are largely unexplained although Dickinson et al. (1980) showed that it is unlikely that any nitrogenous ligands participate in metal binding. We cannot yet explain in detail the separate roles of the "conformational" metal-binding sites and the "catalytic" metal-binding sites. Conformational sites are those to which Mg²⁺ or Mn²⁺ bind in the absence of substrate, producing changes in the UV and circular dichroic spectra of the enzyme (Hanlon and Westhead, 1969a; Brewer and Collins, 1980). A further problem concerns the inhibition of the enzyme by excess metal ion. Since only four Mg²⁺ or Mn²⁺ ions have been found to bind to each dimer, one of these bound metals would seem to be inhibitory. Further information on metal binding and other characteristics of this enzyme have been discussed in an excellent review (Brewer, 1981).

To characterize the divalent cation-binding sites further we have studied the properties of the Co²⁺-substituted enzyme. Potentially, there are four characteristics of Co²⁺ which may yield information not obtainable with the native Mg²⁺ enzyme. First, Co²⁺ gives an electronic absorption spectrum which reflects the geometry of the binding site, and since the spectra of scores of Co²⁺ complexes have been obtained (Carlin, 1965), comparisons to cobalt-enolase complexes may be made readily. Second, Co²⁺ is paramagnetic, so information about its environment may be obtained from electron paramagnetic resonance spectra. Third, it is expected that Co²⁺ will bind more tightly than Mg²⁺ (Irving and Williams, 1948) and, therefore, not require use of concentrations far in excess of stoichiometric quantities to virtually fill specific sites. The enhanced binding to all available sites should facilitate comparison of kinetic and thermodynamically derived binding constants. Finally, it may be possible to get information about the metal-binding sites through use of a fourth property of Co²⁺. The substitution-labile divalent cation, Co²⁺, might be oxidized at its binding site to yield a substitution-inactive cobaltic ion. Co²⁺ labels formed this way have been exploited as electronic absorption spectral probes (Van Wart and Vallee, 1978), as reagents to block catalytic activity (Ryzewski and Takahashi, 1975; Shinar and Navon, 1974; Anderson and Vallee, 1975), or to aid in binding site identification following proteolytic digestion of the protein (Kowalsky, 1969; Grimes et al., 1974).

EXPERIMENTAL PROCEDURES

Materials—Bakers' yeast enolase used in these studies was prepared by a modification of published procedures (Westhead and McLain, 1964; Westhead, 1966). The precipitate from the second ethanol addition was redisolved in an ice-cold solution of 5 mM Tris-Cl, pH 7.7, 1 mM Mg²⁺; conductivity, 0.83 mhm. The enzyme solution was chromatographed on a column of Whatman DE-32 DEAE-cellulose (column dimensions, 1.55 × 3 cm) previously equilibrated with 10 mM Tris-Cl buffer, 1 mM Mg²⁺, pH 7.7, 10 μM EDTA; conductivity, 1.1 mhm. Fractions containing enolase of specific activity 150 or higher were pooled and concentrated by pressure filtration to about 20 mg/ml.

The final purification step was a gradient elution on a DEAE-cellulose column at pH 8.6. In a typical elution, pH 8.6 Tris-Cl buffer solutions were prepared with conductance values of 2.15, 3.64, 4.47, and 7.27 mhm. All four solutions were made 1 mM in Mg²⁺ and 10 μM in EDTA. These solutions were put into a multichambered gradient maker and used to elute the enzyme with a salt concentration gradient from a column (15.5 × 2.1 cm) of Whatman DE-32 DEAE-cellulose equilibrated with the lowest conductance buffer. Fractions containing enolase with specific activity of 300 ± 20 under standard

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Cobalt Enolase

Enolase activity was determined from the rate of increase in absorbance at 240 nm as 2-phosphoglycerate was converted to phosphoenolpyruvate. The standard assay buffer consisted of 50 mM Tris-HCl, with 10 mM EDTA, and 1.2 mM D-2-phosphoglycerate acid at pH 7.8. Specific activity is defined (Westhead and McLain, 1964) as the change in absorbance at 240 nm/min divided by enzyme concentration, expressed as absorbance at 280 nm due to the protein in the assay cuvette. The molar absorptivity of phosphoenolpyruvate under the standard assay conditions is 1475 M⁻¹ cm⁻¹ at 240 nm (Wold and Ballou, 1957). Assays were performed using a recording spectrophotometer with cuvettes thermostated at 30 °C.

Enolase concentrations were routinely estimated by UV absorption at 280 nm using an absorptivity coefficient of 0.89 cm⁻¹ mg⁻¹ (Warburg and Christian, 1941) and a dimmer molecular weight of 93,000 (Chin et al., 1980). Enolase concentrations were occasionally determined by Lowry protein analysis (Lowry et al., 1951) with prior precipitation of protein by trichloroacetic acid. For gel filtration binding measurements at low enolase concentrations, enolase was determined by its intrinsic fluorescence, with excitation at 290 nm and emission at 342 nm. Fluorescence was found to be linear with dilution of protein from 10 to 200 µg/ml and not significantly affected by Co²⁺ concentrations within the range used.

