Formation and Characterization of an Active Phosphoenolpyruvate Carboxykinase−Cobalt(III) Complex†

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ABSTRACT: Avian mitochondrial phosphoenolpyruvate carboxykinase (PEPCK) was incubated with Co^{2+} and H_2O_2 to form a stable Co^{3+}−PEPCK complex. PEPCK, similarly incubated with H_2O_2 and either Mg^{2+} or Mn^{2+}, resulted in no significant loss in activity over 30 min. PEPCK, incubated with Co^{2+} and H_2O_2 at pH 7.4, showed rapid inhibition as observed by a 40% decrease in activity after 5 min. The loss of activity is linear with the incorporation of cobalt into PEPCK, resulting in 15−25% activity for the stoichiometric Co^{3+}−PEPCK complex. The incorporation of and inhibition by Co^{3+} is protected by PEP and GTP (ITP). Treatment of the Co^{3+}−PEPCK complex with β-mercaptoethanol results in a loss of cobalt and full recovery of activity. The reduction and reactivation are protected by PEP and GTP (ITP). EPR, PRR, circular dichroism, and fluorescence studies all indicate that Co^{3+} has been selectively incorporated into the cation site of PEPCK, resulting in a catalytically active enzyme−cation species. The substrates form Michaelis complexes with Co^{3+}−PEPCK, and the catalytic reaction occurs as a second sphere complex as previously suggested [Lee & Nowak (1984) *Biochemistry 23*, 6506); Duffy & Nowak (1985) *Biochemistry 24*, 1152]. Proteolytic digestion of the Co^{3+}−PEPCK complex and isolation of the cobalt-containing peptide by reverse phase HPLC were performed to identify the location of the cation binding site. From mass, amino acid composition, and sequence analyses of the isolated cobalt-peptide, the region Thr276−Lys301 is responsible for metal chelation. This very homologous region, located in the central portion of PEPCK, contains two highly conserved aspartic acids, Asp295 and Asp296, that are the only feasible metal binding ligands.

Chicken liver mitochondrial phosphoenolpyruvate carboxykinase [GTP/ITP: oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] (PEPCK)[1] is a 67 kDa monomeric enzyme that catalyzes the following reversible reaction (Utter & Kurahashi, 1953):

$$\text{OAA} + \text{GTP (ITP)} \overset{Mn^{2+}}{\rightleftharpoons} \text{PEP} + CO_2 + \text{GDP (IDP)} \quad (1)$$

The primary role of this enzyme in higher organisms appears to be the catalysis of the formation of PEP from OAA as the first committed step in gluconeogenesis.

Nucleotide and derived protein sequences of the mature form of the mitochondrial isoenzyme of PEPCK from chicken liver have been derived from cDNA clones (Weldon *et al*., 1990). Crystallographic analysis of PEPCK from animal sources has not been possible due to the current inability to crystallize the enzyme. A recent diffraction study of crystals of the *E. coli* PEPCK has been reported with resultant structural information (Matte *et al*., 1996).

PEPCK shows an absolute requirement for divalent cations for activity. Mn^{2+} is the best activator for avian mitochondrial PEPCK, with Co^{2+} and Mg^{2+} activating to a lesser extent (Lee *et al*., 1981). Mixed metal studies showed a dual cation role for PEPCK (Lee *et al*., 1981). One metal is associated with the enzyme while the other is bound to the nucleotide which serves as the substrate. Results of ^1H and ^{31}P NMR relaxation rate studies were used to describe the formation of Michaelis complexes where substrates bind in the outer sphere of the bound cation and the catalytic reaction occurs as a second sphere complex (Lee & Nowak, 1984; Duffy & Nowak, 1985). PRR studies have suggested that two water ligands are associated with the enzyme-bound metal, indicating that the enzyme provides four ligands to the hexavalent metal (Lee & Nowak, 1984). It was suggested that one of these water molecules serves as a bridge between the substrate and enzyme-bound metal.

Active site residues of avian liver PEPCK have been determined by amino acid and site-selective modification experiments. Proteolytic digests of the modified enzymes followed by chromatographic peptide separation and sequencing have identified the labeled amino acids that may be involved in PEPCK-catalyzed reactions. Using a variety of sulfhydryl reagents, Makinen and Nowak (1989) have located a reactive cysteine in PEPCK although they suggest that it does not directly participate in catalysis. Cys273 was identified as the reactive residue that is labeled by chemical modification with dialdehyde GDP (Nowak *et al*., 1992) suggesting that Cys273 may participate in the binding of the nucleotide. This cysteine is predicted to be at a β-turn and is conserved throughout all species of GTP-utilizing PEPCK.

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[1] Abbreviations: AA, atomic absorption; ArgC, endoproteinase ArgC; β-met, 2-mercaptoethanol; CE, capillary electrophoresis; Co^{3+}−PEPCK, cobalt(III) chemically modified PEPCK; DEAE, diethylaminoethyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EPR, electron paramagnetic resonance; GSH, glutathione; HPLC, high-performance liquid chromatography; LysC, endoproteinase LysC; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PRR, water proton longitudinal relaxation rate.

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Cheng and Nowak (1989a) have demonstrated that a single arginine residue is critical for activity and have proposed that it is involved in CO_2 binding and activation. Dione modification and peptide analysis have shown that Arg289 is the active site residue (Nowak *et al*., 1992). Through the use of the lysine-specific reagent pyridoxal phosphate, Guidinger and Nowak (1991) have shown one reactive lysine at the active site of PEPCK that is involved in the binding and activation of the phosphate-containing substrates. Pyridoxal phosphate modification and inactivation of PEPCK are protected by PEP and GDP. Isolation and sequencing of the labeled protein identified the pyridoxal phosphate label at Lys141 (Nowak *et al*., 1992). Cheng and Nowak (1989b) have also shown that PEPCK has a reactive histidine residue that is at or near the PEP binding site and is involved in catalysis. The identity of this histidine residue is currently unknown. It is believed that most of the active site residues are within the core structure of PEPCK. The identification of the amino acids associated with the metal binding site of avian liver mitochondrial PEPCK is unknown. Crystallographic analysis of the *E. coli* PEPCK (Matte *et al*., 1996) revealed that Asp269 was directly coordinated to the active site metal. It should be noted that there are considerable differences between the *E. coli* and the avian liver enzymes. Ca^{2+} is the best activator for *E. coli* PEPCK, whereas Ca^{2+} is a poor activating cation for the avian liver PEPCK (activity less than 10% that of Mn^{2+}). *E. coli* PEPCK is an ATP-dependent kinase while avian liver PEPCK uses GTP and is not activated by nor does it bind to ATP. No significant sequence homology exists between the *E. coli* and avian liver enzymes.

Recently, the substitution-inert metal Co^{3+} (Cotton & Wilkinson, 1972) has been used to investigate the role that the metal plays in enzyme catalysis. Co^{2+} can be readily oxidized to Co^{3+} by peroxides. When the cobaltous ion binds to an enzyme and is further subjected to oxidation, the resulting cobaltic ion can potentially form ligand exchange-inert complexes with the residues on the enzyme. The *in situ* oxidation of Co^{2+} has been used to study the metal binding sites of several proteins. Kang and Storm (1972) first reported the initial preparation and characterization of Co^{3+}−carboxypeptidase A via the oxidation of the Co^{2+}−enzyme with H_2O_2. The Co^{3+} in carboxypeptidase A is ligand exchange-inert, and the modified enzyme has low peptidase activity. The Co^{3+}−bovine carbonic anhydrase B was prepared in a similar fashion and was reported to be inactive (Shinar & Navon, 1974). Two Co^{3+} atoms were incorporated into alkaline phosphatase with loss of activity (Anderson & Vallee, 1975). The incorporation of Co^{3+} into aspartokinase−homoserine dehydrogenase resulted in one Co^{3+} per subunit and concomitant inactivation of the aspartokinase activity of the enzyme (Rysewski & Takahashi, 1975). Balakrishnan and Villafranca (1979) reported the preparation of both the Co^{3+} and Cr^{3+} glutamine synthetase derivatives. Miziorko *et al*. (1982) reported the stoichiometric incorporation of Co^{3+} into ribulosebisphosphate carboxylase. The Co^{3+}-modified ribulosebisphosphate carboxylase was inactive but still permitted the binding of substrates. When yeast enolase was incubated with Co^{2+} and H_2O_2 at pH 7.5, 2 mol of Co^{3+} was incorporated per monomer. The modified enolase had no catalytic activity. Protection from inactivation was obtained in the presence of the substrate 2-phosphoglycerate. Enolase modified under these conditions retained full catalytic activity and had 1 mol of Co^{3+} incorporated per monomer (Lee, 1988).

This paper discusses the modification of the metal binding site of avian liver mitochondrial PEPCK by Co^{3+} and subsequent formation of a catalytically active Co^{3+}−PEPCK complex. The kinetic and binding properties of the Co^{3+}−PEPCK complex were determined and compared with those of apoPEPCK. Substrate protection studies against the labeling and the removal of the Co^{3+} atom from PEPCK were also examined. The amino acid residues at the metal binding site were identified by protease digestion of Co^{3+}−PEPCK followed by HPLC purification of the cobalt-containing peptide. Mass, amino acid composition, and sequence analyses of the cobalt-containing peptide were performed.

MATERIALS AND METHODS

Materials

Malate dehydrogenase, ArgC, LysC, pyruvate kinase, and lactate dehydrogenase were purchased from Boehringer Mannheim Corp. GTP, ITP, GDP, IDP, ADP, PEP, OAA, NADH, $CaCl_2$, and tetramethylammonium sulfate were purchased from Sigma. $CoCl_2$, $ZnCl_2$, $CuCl_2$, $FeCl_2$, and Tris base were purchased from Mallinckrodt. $CdCl_2$, $MnCl_2$, and $MgCl_2$ were from Baker. Chelex-100, DEAE-Sepharose, and hydroxyapatite A resins were from Bio-Rad. Butyl-Sepharose was purchased from Pharmacia. All other reagents were of the highest purity commercially available. All nonmetal solutions were passed through a Chelex-100 column to remove any contaminating metal ions. Metal solutions were prepared with distilled water which was passed through a mixed-bed deionizing column and then through a Chelex-100 column and adjusted to pH 4.0.

Methods

Chicken liver mitochondrial PEPCK was purified by a modification of the procedure of Lee and Nowak (1984). Mitochondrial isolation, lysis, and ammonium sulfate fractionation were done as previously described. Chromatography was performed on butyl-Sepharose, gel filtration (desalting), DEAE-Sepharose, and hydroxyapatite A columns. After ammonium sulfate precipitation, the protein was solubilized in 1.5 M ammonium sulfate in 5 mM potassium phosphate at pH 7.0 and loaded onto a 100 mL butyl-Sepharose column equilibrated in the same buffer. After the column was washed, a 1 L gradient from 1.5 M to 0 M ammonium sulfate in 5 mM phosphate buffer, pH 7.0, was utilized to elute the PEPCK in a sharp peak at 0.75 M ammonium sulfate. The pooled protein was reconcentrated with 70% ammonium sulfate and desalted using Sephadex G-50 equilibrated in 5 mM phosphate buffer, pH 7.0. The protein was then washed through 50 mL of DEAE-Sepharose resin. The slightly yellow protein that eluted was then washed through 10 mL of hydroxyapatite A resin. PEPCK was not retained on either the DEAE-Sepharose or the hydroxyapatite A resins but contaminating proteins were. The pooled protein was concentrated to 6 mg/mL, diluted in 2 M sucrose, quick-frozen, and stored at −70 °C. This improved procedure gave as much as 70% yield at >95% purity levels. Following the lysis of the mitochondria, the

remainder of the PEPCK purification procedure took less than 3 days. Prior to the studies presented here, PEPCK was desalted using a P6-DG (1 × 20 cm) column having a 2 cm layer of Chelex-100 on top equilibrated in 50 mM Tris-HCl buffer, pH 7.4, and concentrated using a mini-Amicon concentrator with a PM30 membrane. The enzyme used for all studies typically had a specific activity between 4.5 and 7 units/mg (assayed in the "reverse" direction as discussed below) and was >95% pure.

PEPCK Assay. The PEPCK-catalyzed reaction of PEP carboxylation to OAA (the "reverse" direction) was assayed by the method of Noce and Utter (1975) as modified by Hebda and Nowak (1982a). In this continuous assay, PEPCK activity was coupled to malate dehydrogenase, and the oxidation of NADH was spectrophotometrically measured at 340 nm and 25 °C using a temperature-controlled cell. The PEPCK-catalyzed reaction of OAA decarboxylation to PEP (the "forward" direction) was assayed by the method of Hebda and Nowak (1982b). Here, PEPCK activity was coupled to pyruvate kinase and lactate dehydrogenase, and the oxidation of NADH was spectrophotometrically measured at 340 nm. The specific activity is defined as units of enzyme activity per milligram of protein where 1 unit is the amount of enzyme catalyzing the formation of 1 μmol of product mL^{-1} min^{-1} under experimental conditions. All activity assays were performed with a Gilford 240 or 250 spectrometer. Kinetic data were treated by the "EZ-FIT" program, version 2.02, by Perrella Scientific Inc., 1989.

Substrate Concentration. When an accurate determination of substrate concentration was required (i.e., for K_m studies), such determinations were performed enzymatically. The concentration of PEP, OAA, GDP, IDP, GTP, and ITP was determined as the limiting reagent in the PEPCK assays described above. The total absorbance change at 340 nm due to the oxidation of NADH upon addition of a known amount of substrate was determined. The concentration of substrate was calculated by accounting for the extinction coefficient for NADH and the volume of substrate. Metal solution concentrations were determined by AA.

Cobalt(III) Modification of PEPCK. PEPCK was inhibited in the presence of $CoCl_2$ and H_2O_2 at pH 7.4; 50−100 μM PEPCK, in 50 mM Tris-HCl, pH 7.4, and 100 mM KCl, was incubated on ice with 4 mM $CoCl_2$ for 15 min. H_2O_2 was added to give a final concentration of 20 mM. The solution was allowed to sit on ice for an additional 30 min with periodic gentle shaking of the solution. After incubation, excess cobalt and H_2O_2 were removed by chromatography on a P6-DG (1 × 20 cm) column having a 2 cm layer of Chelex-100 on top. The column was equilibrated in 50 mM Tris-HCl buffer, pH 7.4. The modified enzyme was concentrated using a mini-Amicon concentrator with a PM30 membrane. The enzyme concentration was determined by the absorbance at 280 nm or by the Bradford Protein Assay Kit. All absorbance measurements were performed on a Beckman 210 spectrophotometer. Co^{3+} content was determined by AA with a Varian AA-775 spectrometer at 241.7 nm using the Varian CRA-90 Carbon Rod atomizer. This procedure consistently gave 1:1 Co^{3+}−PEPCK ratios. Co^{3+}−PEPCK is stable at 4 °C for at least 1 week with no loss of the cobalt label or activity during this time. All of the experiments discussed within were performed with freshly prepared Co^{3+}−PEPCK. Substoichiometric amounts of Co^{3+} were incorporated into the enzyme by decreasing the $CoCl_2$ concentration in the incubation mixture. Enzyme activity was assayed as described earlier.

Substrate Protection Studies. Substrate protection against Co^{3+} incorporation into PEPCK was determined; 50 μM PEPCK in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM KCl was incubated in the presence of 4 mM $CoCl_2$ and 20 mM H_2O_2 at pH 7.4 with known amounts of substrate. Aliquots (100 μL) were withdrawn at different incubation times and added to an Eppendorf tube containing approximately 25 μL of Chelex-100 resin. The tube was gently shaken and quick-spun using a minicentrifuge to pellet the resin. The residual activity (assayed in the "reverse" direction as described above) and cobalt content were determined from the enzyme in the supernatant.

Substrate protection against Co^{3+} removal from the modified enzyme was performed in an analogous fashion to the Co^{3+} incorporation studies. Co^{3+}−PEPCK in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM KCl was incubated in the presence of known substrate concentrations and 143 mM β-met. At recorded time intervals, aliquots were withdrawn and added to Eppendorf tubes containing Chelex-100 resin. The tubes were shaken and quick-spun as described above, and cobalt content and activity (assayed in the "reverse" direction) were determined.

PEPCK inactivation and reactivation rate constants were calculated as first-order constants from the slope of the line as determined from the plots of log (% activity) vs time. The data were fit using Cricket Graph, V.1.2.

Cysteine Determinations. Cysteine determinations were done using the modification of the method described by Habeeb (1972). Approximately 1 nmol of PEPCK or Co^{3+}−PEPCK, in a volume of 10 μL, was added to a cuvette containing 1.0 mM DTNB in 4 M guanidine hydrochloride, and the absorbance at 412 nm was recorded. The final volume was 0.4 mL. The reaction was complete after 1 min. Using the reported extinction coefficient of 13 600 M^{-1} cm^{-1} (Habeeb, 1972), the number of moles of thiophenolate anions of 5-thio-2-nitrobenzoic acid released upon the reaction with DTNB of PEPCK was calculated. Absorbance values for controls, containing all reagents except enzyme, were subtracted from the observed experimental values.

Circular Dichroism Measurements. Circular dichroism measurements were obtained with a Cary Model 60 recording spectropolarimeter which has been upgraded to a computer-assisted AVIV Model 62DS unit. Samples of 0.26 μM apoPEPCK, Mn^{2+}−PEPCK, and Co^{3+}−PEPCK solutions were prepared in 5 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl. This buffer was used as the reference sample as well as to prepare enzyme solutions. For Mn^{2+}-containing samples, 50 μM $MnCl_2$ was used, and 200 μM PEP was used for samples containing PEP. Prior to analysis, the sample was purged with N_2, and the measurements were conducted in a N_2 atmosphere. Measurements were made in the near-UV (250−300 nm) and far-UV (190-250 nm) regions using a high-quality quartz optical cell with a path length of 1 cm. A CD spectrum of base line was digitally subtracted from the enzyme spectrum of the sample. The resulting values are expressed as differences in the molar extinction coefficient ($\Delta\epsilon$) of the left-handed ($\Delta\epsilon_L$) and right-handed ($\Delta\epsilon_R$) components of circularly polarized light. The observed ellipticity, θ, was converted to ellipticity per mean residue weight, $[\theta]$, by eq 2:

$$[\theta] = \frac{M\theta}{100lc} \quad (2)$$

where M is 109.1, the mean residue weight, l is the optical path length in centimeters, and c is the concentration of enzyme in grams per milliliter. Secondary structure estimations were determined by fitting the mean residual weight ellipticity data to the AVIV program PROSEC V.3.1.

Mn^{2+} Binding to Co^{3+}−PEPCK. The binding of Mn^{2+} to Co^{3+}−PEPCK was determined by measuring the water proton relaxation rates (PRR) using a Seimco-pulsed NMR spectrometer at 24.3 MHz using the Carr−Purcell (1954) 180°−τ−90° sequence. The enhancement values were calculated from the paramagnetic effect of the longitudinal relaxation rates ($1/T_{1p}$). A more rigorous description of this technique has been presented elsewhere (Nowak, 1981). An outline of the method is described here. The contribution of Mn^{2+} to the longitudinal relaxation rate of water ($1/T_{1p}$) is expressed as

$$1/T_{1p} = 1/T_{1(obs)} - 1/T_{1(o)} \quad (3)$$

where $1/T_{1p}$ is the contribution of the paramagnetic species to the relaxation rate, $1/T_{1(o)}$ is the relaxation rate in the absence of the paramagnetic ion, and $1/T_{1(obs)}$ is the observed relaxation rate. The enhancement of the proton relaxation rate resulting from the binding of Mn^{2+} to a macromolecule (specifically PEPCK) is calculated from the following relationship:

$$\epsilon^* = \frac{1/T_{1p}^*}{1/T_{1p}} = \frac{1/T_{1(obs)}^* - 1/T_{1(o)}^*}{1/T_{1(obs)} - 1/T_{1(o)}} \quad (4)$$

where ϵ^* is defined as the observed enhancement of each sample (Eisenger *et al*., 1962; Cohn, 1963). The asterisk indicates the presence of enzyme. $1/T_{1p}$, $1/T_{1(o)}$, and $1/T_{1(obs)}$ have the same definitions as above.