Preparation of Metal-free Enolase—Metal-free enolase was prepared by slow elution through Chelex-100 resin in the buffer to be used in subsequent experiments. Chelex-100 was treated as described by Willard et al. (1969) and equilibrated with the appropriate buffer before use. After Chelex treatment, apoenolase was analyzed for residual Mg²⁺ contamination using a Perkin-Elmer model 403 atomic absorption spectrophotometer. Typically, only 0.02 eq of Mg²⁺/dimer remained. Furthermore, apoenolase assayed in the absence of added Mg²⁺ gave less than 2% of the activity observed in the presence of 1 mM Mg²⁺ in 10 mM HEPES (K⁺), 100 mM KCl, pH 7.5 buffer. The activity of apoenolase reconstituted with Mg²⁺ in the HEPES buffer was identical to that observed in the standard assay buffer.

All buffers and substrate solutions were also freed of metal ions by elution through Chelex-100 columns. Storage containers were acid-washed polyethylene.

Sedimentation Velocity Measurements—Sedimentation velocity measurements were made using a Beckman model E analytical ultracentrifuge. In all experiments, the absorbance of the protein solution was observed at 280 nm through the 30-mm double-sector cell by using the model E split-beam photoelectric scanning system. At least seven scans were recorded at 15-min intervals during each 48,000 rpm run. All sedimentation coefficients were corrected to s₂₀,₅₀ according to Schachman, 1957). A partial specific volume of 0.742 cm³/g for yeast enolase was used for all calculations (Chin et al., 1980).

UV Difference and Electronic Absorption Spectral Measurements—All difference spectra and visible absorption spectra were obtained with a Cary model 14 double beam recording spectrophotometer equipped with a 0.0 to 0.2 absorbance slidewire. For difference spectra measurements, 3 ml of apoenolase solution of known concentration were placed in each of two matched cuvettes. After the base-line was recorded, microliter aliquots of the desired metal ion solution were added and mixed in the sample cuvette. An equivalent volume of deionized water was added to the reference cuvette. Spectra were scanned twice after each pair of additions. Metal ion additions were continued until no further spectral changes were observed. The difference in UV absorption at 298 nm was determined by subtracting the average absorbance at 350 nm, where metal additions cause no spectral changes, from the observed absorbance at 298 nm, where UV changes are maximal.

Electronic absorption spectra were obtained with cylindrical thermostatted 5-cm path length 6-mm matched quartz cuvettes. Aliquots of the apoenolase stock solution placed in both the sample and reference cells. After base-lines were recorded, microliter aliquots of cobaltous

The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PGA, phosphoglyceric acid.

EPR Spectral Measurements—Electron paramagnetic resonance spectra were obtained on a Varian E-9 spectrometer. Integration and subtraction measurements were performed on spectra stored on a Facit-Tek 1072 computer. A Halieran JTD-3-1105 (Air Products Corp.) accessory was used to obtain EPR spectra down to 4.2 K. Cobalt enolase EPR signals were maximum at 4.2 K, though observable up to 24 K. The line shape did not change with temperature nor did the signal show saturation at 200 milliwatts. Magnetic field scan range was calibrated with a Bell digital gauss meter (model 660) to ±1 G. Sample cells were 5-mm outer diameter quartz tubes fitted with ground glass 10/40 ST joints for vacuum tight seals. 0.200 ml of enzyme solution was measured into a small polyethylene vial, further stock solution was added in microliter quantities to this aliquot of enzyme, mixed by gentle tapping, and transferred to the sample tube by means of a 10-inch Pasteur pipette. One cycle of freezing and thawing of the enzyme did not affect the EPR spectrum. In most cases each sample of enzyme was used for two metal ion conditions, e.g. a sample initially frozen and examined with 2.0 Co²⁺/dimer was thawed, Co²⁺ content was increased to 3.5 Co²⁺/dimer, and the sample refrozen and examined. Spot checks on several samples showed no intensity change over two freezings.

Stock enzyme at a concentration of 1.0 mM in dimer was diluted for use to concentrations given in figure legends.

Equilibrium-binding Studies—Measurements of cobaltous ion binding to enolase were made by the equilibrium gel filtration method of Hummel and Dreyer (1962) using Sephadex G-25. Stock solutions of cobaltous ion were labeled with carrier-free Co³⁺; total cobalt ion concentration in stock solutions was occasionally verified by a modification of the Nitroso-Reagent method using sodium 1-nitroso-2-hydroxy-naphthalene-3,6-disulfonate (Vogt, 1962). Radioactivity was measured either with a scintillation spectrometer or a γ-counter. The two methods gave identical values of metal ion bound per mol of enzyme. Specific activities of the eluted protein-containing fractions were measured in standard assay buffer to ensure that the enzyme was not inactivated during elution. Activity after the binding experiment was commonly between 94 and 100% of original activity.