The observed enhancement for enzyme and Mn^{2+} in the absence of ligands will depend on the fraction of Mn^{2+} which is bound to the enzyme and the enhancement for the enzyme-bound Mn^{2+}, ϵ_b:

$$\epsilon^* = \frac{[Mn^{2+}]_f}{[Mn^{2+}]_t}\epsilon_f + \frac{[Mn^{2+}]_b}{[Mn^{2+}]_t}\epsilon_b \quad (5)$$

where ϵ_b is the enhancement of the binary enzyme−Mn^{2+} complex, ϵ_f is the enhancement of the hexaquo cation (defined as unity), and f, b, and t refer to free, bound, and total concentrations of Mn^{2+}, respectively. In treatment of the data, $\epsilon_b = 12.2$ for the Mn^{2+}−PEPCK complex (Hebda & Nowak, 1982b). PRR measurements were taken at room temperature, and the enzyme samples were kept at pH 7.4.

Mn^{2+} binding to Co^{3+}−PEPCK was also studied using EPR following the method of Hebda and Nowak (1982b). Samples were drawn into 1 mm (i.d.) quartz capillary tubes. The free Mn^{2+} concentration of each sample was measured using a Varian E-9 X-band EPR spectrometer at a frequency of 9.52 GHz. The binding of Mn^{2+} to unmodified PEPCK was used as a control in all experiments.

The Mn^{2+} titrations for apoPEPCK and Co^{3+}−PEPCK, using EPR and PRR, were also performed in the presence of 50 μM PEP. The number of binding sites and the binding constants were obtained from a Scatchard plot of the data (Scatchard, 1949) for both the PRR and EPR data. Approximate K_D values of Mn^{2+} for each data point were determined using the equation:

$$K_D = \frac{[Mn^{2+}]_f[L]_f}{[Mn^{2+}-L]} \quad (6)$$

where $[Mn^{2+}]_f$ is the concentration of free Mn^{2+}, determined from the EPR signal height, $[L]_f$ is the concentration of free ligand, PEPCK, and $[Mn^{2+}-L]$ is the concentration of the Mn^{2+}−PEPCK complex.

Substrate Binding As Determined from Fluorescence. The computer-assisted SLM 8100 fluorescence spectrometer was used for all fluorometric measurements. The sample cell holder was maintained at 24 °C using a water bath. Samples of 0.26 μM apoPEPCK and Co^{3+}−PEPCK in the presence and absence of 50 μM $MnCl_2$ were prepared in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM KCl. Using a 1 mL quartz cell, the samples were measured using an excitation wavelength of 297 nm and an emission wavelength of 335 nm. At recorded time intervals, a known concentration of GTP was added directly into the quartz cell containing the enzyme solution. The amount of fluorescence quenching due to each addition of substrate was recorded, and corrections were made for dilution. This process was equivalent to titrating GTP into the enzyme solution. The recorded digital fluorescence data were converted to percent quenching at each GTP concentration using the Microsoft EXCEL 5.0 spreadsheet program. The binding constant was determined using the "EZ-Fit" program, version 2.02, by Perrella Scientific Inc., 1989. This process was repeated for the substrates ITP, GDP, IDP PEP, OAA and CO_2 (treated as HCO_3^-). Each titration was performed in duplicate.

LysC Digestion of ApoPEPCK and Co^{3+}−PEPCK. ApoPEPCK and Co^{3+}−PEPCK solutions were concentrated in 50 mM Tris-HCl, pH 7.4, to approximately 9 mg/mL using a mini-Amicon concentrator with a PM30 membrane. Urea (6 M) was added to the enzyme solutions, and the mixtures were incubated at 90 °C for 30 min. The Co^{3+}−PEPCK solution was then added to an Eppendorf tube containing Chelex-100 resin. The tube was gently shaken and quick-spun using a minicentrifuge to pellet the resin. The cobalt content was determined from the supernatant by AA. After heating, enough 25 mM Tris-HCl, pH 7.4, was added to the solutions to dilute the urea concentration to 2 M. LysC, at 1:50 (w/w) ratio, was added. Nitrogen gas was blown over the solutions. The LysC digest mixture was incubated at 37 °C for 24 h. After digestion, the Co^{3+}−PEPCK solution was treated with Chelex-100 as before to remove any free cobalt ions.

Separation of the Proteolytic Digests by Reverse Phase HPLC. LysC peptides were separated using a C-18 reverse-phase Rainin Microsorb-MV (4.6 × 250 mm) column with a 300 Å pore size. LysC peptides were eluted with the following gradient: 99.9% water with 0.1% trifluoroacetic acid for 10 min; 0−40% acetonitrile in 0.1% trifluoroacetic acid wash for 1 min; 40−99.9% acetonitrile in 0.1% trifluoroacetic acid for 50 min, followed by a 99.9% acetonitrile in 0.1% trifluoroacetic acid wash for an additional 20 min. The flow rate was maintained at 0.8 mL/min throughout the entire run. Peptides were detected at 215 nm for all runs. One milliliter fractions were collected for

the first hour. Cobalt content for each fraction was detected by AA for the Co^{3+}−PEPCK digest.

The cobalt-containing peptide, based on AA measurements, was collected. The cobalt-containing peptide was subjected to CE to test for purity. A 40.0 s injection (approximately 100 pmol) of the peptide was run at 10 kV for 30 min using 50 mM Tris-HCl buffer, pH 7.4, on a Beckman computer-assisted Capillary Electrophoresis P/Ace System 2000 with a D75A800 cartridge.

A 2 nmol sample of the cobalt-containing peptide was subjected to a second HPLC purification using a 20 μm high-capacity PerSeptive POROS HS sulfopropyl column. The peptide was eluted with the following gradient: 99.9% water with 0.1% trifluoroacetic acid for 2 min; 0−99.9% 2 M KCl in 0.1% trifluoroacetic acid wash for 6 min; followed by a 99.9% 2 M KCl in 0.1% trifluoroacetic acid wash for an additional 2 min. The flow rate was maintained at 3.0 mL/min throughout the entire run. One minute fractions were collected. Cobalt content for each fraction was detected by AA.

A 4 nmol sample of the cobalt-containing peptide was treated with 143 mM β-met and Chelex-100 to remove the cobalt which interferes with peptide sequencing. The peptide buffer was then exchanged into distilled deionized water by running the sample through a 10 mL P6-DG column equilibrated in distilled deionized water. This process also eliminated any contaminating primary amines that may interfere with amino acid composition analysis. The sample was submitted to the Mass Spectroscopy and Bio-Core Facilities at the University of Notre Dame, Department of Chemistry and Biochemistry, for mass, amino acid composition, and sequence analyses.

ArgC Digest of the Cobalt−Peptide. Two 4 nmol samples of the peptide with and without cobalt were treated with ArgC at a 1:200 (w/w) ratio of protease to peptide. Nitrogen gas was blown over the solutions. The ArgC digest mixtures were incubated at 37 °C for 24 h. Approximately 100 pmol of each mixture was subjected to CE analyses to determine if the peptides had been digested. The peptides were subjected to mass electrospray analyses. The ArgC-digested peptide without cobalt was submitted to the Bio-Core Facility at the University of Notre Dame, Department of Chemistry and Biochemistry, for amino acid sequence analysis.

RESULTS

H_2O_2 Effects on PEPCK Activity. PEPCK (approximately 100 μM) in 50 mM Tris-HCl, pH 7.4, containing 100 mM KCl was incubated with various concentrations of H_2O_2. H_2O_2 concentrations below 20 mM did not effect enzymatic activity over a period of 30 min as shown in Figure 1A. All subsequent incubations with H_2O_2 were performed at H_2O_2 concentrations below 20 mM.

When PEPCK was incubated with 1 mM $MnCl_2$ or 1 mM $MgCl_2$ and 10 mM H_2O_2, no change in enzymatic activity was seen as compared to untreated enzyme. PEPCK, incubated with 1 mM $CoCl_2$ and 10 mM H_2O_2 at pH 7.4, showed rapid inhibition as observed by a 40% decrease in activity after 5 min and a 75% decrease in activity by 15 min (Figure 1B). No further decrease in activity was observed thereafter. This suggests that a selective oxidation of Co^{2+} to Co^{3+} is occurring at the metal binding site of PEPCK.

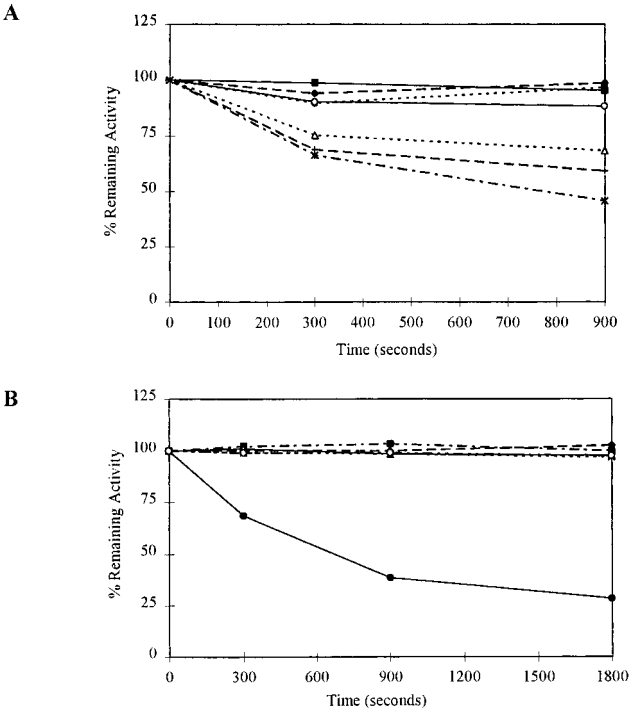

FIGURE 1: Effects of H_2O_2 on PEPCK. (A) PEPCK (approximately 100 μM) in 50 mM Tris-HCl, pH 7.4, containing 100 mM KCl was incubated with either 0 mM (♦), 2.5 mM (■), 5 mM (●), 10 mM (○), 20 mM (△), 40 mM (+), or 80 mM (*) H_2O_2. Aliquots of enzyme were removed at various periods of time and assayed for activity. (B) PEPCK was incubated with either 0 mM H_2O_2 (♦), 10 mM H_2O_2 (■), 1 mM $CoCl_2$ (△), 1 mM $MnCl_2$ and 10 mM H_2O_2 (*), 1 mM $MgCl_2$ and 10 mM H_2O_2 (○), or 1 mM $CoCl_2$ and 10 mM H_2O_2 (●). Aliquots of enzyme were removed at various periods of time and assayed in the reverse direction for activity.

Co^{3+} Modification of PEPCK. Upon incubation of PEPCK with $CoCl_2$ and H_2O_2, as described in Figure 1B, for 30 min, excess cobalt and H_2O_2 were removed from the incubation mixture by passing the solution through a Chelex-100/P6-DG column. The specific activity of PEPCK decreased 70−85% that of untreated enzyme. The Co^{3+}−PEPCK ratio was ascertained to be 1:1 as determined by AA for cobalt content and either A_{280} or Bradford measurements for protein content. Either analytical protein method yields the same values. Table 1 shows the stoichiometry and activity of four representative Co^{3+}-labeled PEPCK complexes from several PEPCK preparations. Each gave a 1:1 Co^{3+}-to-PEPCK stoichiometry. Each complex also exhibited 15−30% the specific activity of unmodified PEPCK. The remaining activity for the modified enzyme was the same in both the forward and reverse reaction assays. These results indicate that stoichiometric incorporation of Co^{3+} into PEPCK leads to the formation of a catalytically reactive enzyme complex with 15−25% activity relative to Mn^{2+}-activated PEPCK. Most preparations for subsequent studies had stoichiometries of 1:1.

Substoichiometric Incorporation of Co^{3+} into PEPCK. By preincubating PEPCK with H_2O_2 and low concentrations of Co^{2+}, it was possible to incorporate substoichiometric amounts of Co^{3+} into PEPCK. Figure 2 shows the residual PEPCK activity versus the number of cobalt atoms incorporated. As the cobalt incorporation into PEPCK increases, the V_{max} values decrease, indicating that PEPCK was modified at the active site. Under the conditions of this experiment, Co^{3+} to PEPCK stoichiometry never exceeds

Table 1: Activity Values for Stoichiometric Co^{3+}−PEPCK Complexes[a]

sample	V_{max} (units/mg)	% control V_{max}	% control V_{max} calcd[c]
0.88 ± 0.04 Co^{3+}−PEPCK	1.25	30.9	25.6
control[b]	4.05		
0.91 ± 0.04 Co^{3+}−PEPCK	0.823	26.1	23.2
control[b]	3.15		
1.00 ± 0.02 Co^{3+}−PEPCK	0.708	15.6	15.6
control[b]	4.55		
0.90 ± 0.03 Co^{3+}−PEPCK	1.33	17.9	24.0
control[b]	7.42		

[a] The formation of Co^{3+}−PEPCK was performed as described under Materials and Methods with various preparations of PEPCK and purified from free cobalt and H_2O_2. Activity measurements were carried out in the reverse direction as described under Materials and Methods. All substrates were at saturating conditions for activity measurements. Co^{3+} concentrations were determined by atomic absorption. PEPCK concentrations were determined either by A_{280} measurements or by Bradford assays with the same results. [b] The control is the same sample of enzyme not treated with Co^{2+} or H_2O_2. The % control is calculated relative to the V_{max} of the untreated enzyme sample. [c] The "% control V_{max} calcd" was calculated from the following equations, where f_1 and f_2 represent the fraction of unmodified and modified enzyme, respectively. 15.6% was used as the maximal activity for fully labeled enzyme:

$$(f_1)(100) + (f_2)(15.6) = \%\ \text{observed}\ V_{max}$$

$$f_1 + f_2 = 1$$

These equations account for the activity due to unmodified enzyme.

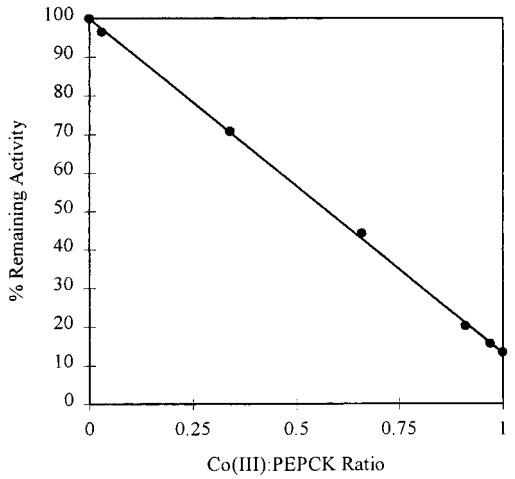

FIGURE 2: Decreasing PEPCK activity with increasing incorporation of Co^{3+}. PEPCK was incubated with H_2O_2 and low (<1 mM) variable concentrations of Co^{2+}. After 30 min, the samples were passed through a P6-DG column to remove free cobalt and H_2O_2. Activity was measured in the reverse direction, and the cobalt content was determined by AA. As the cobalt incorporation into PEPCK increases, the V_{max} values decrease, indicating that PEPCK is specifically modified.

1:1. Since the stoichiometric Co^{3+}−PEPCK complex is active, this suggests the possibility that the metal plays an ionic role in enzymatic activity as opposed to a direct ligand binding function.

Substrate Protection Studies. Protection by substrates against Co^{3+} modification of PEPCK was determined. PEPCK in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM KCl was incubated in the presence of 4 mM $CoCl_2$ and 20 mM H_2O_2 at pH 7.4 and 2 mM of either GTP, ITP, GDP, IDP, PEP, or OAA or 200 mM HCO_3^-. Aliquots were withdrawn at different incubation times, and the Co^{3+} content and the residual activity were determined. Table 2 lists the

Table 2: Inactivation and Reactivation Rate Constants[a] of PEPCK by Co^{3+}

substrate present	k_{inact} ($\times 10^{-3}$ min^{-1})	k_{react} ($\times 10^{-3}$ min^{-1})
none (control)	3.04	5.19
IDP	2.72	5.08
GDP	2.43	5.00
OAA	2.08	4.67
HCO_3^-	2.07	5.29
PEP	1.51	1.96
ITP	0.86	1.28
GTP	0.74	1.22

[a] The k_{inact} was determined by measurement of the loss of activity with time in the presence of Co^{2+} and H_2O_2 as described. The k_{react} was determined by measurement of the recovery of PEPCK activity upon incubation of Co^{3+}−PEPCK with β-met. Activity measurements were carried out in the reverse direction as described under Materials and Methods. PEPCK inactivation and reactivation rate constants were calculated from the slopes as determined from the plots of log (% activity) vs time. The data were fit using Cricket Graph, V.1.2. Standard deviations were not greater than 3% for all fits.

inactivation rate constants of PEPCK by Co^{3+} as calculated from the slope of the line determined by plotting log (% remaining activity) vs time.

A k_{inact} of 3.04×10^{-3} min^{-1} was measured for enzyme incubated in the presence of Co^{2+} and H_2O_2 only. The k_{inact} values for PEPCK in the presence of either GDP, IDP, OAA, or HCO_3^- were only slightly lower. AA data (not shown) show that in the presence of these substrates, near-stoichiometric incorporation of Co^{3+} into PEPCK occurs by 10 min. These results suggest that GDP, IDP, OAA, and HCO_3^- offer little protection against Co^{3+} incorporation into and inactivation of PEPCK. Enzyme incubated in the presence of GTP, ITP, or PEP showed a significantly slower loss in enzymatic activity after 10 min. The k_{inact} values for PEPCK in the presence of these substrates were 2−4 times smaller than the k_{inact} value for PEPCK in the absence of any substrates. AA data (not shown) show that in the presence of GTP, ITP, or PEP, only 50% incorporation of Co^{3+} into PEPCK occurred after 10 min. These results suggest GTP, ITP, and PEP protect against Co^{3+} incorporation into PEPCK.

The above experiments were performed with excess Co^{2+} such that all nucleotide would be in a Co^{2+}−nucleotide complex. To determine if free GTP, Mn^{2+}−GTP, or Mg^{2+}−GTP would also offer protection, the nucleotide protection experiments were repeated using 500 μM Co^{2+}, 500 μM Co^{2+} with 4 mM Mn^{2+}, and 500 μM Co^{2+} with 4 mM Mg^{2+}. The GTP concentration was 2 mM for all experiments. PEPCK was incubated with the metals prior to the addition of GTP and H_2O_2. Previous studies have found that Co^{2+} binds tightly to PEPCK such that even large excesses of either Mn^{2+} or Mg^{2+} cannot readily displace it. The inactivation rate results for these studies (not shown) were nearly identical to the GTP sample discussed above. This indicates that free GTP as well as all metal−GTP complexes behave similarly in protecting PEPCK from Co^{3+} modification. Additionally, when OAA and GTP were both added to the incubation mixture, no significant change in the inactivation rates was observed as compared to GTP alone. This indicates that only the nucleotide in this experiment offers significant protection against Co^{3+} modification of PEPCK.

When Co^{3+}−PEPCK was treated with 143 mM β-met, a 97% reduction in the amount of Co^{3+} label in PEPCK was seen with a concomitant restoration in PEPCK activity. This

reversibility suggests no oxidative damage to the protein after modification. Substrate protection studies against Co^{3+} removal from the modified enzyme were also performed. Co^{3+}−PEPCK in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM KCl was incubated in the presence of 2 mM of either GTP, ITP, GDP, IDP, PEP, or OAA or 200 mM HCO_3^- and 143 mM β-met. The substrate concentrations used for these experiments are 10−20 times greater than their respective K_m values for unmodified PEPCK (see Table 4). At recorded time intervals, aliquots were withdrawn, and Co^{3+} content and activity were determined. PEPCK reactivation rate constants were calculated from the slope of the line as determined by plotting log (% activity) vs time (see Table 2).

Co^{3+}−PEPCK incubated with 143 mM β-met but in the absence of any substrate showed an activity increase from 0.5 unit/mg to 3.5 units/mg after 10 min with a k_{react} of 5.19 $\times$ 10^{-3} min^{-1}. As shown in Table 2, only GTP, ITP, or PEP offers substantial protection against the removal of Co^{3+}. After 10 min, Co^{3+}−PEPCK incubated with 143 mM β-met and either GTP, ITP, or PEP showed only a 10% reduction in the amount of Co^{3+} label, which contrasts to the 60% loss in the Co^{3+} label observed in the absence of substrates. These results indicate that considerable protection against Co^{3+} removal from PEPCK is seen with GTP, ITP, and PEP.

All protection experiments indicate that only GTP, ITP, and PEP offer considerable protection against either oxidation or reduction of the PEPCK-bound cobalt.