RESULTS

Co²⁺ and Mg²⁺ Activation of Yeast Enolase—Co²⁺ is an effective activator of yeast enolase. Initial velocities of catalysis in the presence of varying concentrations of Mg²⁺ or Co²⁺ are shown in Fig. 1. A double reciprocal plot of the data forming the Co²⁺ activation portion (0–3.5 µM) of Fig. 1 gave

![Figure 1](http://www.jbc.org/)
a linear region from which we estimated an apparent activation constant $K_a = 1.1 \ \mu M \ \text{Co}^{2+}$. Maximum activity with Co$^{2+}$ occurs at 3.5 $\mu M \ \text{Co}^{2+}$, more than two orders of magnitude lower than the maximally activating Mg$^{2+}$ concentration, 1 mm (Fig. 2). The maximum activity with Co$^{2+}$ is 15% of that observed when Mg$^{2+}$ is the activating cation.

Beyond 3.5 $\mu M \ \text{Co}^{2+}$, a decrease in activity is observed, an effect also observed for Mg$^{2+}$-activated enolase when Mg$^{2+}$ concentrations exceed 1 mm. $K_i$ for Co$^{2+}$ is 18.5 $\mu M$ at saturating substrate concentration (1.2 mm D-2-PGA). The extrapolated maximum velocity, after correction for Co$^{2+}$ inhibition, is about 35% higher than the highest velocity observable, i.e. 20% of the Mg$^{2+}$ value.

With Co$^{2+}$ concentration held constant at 3.5 $\mu M$, the apparent $K_a$ for D-2-PGA was 31 $\mu M$ when assayed in 10 mm HEPES (K$^+$), 100 mm KCl, pH 7.5, at 30 $^\circ$C. This constant is more than four times lower than the $K_a$ for Mg$^{2+}$-activated enolase (Cardenas and Wold, 1968; Fuller et al., 1977).

**Sedimentation Velocity of Cobalt(II)-Enolase**—Sedimentation velocity studies were undertaken to determine whether the Co$^{2+}$-activated enzyme remained dimeric under the conditions of the subsequent physical measurements. Sedimentation velocities for yeast enolase were determined at four different Co$^{2+}$ concentrations as shown in Table I. The data of Table I, at low protein concentration, are in agreement with previously reported values of 5.85 S (Holleman, 1973) and 5.9 S (Malmstrom, 1961) for the Mg$^{2+}$ enzyme. Holleman (1973) reported that an enolase solution with equal parts monomer and dimer would have an approximate sedimentation coefficient of 4.8 S so it is apparent that under our conditions the enzyme is not appreciably dissociated.

The sedimentation coefficient found here for apoenolase (5.42 S) is 7% lower than the literature value of 5.85 S obtained in 1 mm Mg$^{2+}$ and extrapolated to zero protein concentration (Hullemann, 1973). Brewer and Weber (1966) reported a similar 5% decrease in $s_{20,w}$ (from 5.90 to 5.58 S) as 2 mm EDTA was added to a 3 mg/ml enolase solution containing endogenous Mg$^{2+}$. This decrease in sedimentation coefficient was interpreted as due to a protein conformational transition to a less compact form upon metal ion removal.

Co$^{2+}$- and Mg$^{2+}$-induced Ultraviolet Difference Spectra—UV difference spectroscopy is a convenient technique to monitor enolase conformational changes induced by divergent cations (Hanlon and Westhead, 1965; Brewer and Collins, 1980). Titration of an apoenolase solution with Co$^{2+}$ results in a characteristic UV difference spectrum, essentially the same as those previously reported for other divalent cations (Hanlon and Westhead, 1969a; Brewer, 1981). The difference spectrum exhibits positive absorption maxima at 298 and 287 nm and a broad absorption minimum at 265 nm.

Plots of absorbance change at 298 nm versus gram atoms of metal ion per dimer are shown for Mg$^{2+}$ (Fig. 2) and for Co$^{2+}$ (Fig. 3). It is apparent in the case of Co$^{2+}$ that the absorbance change at 298 nm increases as metal ions are added only up to 2 eq of metal ion/dimer. No further spectral changes are observed as excess equivalents of metal ion are added.

We tested the fit of the data points in Fig. 2 to theoretical curves by assuming two independent sites (i.e. one per monomer) with identical dissociation constants and equal effect on the spectrum (solid line) or, alternatively, by assuming that the absorbance change is due to the filling of only one site per dimer (dashed line). The equation used to determine the concentration of filled sites was the standard quadratic equation resulting from the equality $(E_t - EM) (M_t - EM)/EM = K_d$ where $E_t$ and $M_t$ are the total concentrations of relevant enzyme sites and metal ions, $EM$ is the concentration of filled sites, and $K_d$ the dissociation constant. We assumed that the absorbance change is proportional to $EM$. Mg$^{2+}$

### Table I

| Run | [Co$^{2+}$] (um) | [Enolase] mg/ml | Co/enolase | Temperature $^\circ$C | $s_{20,w}$ |
|-----|----------------|-----------------|------------|---------------------|-----------|
| 1   | 0.0           | 0.30            | 7.1        | 30.0                | 5.42      |
| 2   | 3.5           | 0.21            | 5.0        | 30.25               | 5.96      |
| 3   | 18.5          | 0.29            | 6.9        | 3.7                 | 5.72      |
| 4   | 18.5          | 0.21            | 5.0        | 3.7                 | 5.74      |
| 5   | 200.0         | 0.29            | 6.9        | 29.0                | 5.63      |
| 6   | 500.0         | 0.29            | 6.9        | 7.0                 | 5.56      |
Equilibrium Binding of Co\(^{2+}\) to Apoenzyme—Further quantitative measurements of the interaction of Co\(^{2+}\) with apoenolase were made by the gel filtration method (Hummel and Dreyer, 1961). The stoichiometry of binding of \(^{57}\)Co\(^{2+}\) to apoenolase was determined at several free Co\(^{2+}\) concentrations. The results of these individual experiments are summarized in Fig. 4 as \(n\), the number of gram atoms of Co\(^{2+}\) bound per mol of enolase dimer versus free Co\(^{2+}\) concentration. A maximum of four Co\(^{2+}\) are bound per enolase dimer at the highest free metal ion concentration studied (1 mM Co\(^{2+}\)) in the absence of substrate. It is apparent that 2 mol of cobalt are bound much more tightly than the other two. The shape of the curve beyond 0.1 mM Co\(^{2+}\) suggests marked interactions among binding sites, characterized by cooperative and anti-cooperative binding. In the presence of substrate (dotted line) binding of Co\(^{2+}\) is so strengthened that constants for the first three sites cannot be estimated. To get binding constants under conditions more similar to assay conditions we performed other experiments with \(^{57}\)Co at lower enolase enzyme concentrations were chosen so that bound Co\(^{2+}\) was from 15 to 30% of the free Co\(^{2+}\) and so were generally between 3 and 20 mg/ml. Inset data were obtained at enolase concentrations between 0.2 and 2.0 \(\mu\)M.

Note that although the data of Fig. 1 show cobalt binding to an inhibitory site with a \(K_d\) near 20 \(\mu\)M, the data of Fig. 4 suggest that this would have to be the fourth site per dimer. We see no evidence under these conditions for binding of more than 4 ions even in the presence of substrate. The buffer and salt concentrations in these experiments were identical to those used for kinetic measurements (Fig. 1).

Electronic Absorption Spectra of Co\(^{2+}\)-Enolase Complexes—The electronic absorption spectra were obtained by visible difference spectroscopy. A flat spectral baseline was determined with 60 \(\mu\)M apoenolase in both the sample and reference cuvettes. Upon addition of 2 eq of Co\(^{2+}\) per dimer, a weak broad spectrum was observed with an absorption maximum at 18.4 kilokaisers (545 nm, \(E = 34 \text{ M}^{-1} \text{ cm}^{-1}\)). Weaker maxima appear to be present at 17.2 kilokaisers (580 nm, \(E = 27 \text{ M}^{-1} \text{ cm}^{-1}\)) and 20.8 kilokaisers (480 nm, \(E = 17 \text{ M}^{-1} \text{ cm}^{-1}\)) (Fig. 5, Curve A). All molar absorptivities are calculated with reference to Co\(^{2+}\) concentration. The addition of 2 more equivalents of Co\(^{2+}\), a total of 4 eq of Co\(^{2+}\)/dimer, causes only a slight absorbance increase in the spectrum. The difference between the spectra observed with 2 and 4 mol of Co\(^{2+}\)/mol of enolase dimer is shown as Curve B in Fig. 5. From dissociation constants estimated from data of Figs. 3 and 4, we calculate that over 90% of the Co\(^{2+}\) is bound when 2 eq of Co\(^{2+}\) are added at the concentrations of Fig. 5. The data of Fig. 4 also suggest that under the same conditions only about one-fourth of the additional Co\(^{2+}\) would be bound when the 3rd and 4th equivalents are added. Therefore, no quantitative interpretation of the spectrum due to those additional equivalents is warranted, but it is likely that most of the additional absorption is due to solvated Co\(^{2+}\). The spectrum of Co(H\(_2\)O)\(_6\)\(^{2+}\) exhibits a spectral maximum at 515.5 nm (\(E = 4.6 \text{ M}^{-1} \text{ cm}^{-1}\)) with shoulders at 465.1 nm (\(E = 2.6 \text{ M}^{-1} \text{ cm}^{-1}\)) and at 625 nm (\(E = 0.3 \text{ M}^{-1} \text{ cm}^{-1}\)) (Sutton, 1968).

Electron Paramagnetic Resonance Spectra of Enolase-Coaltous Complexes—Fig. 6A shows the EPR spectrum of a sample containing 2.0 Co\(^{2+}\)/yeast enolase dimer. Immediately apparent is a perpendicular feature with a crossing point at \(g_{\text{max}} = 3.968, g_{\text{min}} = 2.670,\) and \(g_{\text{min}} = 2.056\). A further increase of cobalt content in the sample to 3.5 Co/dimer results in the
similar spectrum shown in Fig. 6A. However, the \( g_{\text{max}} \) and \( g_{\text{min}} \) features have not changed with the increase in Co content. The difference spectrum, i.e. spectrum 6A minus spectrum 6A is shown as Fig. 6C and shows a broad "parallel" feature at \( g = 2.331 \).