Cysteine Determination of Co^{3+}−PEPCK. When PEPCK is modified by Co^{3+}, 85% of the activity is lost. Treatment of Co^{3+}−PEPCK with β-met results in cobalt removal and restoration of activity. While it appears that the modification process does not cause any structural damage to PEPCK, disulfide bonds may form during the oxidative modification process. PEPCK contains 13 cysteine residues, none of which are in disulfide bonds (Hebda *et al*., 1982a). It was possible that under the oxidizing conditions present in the modification process, disulfide bonds were formed, and this may account for the decrease in activity. When the enzyme was treated with β-met, it was possible that these newly formed disulfide bonds were also reduced. This could account for the observed increase in activity.

To explore this possibility, cysteine determinations were done on apoPEPCK and Co^{3+}−PEPCK. The free sulfhydryls were determined by treating PEPCK with DTNB and calculating the number of moles of thiophenolate anions of 5-thio-2-nitrobenzoic acid released. Measurements were done at 412 nm.

Three separate measurements each were made with apoPEPCK and Co^{3+}−PEPCK. The absorbance values for all samples were identical and indicated that 13 free sulfhydryls are present in apoPEPCK as well as in Co^{3+}−PEPCK. This indicates that Co^{3+}−PEPCK contains no disulfide bonds. Therefore, the decrease in activity upon Co^{3+} modification is not due to the oxidation of cysteine residues.

Circular Dichroism Spectra. Solutions of 0.26 μM apoPEPCK and Co^{3+}−PEPCK in 5 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl in the presence and absence of 50 μM Mn^{2+} and 200 μM PEP were measured from 190 to 300 nm. The observed ellipticity, θ, was converted to the ellipticity per mean residue weight, [θ], and plotted versus wavelength. Secondary structure information was then determined from the CD spectra by fitting the mean residual weight ellipticity data to the AVIV program PROSEC V.3.1 (Table 3).

Table 3: Secondary Structure of ApoPEPCK and Metal−PEPCK Complexes in the Presence or Absence of PEP As Determined by Circular Dichroism Spectroscopy

	% secondary structure[a]				
sample	α helix	β sheet	β turn	random coil	% error
native PEPCK	27.2	20.6	16.8	35.4	9.1
Mn^{2+}−PEPCK	21.1	23.5	16.6	38.8	7.0
Co^{3+}−PEPCK	19.3	25.0	17.1	38.6	13.0
PEPCK−PEP	27.7	20.7	16.9	34.7	9.8
Mn^{2+}−PEPCK−PEP	28.8	19.5	17.0	34.7	14.0
Co^{3+}−PEPCK−PEP	29.0	19.0	17.0	35.0	7.5

[a] Secondary structure estimations were determined by fitting the CD data to the AVIV program PROSEC, V.3.1.

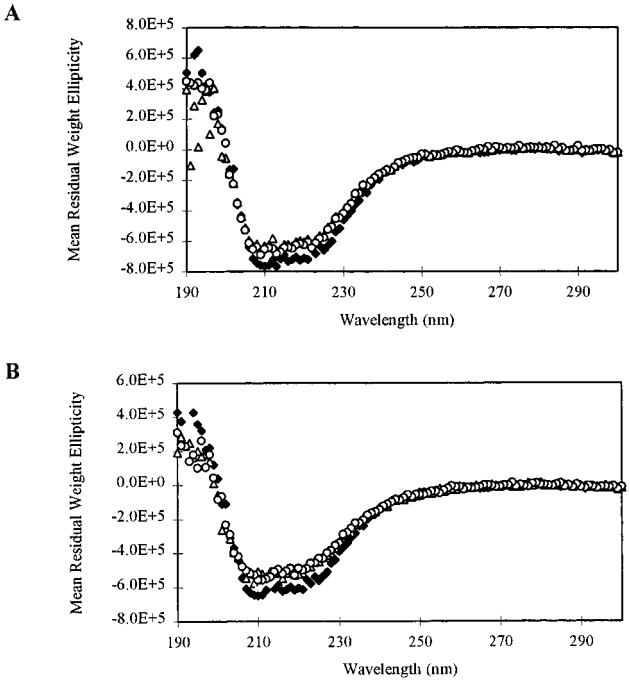

FIGURE 3: Circular dichroism spectra for apoPEPCK, Mn^{2+}−PEPCK, and Co^{3+}−PEPCK. The apoPEPCK and metal−PEPCK solutions were prepared in 5 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl. This buffer was also used as the reference sample. Measurements were made from 190 to 300 nm. (A) CD spectra taken in the absence of PEP. ApoPEPCK is represented by the darkened diamonds (♦), Mn^{2+}−PEPCK is represented by the open triangles (△), and Co^{3+}−PEPCK is represented by the open circles (○). (B) CD spectra taken in the presence of PEP. PEPCK−PEP is represented by the darkened diamonds (♦), Mn^{2+}−PEPCK−PEP is represented by the open triangles (△), and Co^{3+}−PEPCK−PEP is represented by the open circles (○).

Figure 3A shows the circular dichroism spectra of apoPEPCK, Mn^{2+}−PEPCK, and Co^{3+}−PEPCK. The two PEPCK−metal complexes gave identical spectra and were slightly different from that of apoPEPCK. Based on secondary structure estimations, shown in Table 3, both Mn^{2+}−PEPCK and Co^{3+}−PEPCK show a 3−5% increase in β-sheet content as well as a 3% increase in random coil content, obtained at the expense of α-helical content in apoPEPCK. This contrasts with the previous report (Hwang & Nowak, 1989) that showed a 7% increase in the α-helical content of PEPCK, at the expense of β-sheet content, upon metal binding. No change was observed in the CD spectra of

Table 4: PRR and EPR Mn^{2+} Binding Constants for Various PEPCK Complexes

	PRR measurements		EPR measurements	
sample	n[a]	K_D (μM)	n[a]	K_D (μM)
apoPEPCK	0.95 ± 0.06	46.7 ± 5.1	1.02 ± 0.10	48.5 ± 6.2
PEPCK−PEP	0.98 ± 0.09	12.0 ± 4.7	1.08 ± 0.13	13.6 ± 6.2
Co^{3+}−PEPCK	0[b]		0[b]	
Co^{3+}−PEPCK−PEP	0[b]		0[b]	

[a] n represents the stoichiometry of Mn^{2+} to enzyme as determined from Scatchard analysis. Data were fit using the "EZ-Fit" program, version 2.02, by Perella Scientific Inc., 1989. [b] No measurable Mn^{2+} binding was observed for these samples.

Co^{3+}−PEPCK upon the addition of Mn^{2+} (spectrum not shown).

Similar changes were observed in the CD spectra of Mn^{2+}−PEPCK and Co^{3+}−PEPCK complexes upon the addition of PEP (Figure 3B). Secondary structure estimations (Table 3) show that both Mn^{2+}−PEPCK and Co^{3+}−PEPCK have a slight increase in α-helical content, at the expense of β-sheet, upon binding PEP. This is consistent with the observations of Hwang and Nowak (1989) which also showed increases in the α-helical content of PEPCK in the presence of PEP. It is important to stress that both Mn^{2+}−PEPCK and Co^{3+}−PEPCK showed similar changes in their respective CD spectra. These spectra differ from that obtained for the PEPCK−PEP binary complex. This suggests that Co^{3+} is at the Mn^{2+} binding site on PEPCK and that Co^{3+}-modified PEPCK can form a Co^{3+}−PEPCK−PEP complex with conformations similar to Mn^{2+}−PEPCK−PEP.

Mn^{2+} Binding to the Co^{3+}−PEPCK Complex. In order to further verify that the modification of PEPCK by Co^{2+} and H_2O_2 was specific, the binding of Mn^{2+} to Co^{3+}−PEPCK was determined by PRR and EPR. Mn^{2+} titrations were performed for apoPEPCK and Co^{3+}−PEPCK in the presence and absence of 50 μM PEP. The number of binding sites and binding constants as obtained from Scatchard analyses of the PRR and EPR data are presented in Table 4.

For PRR measurements, Mn^{2+} was titrated into 50 μM apoPEPCK and Co^{3+}−PEPCK solutions, and the enhancement for each addition was measured. As shown in Figure 4A, apoPEPCK had a significant change in enhancement upon addition of Mn^{2+}, indicating Mn^{2+} binding to the enzyme. In contrast to apoPEPCK, Co^{3+}−PEPCK showed no change in enhancement over the Mn^{2+} concentration range tested, indicating that Mn^{2+} does not associate to Co^{3+}−PEPCK. Similar results are observed when the titration is done in the presence of PEP (titration results not shown). PEP enhances the binding of Mn^{2+} to PEPCK (Hebda & Nowak, 1982b).

To directly measure the binding of Mn^{2+} to the Co^{3+}−PEPCK complex, EPR measurements were performed. No significant Mn^{2+} associates with the Co^{3+}−PEPCK complex as shown in Figure 4B and in Table 4. Similar results are observed when the titration is done in the presence of PEP (titration results not shown). This contrasts with the results of apoPEPCK that show a saturation effect of Mn^{2+} into PEPCK (Figure 4B) with a K_D of 49 μM in the absence of PEP and a K_D of 14 μM in the presence of PEP (Table 4). These results indicate that Co^{3+} has been specifically incorporated into the metal binding site on PEPCK excluding binding of Mn^{2+}.

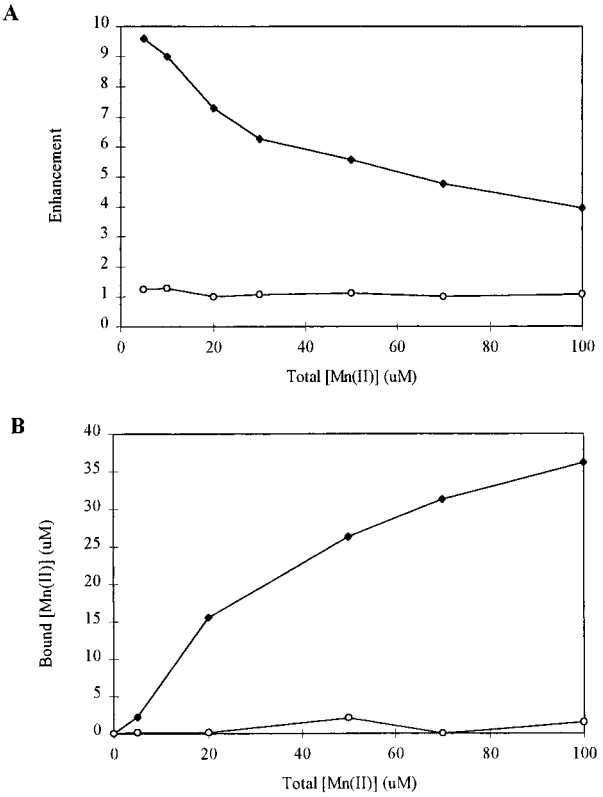

FIGURE 4: EPR and PRR titrations of Mn^{2+} into apoPEPCK and Co^{3+}-modified PEPCK. (A) PRR titration of Mn^{2+} into native and Co^{3+}−PEPCK. Enhancement values were plotted as a function of Mn^{2+} concentration. (B) Mn^{2+} binding curve obtained by EPR titration of Mn^{2+} into native and Co^{3+}−PEPCK. Bound Mn^{2+} is plotted as a function of total Mn^{2+} added. ApoPEPCK is represented by the darkened diamonds (♦), and Co^{3+}−PEPCK is represented by the open circles (○).

Kinetic Properties of Co^{3+}-Modified PEPCK. Kinetic studies showed that the order of cation activation for native PEPCK was $Mn^{2+} > Co^{2+} > Ca^{2+} > Mg^{2+}$. No activity was observed when Cu^{2+}, Fe^{2+}, Zn^{2+}, or Cd^{2+} was the only metal present. Several of these observations have been previously reported (Lee *et al*., 1981). The Co^{3+}−PEPCK complex has a requirement for the addition of divalent cation for activity. The order of cation activation for Co^{3+}−PEPCK based on maximal velocity was $Mn^{2+} > Ca^{2+} > Co^{2+} > Cu^{2+} > Mg^{2+} > Zn^{2+}$. No activity was observed when Fe^{2+} or Cd^{2+} was added. It is interesting to note that more metals activate Co^{3+}−PEPCK as compared to native PEPCK. This appears to be due to the presence of Co^{3+} at the metal site of the protein which prevents the interaction of nonactivating or inhibiting metals with PEPCK. The observed activity of Co^{3+}−PEPCK with these "inhibiting metals" is due to the formation of metal·nucleotide complexes that serve as substrates.

The kinetic properties of native PEPCK and of the Co^{3+}−PEPCK complex were studied at pH 7.4 in the absence of β-met. A comparison of the kinetic constants for the substrates is summarized in Table 5. Kinetic constants for Mn^{2+}, Co^{2+}, and Mg^{2+} activation are presented in Table 6. While the kinetic constants for all substrates and metals are similar for both enzymes, PEP, OAA, GTP, ITP, GDP, IDP, Mn^{2+}, and Co^{2+} all show decreased $K_{m,app}$ values for Co^{3+}−PEPCK as compared to native PEPCK, suggesting a stronger interaction of these substrates and cofactors with the modified enzyme. The reduced $K_{m,app}$ values reflect a change in the

Table 5: Kinetic Parameters for Various Substrates with ApoPEPCK and Co^{3+}−PEPCK

sample	variable substrate[a]	$K_{m,app}$ (mM)	V_{max} (units/mg)	k_{cat}/K_m ($\times 10^6$ $min^{-1}\cdot M^{-1}$)
apoPEPCK (control)	PEP	0.193 ± 0.035	4.55	1.75
	OAA	0.131 ± 0.016	21.3	12.1
	HCO_3^-	14.0 ± 1.3	4.30	0.023
	IDP	0.162 ± 0.011	4.28	1.96
	GDP	0.089 ± 0.008	4.53	3.78
	ITP	0.090 ± 0.008	20.7	17.1
	GTP	0.077 ± 0.011	21.0	20.2
Co^{3+}−PEPCK (1:1)	PEP	0.093 ± 0.015	0.708	0.57
	OAA	0.123 ± 0.009	3.48	2.10
	HCO_3^-	47.3 ± 6.3	0.681	0.001
	IDP	0.103 ± 0.016	0.566	0.41
	GDP	0.060 ± 0.006	0.744	0.92
	ITP	0.044 ± 0.004	3.29	5.55
	GTP	0.042 ± 0.004	3.44	6.08

[a] Kinetic assays were performed in the forward and reverse directions as described under Materials and Methods. 2 mM Mn^{2+} was added to the assay mix. No measurable velocity was observed when additional metal was not added to the assay mix. No β-met was present in the assay mix. Fixed substrates are saturating.

Table 6: Kinetic Parameters for Various Metals with ApoPEPCK and Co^{3+}−PEPCK

sample	cation[a]	K'_a[b] (mM)	V_{max} (units/mg)	k_{cat}/K_a ($\times 10^5$ $min^{-1}\cdot M^{-1}$)
apoPEPCK	Mn^{2+}	0.264 ± 0.022	7.42	20.8
	Co^{2+}	0.505 ± 0.106	2.28	3.34
	Mg^{2+}	1.51 ± 0.37	0.470	0.23
Co^{3+}−PEPCK (1:1)	Mn^{2+}	0.158 ± 0.022	1.33	6.23
	Co^{2+}	0.280 ± 0.036	0.544	1.44
	Mg^{2+}	1.75 ± 0.42	0.151	0.06

[a] Kinetic assays were performed in the reverse direction as described in the text. No measurable velocity was observed when additional metal was not added to the assay mix. No β-met was present in the assay mix. [b] Calculated based on the concentration of added cation giving $0.5V_{max}$ analogous to a K_m determination.

Table 7: Binding Constants of Nucleotides to PEPCK and PEPCK−Metal Complexes As Determined by Fluorescence[a]

PEPCK complex	GTP K_D (μM)	ITP K_D (μM)	GDP K_D (μM)	IDP K_D (μM)
native PEPCK	5.9 ± 0.5	13.2 ± 1.5	10.7 ± 1.5	10.6 ± 1.4
Mn^{2+}−PEPCK	6.9 ± 0.4	14.2 ± 1.6	10.4 ± 1.8	10.6 ± 1.5
Co^{3+}−PEPCK	6.9 ± 0.4	13.2 ± 1.5	9.6 ± 1.8	8.9 ± 1.6

[a] Fluorescent titrations were performed as described in the text. An excitation wavelength of 297 nm was used, and an emission wavelength of 335 nm was measured. After correcting for dilution, the recorded digital fluorescence data were converted to percent quenching at each substrate concentration using the Microsoft EXCEL 5.0 spreadsheet program. The binding constant was determined using the "EZ-Fit" program, version 2.02, by Perella Scientific Inc., 1989. Each K_D value is an average of two titrations. Free nucleotide and metal−nucleotide caused similar quenching of PEPCK fluorescence.

kinetic parameters for the modified enzyme. With the exception of HCO_3^-, Co^{3+}−PEPCK is between 3 and 5 times less kinetically efficient than native PEPCK for all substrates examined. Co^{3+}−PEPCK is 21 times less kinetically efficient as native PEPCK for HCO_3^-.

Substrate Binding As Determined from Fluorescence. Fluorescence measurements were obtained using an excitation wavelength of 297 nm. This was done to ensure that the emission fluorescence arises nearly entirely from tryptophan residues and that any contributions from tyrosine or phenylalanine were negligible.

GTP, ITP, GDP, and IDP (in the presence and absence of Mn^{2+}) all quench PEPCK fluorescence by approximately 30% for each PEPCK complex studied. Free nucleotide and metal−nucleotide quench PEPCK fluorescence similarly. No change in fluorescence intensity was observed by the addition of PEP, OAA, or HCO_3^- to apoPEPCK, Mn^{2+}−PEPCK, or Co^{3+}−PEPCK. From measurements of percent quenching versus substrate concentration, K_D values were obtained for the formation of nucleotide−enzyme complexes. These data are summarized in Table 7. The observed K_D values are between 6 and 14 μM for all enzyme−nucleotide complexes tested. These K_D values are in good agreement with previously obtained binding constants determined either by PRR or by direct binding studies (Hebda & Nowak, 1982b; Lee & Nowak, 1984). The identity of the K_D values for apoPEPCK, Mn^{2+}−PEPCK, and Co^{3+}−PEPCK suggests that GTP, ITP, GDP, and IDP bind similarly to the three enzyme forms.

Separation of the Cobalt-Containing Peptide by Reverse Phase HPLC. The identification of the cation sites in the PEPCK sequence is feasible by the isolation and characterization of the cobalt-containing peptide(s). ApoPEPCK and Co^{3+}−PEPCK were first denatured by treatment with 6 M urea at 90 °C for 30 min. The enzyme solutions were then digested with LysC at a 1:50 w/w ratio at 37 °C for 24 h. No loss of the cobalt label was observed after denaturation and digestion.

Peptides for the LysC digestion were separated by reverse phase HPLC. PEPCK gave approximately 13 detectable peptides as shown in Figure 5A,B (16 total peptides are possible from the LysC digest). The cobalt-containing fractions were found in the void volume as well as in a peak that eluted at 12 min (see Figure 5C). The cobalt content in these two fractions equaled the total amount of cobalt injected. The cobalt-containing fraction at 4 min (void volume) was not subjected to further investigation since free cobalt elutes in the void volume. The cobalt-containing peptide at 12 min is not present in the chromatographic profile for the unmodified enzyme and appears to be a migration of a peptide that elutes at 48 min for the unmodified enzyme (see Figure 5A,B). It appears that the presence of cobalt causes this shift in retention times. The cobalt-containing peptide was subjected to CE to test for purity. A single peak was observed at approximately 19.8 min (Figure 6A). No other peak was observed, indicating that the peptide fraction is pure.