The addition of 2.0 Mg\(^{2+}\)/dimer of enolase containing 1.5 Co\(^{2+}\)/dimer results in the complex EPR spectrum of Fig. 7A. Most prominent in the changes with Mg addition is the appearance of eight resolved Co\(^{2+}\) hyperfine lines centered at \( g = 5.872 \) split by 76 G. However, the sample is clearly not a single species because there are more than three \( g \) features. Above and below the hyperfine features and at \( g_{\text{max}} \) of 3.90 are features similar to those of Fig. 6 and which probably come from Co-enolase to which Mg\(^{2+}\) is not bound. The features at \( g = 2.737 \) and 2.064 probably also belong to the Mg-free enolase. Addition of substrate (1.0 PGA/dimer) to this sample causes a great increase in prominence of the lower \( g \) values at 2.12 and 2.74 and a slight shift of \( g_{\text{max}} \) to 6.512 with the hyperfine splitting remaining unchanged at 76 G. Enough substrate was added to fill only one site in order to see the simplest possible change, uncomplicated by possible interactions between the two substrate sites per dimer. Some features of the 1.5 Co\(^{2+}\)/2.0 Mg\(^{2+}\) species are evident in the spectrum 7B. As can be seen in Fig. 7C, the addition of 1 eq of PGA/dimer with 1.5 eq of Co\(^{2+}\) causes appearance of a spectrum very similar to that of the sample which also contained Mg. Further addition of Co to the level of 3.5 Co/dimer in the presence of PGA yields the spectrum of Fig. 7D which is dissimilar to the previous 1.5 Co\(^{2+}\)/1 PGA/dimer spectrum. In addition to the reduction of the clarity of the hyperfine splitting there are slight shifts to the higher field features of the 3.5 Co\(^{2+}\)/1 PGA/dimer spectrum.

Oxidation of Co\(^{2+}\)-Enolase by \( \text{H}_2\text{O}_2 \)---Studies were undertaken to determine whether Co\(^{2+}\) bound to yeast enolase could be oxidized to yield covalently bound cobaltic ion labels at the metal ion-binding sites. The results of one such experiment and associated controls are shown in Fig. 8. Upon addition of 50 mM \( \text{H}_2\text{O}_2 \) to a 4.9 \( \mu \text{M} \) enolase solution containing 0.5 mM Co\(^{2+}\) (4 Co\(^{2+}\) are bound per dimer at this concentration), the enzyme is inactivated with a half-time of about 7 min. Control experiments showed that the time required for complete inactivation with Co\(^{2+}\) plus \( \text{H}_2\text{O}_2 \), no significant activity loss was observed when enolase was incubated with 0.5 mM Co\(^{2+}\) alone, with 50 mM \( \text{H}_2\text{O}_2 \) alone, or with 0.5 mM Mg\(^{2+}\) plus 50 mM \( \text{H}_2\text{O}_2 \). The half-time for inactivation was unaffected when the \( \text{H}_2\text{O}_2 \) concentration was raised from 50 to 100 mM. When the Co\(^{2+}\) concentration was lowered to 0.2 mM, however, \( t_{1/2} \) increased to 18 min at [\( \text{H}_2\text{O}_2 \)] = 0.1 M.

At low ratios of Co\(^{2+}\) to enolase, the rate of enzyme inactivation is not linear with cobalt concentration. At an ionic strength of 10 mM HEPES buffer, pH 7.4, this effect was so pronounced that no inactivation of 1.1 \( \mu \text{M} \) enolase occurred in half an hour in the presence of 1.5 eq of Co\(^{2+}\) per dimer in
the ±H₂O₂ spectra agree within 1%. Note that the g₁₀ feature is more clearly seen in this spectrum than in the spectra of 2.0 Co/dimer. Fig. 10 shows the spectrum of 3.5 Co/dimer plus H₂O₂. The double integrated spectra show an intensity loss of 34% after the 1-min exposure to H₂O₂, indicating conversion of Co²⁺ to Co³⁺. No further change took place during an additional 4-min exposure. The lack of such a change when there are fewer than 2 Co²⁺ per dimer indicates that the conformational metal is inaccessible to H₂O₂. Furthermore, there is apparently no electron transfer from the conformational site Co²⁺ to the catalytic site Co³⁺ since the loss of signal intensity is not complete. Such electron transfer would not have been too surprising since close interactions between the sites have been deduced from EPR observation on the Mn⁴⁺ enzyme (Chien and Westhead, 1971) and on the Cu²⁺ enzyme (Dickinson et al., 1980). It appears that inactivation of the enzyme is due directly to the formation of Co⁴⁺ at the catalytic sites although we have seen spectral changes during oxidation that must result from cobalt-catalyzed oxidation of protein residues. Ultraviolet and visible spectra were recorded during and after oxidation reactions, and the most prominent changes were the growth of an absorbance peak at 305 nm and increasing absorbance at low wavelengths, seen as a rapidly rising absorbance below 260 nm. Comparison of the rate of change at 305 nm with the rate of inactivation showed that the latter was far faster than the absorbance change. The absorbance change in one experiment was linear during an hour of observation; 5 min after H₂O₂ addition, the enzyme was inhibited 80%, but the absorbance increase at 305 nm had reached only about 10% of its 60-min value. Apparently oxidation of protein residues can occur, but those detectable by UV spectroscopy are much slower than inactivation or loss of the EPR spectral intensity.