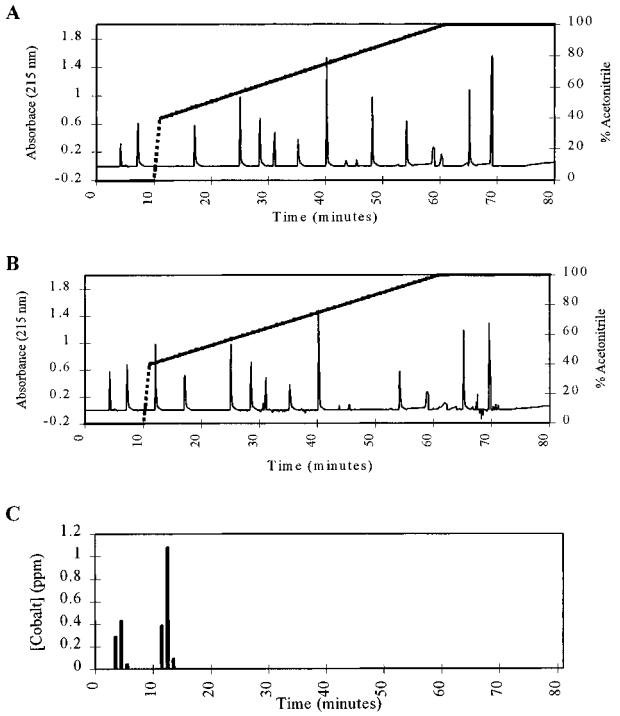

FIGURE 5: HPLC chromatographic profile of LysC-generated peptides of apoPEPCK and Co^{3+}−PEPCK. (A) ApoPEPCK was treated with 6 M urea and LysC (1:50 w/w) and incubated at 37 °C for 24 h. A reverse phase C-18 HPLC column was used to separate the LysC peptides. Peptides were eluted with the following gradient: 99.9% water with 0.1% trifluoroacetic acid for 10 min; 0−40% acetonitrile in 0.1% trifluoroacetic acid wash for 1 min; 40−99.9% acetonitrile in 0.1% trifluoroacetic acid for 50 min, followed by a 99.9% acetonitrile in 0.1% trifluoroacetic acid wash for an additional 20 min. The solid line (—) represents the HPLC chromatographic profile. The dashed line (- - -) represents the acetonitrile gradient. (B) Co^{3+}−PEPCK was treated identically to that of native PEPCK, described above in (A). (C) The cobalt content for the entire elution profile in (B) was assayed by AA.

A second test of peptide purity was performed by subjecting 2 nmol of the cobalt-containing peptide to a second HPLC purification using a 20 μm high-capacity PerSeptive POROS HS sulfopropyl column. This column is designed to separate cationic species at high flow rates. Only one peak was observed in the chromatographic profile (not shown) between 3 and 4 min, shortly after the KCl gradient began. The cobalt-containing fraction was found in this peak. Since both the CE and secondary HPLC data indicated that the cobalt-containing peptide is a single pure peptide, the peptide was not subjected to further purification.

Mass and Amino Acid Analyses of the Cobalt-Containing Peptides. The cobalt-containing peptide was submitted for an electrospray mass spectroscopy analysis at the departmental Mass Spectroscopy Facility at the University of Notre Dame, Department of Chemistry and Biochemistry. The peptide was first passed through a Dowex cation exchange resin 3 times to eliminate salts and buffers that may interfere with mass analysis. A molecular mass of 2989.6 Da (spectrum not shown) was obtained. There is only one peptide from a LysC digest that could have that molecular mass, the peptide in the region from Thr276 to Lys301. This fragment has a calculated molecular mass of 2943.5 Da. The presence of cobalt would give a mass of 3002.7 Da. The peptide-bound cobalt appears to be stable during the mass spectroscopy analysis. While the mass from electrospray spectral analysis does not agree precisely with this weight, there is no other LysC peptide that is close to this mass. There are several explanations for this slight difference in the observed mass and the theoretical mass. The accuracy of mass determinations is approximately ±0.5%. The observed mass for the cobalt−peptide falls well within this error range. Slight differences between observed and theoretical masses are not uncommon. Ettner *et al*. (1995) showed a series of peptides that had slight discrepancies between the theoretical mass and the observed mass spectroscopy results. It is also possible that the obtained mass of 2989.6 Da represents the apopeptide in an adduct formation with two sodium atoms, where the cobalt label dissociated from the peptide during analysis. Adduct formation of a compound with sodium or potassium atoms is a common occurrence in electrospray analysis. The slight differences between the theoretical and observed masses may also be due to a modified N-terminus (*vidae infra*).

An 18% SDS−PAGE containing the sample and various molecular weight markers was run. The gel (not shown) gave an estimated molecular mass of 3000 Da for the peptide. This mass agrees with that from the mass spectroscopy analysis.

An amino acid composition analysis was performed on the cobalt-containing peptide. The amino acid composition analyses were performed on three different peptide samples obtained from three different HPLC runs. In each case, the composition analysis was the same (data not shown). The amino acid compositions of the cobalt-containing peptide are similar to the predicted LysC peptide Thr276−Lys301 of PEPCK.

An amino acid sequence analysis of the cobalt−peptide using Edman chemistry failed. Sequencing also failed to give results after the cobalt was removed from the peptide (trace amounts of transition metals may interfere with sequencing). It is possible that the peptide is N-terminally altered which would prevent sequencing.

The cobalt was removed from the peptide by treatment with β-met followed by gel filtration. The peptide was then treated with ArgC (1:200 w/w) which specifically cleaves at arginine residues. If Thr276−Lys301 is the cobalt-containing peptide, digestion would occur at Arg289, creating a new N-terminus. The CE profile of the untreated peptide is shown in Figure 6A. A single peak is observed at 19 min. Figure 6B shows the CE profile of the ArgC-treated peptide. The peak at 19 min is no longer present, and two new peaks at 6 and 11 min are formed. This indicates that the peptide was cleaved at one position. The digested peptide sample was then submitted for sequence analysis with no attempts at additional purification. ArgC digestion was also performed on the cobalt-labeled peptide. CE analysis (not shown) of ArgC digestion of the cobalt-labeled peptide also showed that two peaks at 6 and 10 min were formed, indicating that the cobalt-labeled peptide was also cleaved at one position. The two peptides were separated using the 20 μm high-capacity PerSeptive POROS HS sulfopropyl column described above. Two peaks were observed in the chromatographic profile (not shown) with retention times of 1.5 and 3.5 min. AA analysis showed that only the first peak contained cobalt. The peaks were collected and subjected to mass electrospray analyses (not shown). The peak at 1.5 min was found to have a mass of 1444.9 Da, and the peak at 3.5 min had a mass of 1575.8 Da. The mass of 1444.9 Da corresponds well to the predicted mass for the

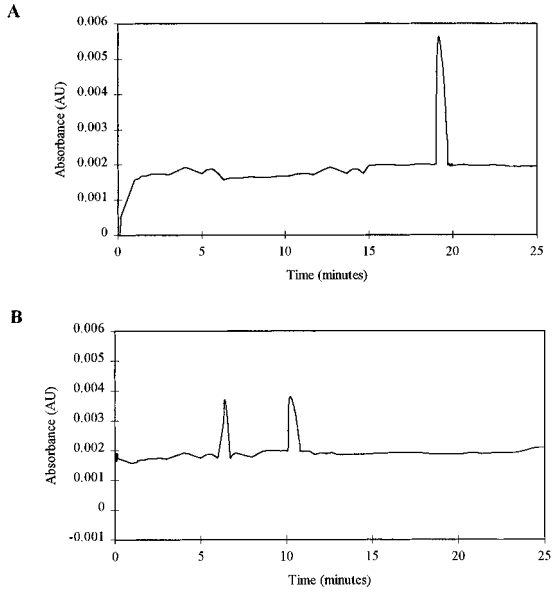

FIGURE 6: Capillary electrophoresis profiles of the untreated and ArgC-treated cobalt-containing peptide. Co^{3+} was removed from the peptide prior to injection as described. (A) A 40.0 s injection (approximately 2 nmol) of the peptide was run at 10 kV for 30 min using 50 mM Tris-HCl buffer, pH 7.4, on a Beckman computer-assisted Capillary Electrophoresis P/Ace System 2000 with a D75A800 cartridge. A single peak was observed at 19 min. (B) A 40.0 s injection of the peptide after treatment with ArgC run identically to that in (A).

region Ile290−Lys301 (1385.9 Da) if cobalt (58.9 Da) is associated. The mass of 1575.8 Da corresponds well to the predicted mass for the region of Thr276−Arg289 which has a calculated mass of 1575.3 Da. The obtained masses are both within the ±0.5% error range of the calculated masses. Since no cobalt was found associated with the region of Thr276−Arg289 after ArgC digestion as determined by AA and electrospray mass analyses, this indicates that amino acids Thr276−Arg289 are not involved in metal binding. The metal associates only with amino acids Ile290−Lys301.

Amino acid sequence results for the ArgC-digested peptide (cobalt removed) appear in Table 8. A total of 12 cycles were run. No amino acid was observed in the last cycle. The amino acids identified by sequencing exactly match the PEPCK region Ile290−Met300. This indicates that the peptide from the LysC digest must be Thr276−Lys301. Lys275 is the preceding amino acid in the sequence. The ArgC digest occurred at Arg289. Since none of the cycles during the sequencing identified the region Thr276−Arg289, but rather identified only the region after Arg289, the cobalt-containing peptide must be N-terminally altered at Thr276. N-Terminal blockage with a threonine residue is rare. N-Terminal alteration would explain why initial sequencing could not be performed on the peptide and why there is a slight discrepancy between the theoretical mass and the observed mass (*vide supra*).

This peptide is located in the central portion of the enzyme and close to the putative nucleotide binding site. Table 9 shows a sequence alignment of seven different species of PEPCK from the region Thr276−Lys301 of avian liver mitochondrial PEPCK. This is a highly homologous region of PEPCK with 23 out of the 26 amino acids well conserved throughout the 7 PEPCK sequences. This region contains two totally conserved aspartic acid residues, Asp295 and Asp296 (using the avian liver mitochondrial PEPCK numbering system), that appear to be the only feasible ligands to the metal. Residues His291 and Cys292 may also serve as metal ligands; however, they are both less well-conserved residues.

Table 8: Amino Acid Sequence Analysis of the ArgC Digest of the Cobalt-Containing Peptide[a]

cycle	amino acid	peptide[a] (pmol of amino acid)	PEPCK Ile290−Lys301
1	Ile	6076.4	Ile
2	His	2274.8	His
3	Cys	—[b]	Cys
4	Val	238.4	Val
5	Gly	101.2	Gly
6	Asp	91.9	Asp
7	Asp	76.0	Asp
8	Ile	16.9	Ile
9	Ala	14.7	Ala
10	Trp	3.1	Trp
11	Met	1.4	Met
12	—[c]	—	Lys

[a] The cobalt-containing peptide was sequenced at the Bio-Core Facility at the University of Notre Dame. This sample was obtained from an ArgC digest of the Co−peptide after Co^{3+} had been removed. Two peptides were obtained from the ArgC digest; however, only one peptide produced sequencing results. [b] Sample not detected because neither the enzyme nor the peptide was modified to block free cysteines (*e.g.*, with iodoacetate or *N*-ethylmaleimide) prior to sequencing. The chromatographic profile for this cycle was typical for unmodified cysteine. [c] Although a cycle was attempted, too little peptide remained for the identification of the amino acid.