Cobalt Binding to H₂O₂-inactivated Enolase—Enolase inactivated by treatment with 50 mM H₂O₂ in the presence of 0.5 mM Co²⁺ retained bound cobalt after elution over a column of metal-free Sephadex G-25. An average of 3.45 eq of cobalt/dimer (average of five experiments) remained bound following column elution. By contrast, no cobalt remained bound if the H₂O₂ treatment was omitted. These results were obtained by A₂₈₀ and Lowry protein concentration measurements and by ⁵⁷Co radioactivity and Nitroso-R-Salt total Co concentration measurements. The results clearly indicate that the H₂O₂ treatment is required for cobalt binding to enolase during nonequilibrium gel filtration.

The long term stability of the cobalt-enolase complex prepared by reaction with H₂O₂ was tested. Enolase (60.8 µM) was incubated with 0.5 mM ⁵⁷Co²⁺ and 50 mM H₂O₂ until it...
lost greater than 99% of the original activity. It was then eluted through a Sephadex G-25 column (1 x 40 cm) at t = 0 to remove unbound cobalt. The protein peak fractions were combined and incubated at 30 °C. At this point, Lowry protein analysis and 153CoO2+ radioactivity measurements showed that the enzyme retained 3.2 eq of cobalt bound per dimer. Aliquots of this incubation solution (with 3.2 cobalt/dimer) were passed over new identical Sephadex G-25 columns at 4.0, 16.0, and 72 h. These columns were previously equilibrated with metal-free 10 mM HEPES (K+), 100 mM KCl, pH 7.5, buffer. Elution profiles from these three columns showed two well separated peaks of radioactivity, the first associated with protein and the second that of free cobalt ion. The stoichiometry of bound cobalt per dimer was determined as 3.2 at t = 0, 2.2 at 4 h, 1.1 at 16 h, and 0.8 at 72 h. These results show that cobalt spontaneously dissociates from the protein until the stoichiometry becomes slightly less than one ion per dimer after 3 days. Equivalent results were obtained by dialysis; after 3 days retained cobalt was down to about 0.9 atom/enzyme dimer, and this was retained for an additional 4 days. Under identical dialysis conditions, cobalt added to enolase as CoO2+ was almost completely removed in less than 2 days.

Very limited chymotryptic and tryptic digestion of enzyme containing the one tightly bound atom of cobalt produced a lower molecular weight form of the enzyme still containing bound cobalt. Further digestion to produce small peptides, however, led to complete loss of the label from peptide material.

**DISCUSSION**

This discussion will be concerned chiefly with three questions regarding the metal ion sites on enolase. How many binding sites are there? Are the sites independent with apparent symmetry between the two halves of the dimer? What do the data tell us about the environment of the metal ion sites?

The characteristic difference spectrum produced by the binding of cations to enolase in this buffer is evidentially due to a conformational change which brings tryptophan and tyrosine rings into a less polar environment, therefore, a "tightening" of the protein structure (Brewer 1981). There has been some question about the stoichiometry of the spectral change with metal ion binding because higher than stoichiometric concentrations of metals previously used are required to obtain saturation (Fig. 2; see also Brewer and Collins, 1980). The strongly bound CoO2+ ion, however, shows clear stoichiometric binding at 2 sites/dimer to produce the conformational change (Fig. 3). Since cobalt ions are readily bound to more than 2 sites/dimer (Fig. 4), the filling of 2 sites/dimer is necessary and sufficient to produce all of the conformational change detected spectrally. The tightness of binding of course makes it impossible to detect the nonequivalent equilibrium constants observed for binding of the first two eq of Mg2+ (Brewer and Collins, 1980, and references therein).

In the case of Mg2+ and Mn2+, the dissociation constants from sites other than the first two/dimer are so weak that no binding is measurable in the absence of substrate (Hanlon and Westhead, 1969a) although addition of Mn2+ in excess of two/dimer caused striking exchange splitting in the EPR spectrum of the first two ions bound, suggesting that low level occupancy of a 3rd site was responsible (Chien and Westhead, 1971). With Cu2+ EPR, however, no evidence for such interaction was observed (Dickinson et al., 1980). It seems probable that the metal site interactions are affected critically by the nature of the metal ions present. For example, early work (Hanlon and Westhead, 1969a) which showed nonequivalent binding of either Mn2+ or Mg2+ to the two subunits also showed that when both metals were present they each bound with the lower dissociation constant. Our new interpretation of this phenomenon is that negative interactions between the metal sites on the two subunits occurs when the same ion is present on both subunits but not when Mn2+ is at one site and Mg2+ is available to fill the second site. A further indication of asymmetry in the subunits from the present work is that just about 1 eq of cobalt/dimer remains tightly bound during dialysis of the H2O2-treated enzyme.

The presence of substrate produces readily detectable binding of two additional Mn2+ or Zn2+ ions per dimer. However, four Cu2+ ions are bound per dimer of enolase in the presence or absence of substrate, and we see that this is true also of CoO2+ (Fig. 4). In the latter case the data show that binding is greatly enhanced in the presence of substrate as it is for Mg2+ or Mn2+. Our data showing a maximum of four CoO2+ ions bound at millimolar concentrations of CoO2+ agrees with previous data for Mn2+ and Mg2+. We consider this a very important point since these metals all cause inhibition of catalytic activity at higher concentrations (e.g. Fig. 1). Convincing data (Faller et al., 1977; Shen, 1973) show that at least the third metal ion is required for activity of the enzyme. We have to deduce that negative interactions between the subunits allow the 3rd ion per dimer to be activating and cause inhibition of the enzyme when the fourth site is filled.

This conclusion is controversial since Elliott and Brewer (1980) have reported the binding of 4 eq of CoO2+/dimer at 0.1 mM free CoO2+ and find a total of 6 eq of CoO2+ or Zn2+ bound in the presence of substrate. The fifth or sixth equivalents would be responsible for inhibition in their scheme. Our calculated inhibition constant of 18 µM does not have an obvious correspondence with a binding constant in the two sets of data of Fig. 4, but it is not inconsistent with the binding of the fourth equivalent of CoO2+ substrate. It would be difficult to get data of sufficient precision to measure the correspondence with certainty. Nevertheless, there is a clear difference between our finding of four metal ion sites and the finding of six sites by Elliott and Brewer.

The difference in the nature of the two "conformational" metal sites on the two "catalytic" metal sites is apparent both in the visible spectra (Fig. 5) and the EPR spectra (Fig. 6). Without a complete electronic absorption spectrum for these species, including near-infrared transitions, assignment of the CoO2+ coordination number and geometry for 2 Co(II)-enolase must remain tentative. Both tetrahedral and octahedral geometries for high spin Co3+ can give rise to bands at approximately the same energy, around 500 nm. However, tetrahedral complexes more frequently give rise to bands maxima at lower energy (700 nm) (Carlin, 1965). Five coordinate CoO5 complexes also exhibit bands throughout the spectral region of the Co(II)-enolase transitions (700 to 460 nm). Spectral intensity is a better indicator of geometry. The spectrum of tetrahedrally coordinated Co3+ is much more intense than that of CoO2+ in an octahedral geometry, usually by a factor of 100 in molar absorptivity (Carlin, 1965). The most intense visible absorption bands in distorted tetrahedral CoO5 complexes generally have molar absorptivities that exceed 250 M-1 cm-1; those for five-coordinate CoO7 complexes are between 60 and 225 M-1 cm-1 (Rosenberg et al., 1973; Rosenberg et al., 1976; Dori and Gray, 1968; Ciampolini and Nardi, 1967); regular octahedral complexes, such as hexaquocobalt II, have absorptivities of about 5 M-1 cm-1.

The absorptivity of CoO2+ enolase most probably indicates a distorted octahedral geometry. Either inherent disymmetric
or dynamic vibration of ligands about the symmetric position (vibronic coupling) can increase band intensity an order of magnitude above that of a regular octahedral Co\textsuperscript{2+} complex (Ballhausen, 1960). The spectra of the second pair of cobalt ions bound are not distinguished from that of hexaquacobaltous ions so they appear to form more regular octahedral complexes with oxygen ligands. It is interesting that the enzymes usually classified as metalloenzymes form Co\textsuperscript{3+} complexes that have much greater spectral intensity than that of enolase. They are generally thought to be tetrahedral (Haffner and Coleman, 1973; Fee, 1973; McMillin et al., 1974a, 1974b; Cockle et al., 1974; Vallee and Wacker, 1970) or 5-coordinate (Rosenberg et al., 1975). The EPR spectra reinforce the deduction that cobalt enolase forms distorted octahedral complexes at the first two sites.

The EPR spectra of the first two cobalt ions to bind to yeast enolase (Fig. 6A) show apparent g values of 2.06, 2.67, and 3.97. Except for the splitting of the lower g values, the spectra resemble rather closely those of known tetrahedrally distorted octahedral complexes of high spin Co(II) (Bencini et al., 1980). There has been rather great interest in high spin cobalt (II) EPR parameters recently (Bencini et al., 1981; Banci et al., 1980) but because the g\textsubscript{app} tensor reflects g\textsubscript{\perp}, g\textsubscript{D} and g\textsubscript{E} deduced from the three observed g\textsubscript{app} values are rather limited. A number of useful calculations have been made but the calculations require a number of rather arbitrarily chosen ligand field parameters. The range of expected g\textsubscript{app} values for octahedral cobalt coordination for a range of axial and tetragonal distortion parameters has been calculated (Bencini et al., 1981). However the above g values cannot be fit clearly into that scheme suggesting that the distortions in cobalt enolase for the first two metal sites per dimer are of symmetry lower than 4-fold. Nevertheless, considering Fig. 4 in Bencini et al., 1981, the g values near 4 and 2 suggest that the tetragonal component of the distortion is quite large but the axial compression is small. Without single crystal data, of course, the directional meaning of “axial” is indefinite, but the term does carry symmetry information about the ligand environment of the Co ion.

The addition of a third and fourth cobalt ion to yeast enolase contributes the spectrum of Fig. 6C to the total EPR spectrum. This spectrum is only slightly different from that of the first two cobalt ions bound, but because of the intermediate g values of the latter, it is quite distinguishable from the former. However, these latter cobalt sites give an EPR spectrum hardly distinguishable from that of an aqueous solution of Co\textsuperscript{3+} ion, just as in the case of the visible spectrum (Fig. 5). The binding data reassure us that these latter sites are protein bound. It is not surprising that the environment of these metal ions is similar to Co\textsubscript{2+}(H\textsubscript{2}O)\textsuperscript{6+} because the parameters of the EPR for Cu binding to enolase were found to reflect only oxygen ligands, also in a distorted octahedral environment.

The line widths of all cobalt enolase samples reported in this work are much larger than those of corresponding simple inorganic complexes. This must reflect a certain “indefiniteness” in the geometry of the metal site in the protein; there may be a range of “conformational substances” as were found for myoglobin (Frauenfelder et al., 1979). These substances could be small variations of ligand geometry at the metal site although there is not sufficient resolution to describe the EPR line widths in terms of certain definite substances as opposed to a continuum of states.

The addition of magnesium (Fig. 7A) or 2 PGA (Fig. 7B) increases the g anisotropy of the spectrum of the first two Co\textsuperscript{2+} ions bound and causes the appearance of cobalt hyperfine splitting of 76 G on the low field g value. This increase in g\textsubscript{max} to about 6 with g\textsubscript{min} = 2.7, according to the scheme of reference (Bencini et al., 1981), indicates an increase in the ratio of tetragonal distortion to axial distortion. The effects of addition of Mg\textsuperscript{2+} and PGA are apparently very similar but are distinguishable giving g = 5.87 and 6.64, respectively, with A\textsubscript{iso} = 76 G in both cases. The sample containing 2.0 Mg\textsuperscript{2+} and 1.0 PGA gives an EPR spectrum most similar to that of the sample containing only 1.0 PGA, but in the low field region peaks belonging to the spectrum of Fig. 7A are evident. These changes indicate that the PGA has a definite effect on the structure of the metal site. Whether this is mediated through the protein conformation change or through direct binding of PGA to the cobalt ion cannot be decided from these experiments. Further addition of cobalt (Fig. 7D) seems not to increase the g = 4 region. This indicates a site rather similar to that giving the spectrum of Fig. 6B indicating that in the presence of PGA or Mg, the second binding sites remain weakly tetragonally distorted.

Our attempts to provide a covalent metal site probe by oxidation of Co\textsuperscript{3+} in situ with H\textsubscript{2}O\textsubscript{2} was not successful in its primary objective. Formation of very stable Co\textsuperscript{3+} complexes is greatly favored by nitrogenous ligands and disfavored by a preponderance of oxy ligands. The failure of enolase to form stable Co\textsuperscript{3+} complexes is in accord with deductions from Cu\textsuperscript{2+} EPR spectra (Dickinson et al., 1980) that enolase metal sites do not include nitrogenous ligands. At low ratios of Co\textsuperscript{3+} to enolase, especially at low ionic strength there is a striking resistance of the enzyme to oxidation in the presence of H\textsubscript{2}O\textsubscript{2}. EPR spectral data (Figs. 9 and 10) show conclusively that lack of inactivation is the consequence of lack of Co\textsuperscript{3+} oxidation and not to insensitivity of the enzyme to the oxidation state of cobalt in the first two sites. Apparently the first two metal ions bound (the “conformational” metal ions) are buried in a tight environment inaccessible to H\textsubscript{2}O\textsubscript{2}, especially at low ionic strength. The change in the metal ion environment as a result of H\textsubscript{2}O\textsubscript{2} treatment (Fig. 9) is very likely correlated with the slow oxidation of organic residues seen as a change in the optical spectra of the protein.

The second pair of Co\textsuperscript{2+} ions bound is readily accessible to H\textsubscript{2}O\textsubscript{2} and readily oxidized to Co\textsuperscript{3+}. The difference in ease of removal of these ions during subsequent dialysis is a fourth suggestion that the enolase dimers do not behave symmetrically.

Throughout our discussion we have assumed that the addition of substrate causes increased affinity of metal ions to the enzyme, at all metal-binding sites, but does not cause redistribution of metal ions from “conformational” to catalytic sites. A forthcoming study \(^2\) in which Cr\textsuperscript{3+} ion has been fixed to specific sites as Cr\textsuperscript{3+} will provide more unambiguous data on the effects of metal occupancy of specific sites.

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Note Added in Proof—Brewer et al. (1983) have observed effects of substrate additions on Co\textsuperscript{2+}-enolase EPR spectra; our data and conclusions are in agreement with theirs. Estimates of numbers of binding sites/mol of enzyme remain different, however (c.f., Elliot and Brewer, 1980).

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