DISCUSSION

Avian liver mitochondrial PEPCK requires two divalent metal ions for activity; one is directly associated with the enzyme. Mn^{2+} has been proposed as a regulator for this enzyme *in vivo* (Lee & Nowak, 1984). The location of the metal site and the direct role that the metal plays in catalysis are still unclear.

Reports of stable cation−protein complexes with Co^{3+} (Kang & Storm, 1972; Shinar & Navon, 1974; Anderson & Vallee, 1975; Balakrishnan & Villafranca, 1975; Miziorko *et al*., 1982) have suggested the utility of Co^{3+} as a probe of the PEPCK metal activator site. The spectral properties, preference for nitrogen and oxygen donors, and inertness to substitution reactions render Co^{3+} a potentially valuable probe for such a study. One important difference between the properties of Co^{2+} and Co^{3+} complexes is that the rate of ligand exchange is much slower for Co^{3+} complexes. If metal−ligand chemistry plays an active role in the catalytic process, an inactive enzyme catalyst is expected.

A limitation of Co^{3+}−protein modification is that stabilization of a Co^{3+} complex requires careful regulation of the coordination environment. A set of six ligands of sufficient field strength (ligands relatively high in the electrochemical series) must be provided. A $Co^{III}(NH_3)_6^{3+}$ complex is very stable to reduction compared to $Co^{III}(H_2O)_6^{3+}$ since ammonia is considerably higher than water in the electrochemical series (Cotton & Wilkinson, 1972). An octahedral coordination geometry is also necessary. Few 5-coordinate Co^{3+} complexes have been reported to exist in the solid state. A solution five-coordinate Co^{3+}-complex would not be expected to be exchange-inert due to the differences in crystal field stabilization (Cotton & Wilkinson, 1972). These limitations can inhibit the use of Co^{3+} as a protein modifica-

Table 9: PEPCK Sequence Alignment[a]

Source	Position 276			280				285				290				295					300	301
Chicken mito.	**T**	**N**	**L**	**A**	**M**	**M**	T	**P**	**S**	**L**	**P**	**G**	**W**	**R**	**I**	H	**C**	**V**	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**
Chicken cyto.	**T**	**N**	**L**	**A**	**M**	L	N	**P**	**S**	R	**P**	**G**	**W**	**R**	**I**	E	**C**	**V**	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**
A. suum	**T**	**N**	**L**	**A**	**M**	L	E	**P**	T	I	**P**	**G**	**W**	K	V	R	V	I	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**
H. contort.	**T**	**N**	**L**	**A**	**M**	L	N	**P**	A	**L**	**P**	**G**	**W**	**R**	**I**	R	**C**	**V**	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**
Drosophila	**T**	**N**	**L**	**A**	**M**	L	N	**P**	**S**	**L**	A	N	Y	**R**	**I**	E	**C**	**V**	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**
Rat	**T**	**N**	**L**	**A**	**M**	L	N	**P**	T	**L**	**P**	**G**	**W**	**R**	**I**	E	**C**	**V**	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**
Human	S	**N**	**L**	**A**	**M**	**M**	N	**P**	**S**	**L**	**P**	**G**	**W**	K	V	E	**C**	**V**	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**

[a] Comparison of the amino acid sequences of PEPCK from various species in region of 276−301 from avian liver mitochondrial PEPCK. The amino acid sequences of PEPCK from chicken liver mitochondria (Chicken mito.) (Weldon *et al*., 1990), chicken liver cytosol (Chicken cyto.) (Cook *et al*., 1986), *Ascaris suum* (*A. suum*) (Geary *et al*., 1993), *Haemonchus contortus* (*H. contort*.) (Klein *et al*., 1992), *Drosophila melanogaster* (*Drosophila*) (Gundelfinger *et al*., 1987), rat liver cytosol (Rat) (Beale *et al*., 1985), and human liver cytosol (Human) (Ting *et al*., 1993) are shown. Sequences were aligned to maximize overall identity between all of the sequences shown. The numbering system is based on the sequence of chicken mitochondrial PEPCK (Weldon *et al*., 1990). The preceding amino acid in chicken mitochondrial PEPCK is ^{275}Lys. Identities between these enzymes and the Chicken mito. PEPCK are indicated in boldface type. The aspartic acids proposed to be involved in metal chelation are shaded.

tion agent since not all proteins can accommodate the "demands" required of the cobaltic ion. These requirements may affect the stability and lifetime of a Co^{3+}−protein complex, limiting the usefulness of Co^{3+} as a probe. Rose *et al*. (1984) showed the half-life of Co^{3+}-modified yeast enolase was only 16 h. This short half-life severely limited the investigations of the modified enzyme.

Co^{3+} modification was performed with avian liver mitochondrial PEPCK to gain insight into the role of the metal in enzyme catalysis. The nature of the ligands furnished by PEPCK was not known. Techniques such as ^{113}Cd NMR are not feasible with PEPCK due to problems of limited solubility. Only recently has PEPCK been crystallized from *E. coli* and its X-ray diffraction patterns measured (Matte *et al*., 1996). This work identified Asp269 as a Ca^{2+} ligand. PEPCK from other sources has not yet been crystallized. Since Mn^{2+} is the most activating cation for avian mitochondrial PEPCK, several assumptions can be made about the metal site on PEPCK. Mn^{2+} is an octahedral, 6-coordinate ion that favors oxygen ligands. This selectivity indicates that PEPCK would be very suitable for Co^{3+} modification, since Co^{3+} prefers an octahedral arrangement and has a high preference for nitrogen and, to a lesser extent, oxygen ligands.

PEPCK incubated with Co^{2+} and H_2O_2 at pH 7.4 results in stoichiometric incorporation of Co^{3+}, and the enzyme is partially inactivated with a k_{inact} of 3.04×10^{-3} min^{-1}. The formation of a catalytically active Co^{3+}−PEPCK complex is demonstrated by the analytical determination of stoichiometric incorporation of Co^{3+} into PEPCK with retention of approximately 15−20% activity compared to unmodified PEPCK. To the best of the authors' knowledge, this is only the third report that an active enzyme−Co^{3+} complex has been formed. Kang and Storm (1972) reported that the Co^{3+}−carboxypeptidase A complex retained full esterase activity (as compared to the Co^{2+}−carboxypeptidase A complex). It was suggested that the ester substrates did not interact in the first coordination sphere of the enzyme-bound metal. Almost all peptidase activity was lost for the Co^{3+}-modified carboxypeptidase A. This led to the possibility either that the two types of substrates (esters or peptides) are hydrolyzed by different mechanisms or that a common mechanism is involved in which scission of a bond within the first coordination sphere of the metal ion does not occur. Lee (1988) showed that one metal site on yeast enolase can be occupied with Co^{3+} with no decrease in catalytic activity. The role of this metal in catalysis has not been unambiguously clarified. The formation of a catalytically active Co^{3+}−PEPCK suggests that catalysis is in the second coordination sphere of the PEPCK-bound metal. This is consistent with the previous proposal based on ^{1}H and ^{31}P nuclear relaxation rate studies (Lee & Nowak, 1984; Duffy & Nowak, 1985) that substrates bind in an outer sphere complex to the PEPCK−metal. The catalytic reaction must occur in this outer sphere complex.

One limitation of *in situ* Co^{3+} modification is that Co^{2+} has been shown to react with H_2O_2 under physiological conditions to form "reactive species" (hydroxyl radicals) that can cause free radical oxidative damage, specifically the hydroxylation of aromatic compounds. This is equivalent to the Fenton chemistry process that utilizes Fe^{2+} and H_2O_2 (O_2) (Fenton, 1894). Cobalt salts have been reported to promote the oxidation and destruction of the protein cytochrome P-450 and GSH (Moorhouse *et al*., 1985). Metal-catalyzed free radical damage was detected during the Co^{3+} modification of arsanilazotyrosine-248 carboxypeptidase A (Urdea & Legg, 1979). The addition of free radical scavengers such as phenol prevented the free radical oxidative damage. Since it is possible to generate a highly reactive hydroxyl radical during the Co^{3+} oxidation, there was concern that the observed loss of PEPCK enzymatic activity upon modification may be due to oxidative damage to the enzyme. When the Co^{3+}-modified enzyme was treated with 143 mM β-met, 97% of the Co^{3+} label was removed with a concomitant restoration of full PEPCK activity. This demonstrates that the labeling process is reversible and that no deleterious irreversible damage by oxidation of PEPCK has occurred during the modification procedure. Native PEPCK contains 13 cysteine residues, none of which are in disulfide bonds (Makinen *et al*., 1983; Weldon *et al*., 1990). Co^{3+}−PEPCK has 13 free cysteine residues, the same number as apoPEPCK. This demonstrates that the decrease in activity upon PEPCK modification is not due to disulfide bond formation. While no observable oxidative damage was detected with Co^{3+}-modified PEPCK, these potential reactions suggest that Co^{3+} may not be a feasible probe with

some other proteins due to the potentially harsh oxidative treatment necessary for *in situ* Co^{3+}−protein modification.

Protection from Co^{3+} incorporation into PEPCK occurs with GTP, ITP, and PEP. Little protection from cobalt oxidation and incorporation occurs with the other substrates. The lack of protection by GDP and IDP suggests that the effect by GTP and ITP is not due to indirect effects of (Co^{2+}, Co^{3+})−nucleotide coordination. Furthermore, free GTP as well as metal−GTP protects against Co^{3+} modification of PEPCK. The lack of protection by the substrates GDP and CO_2 agrees with previous PRR studies that showed that GDP and CO_2 do not significantly alter the interaction of PEPCK-bound Mn^{2+} with solvent (Hebda & Nowak, 1982b). The substrates GTP, ITP, and PEP are those that contain the phosphoryl group that undergoes transfer during catalysis. These results suggest that the phosphoryl group of PEP or the γ-phosphoryl group of GTP (ITP) interacts with the enzyme-bound metal in such a way that it protects against H_2O_2 (and H_2O) accessibility to Co^{2+} at the metal binding site of PEPCK. GTP, ITP, and PEP also offer substantial protection against the removal of Co^{3+} from the enzyme. This also suggests that the phosphoryl group of PEP or the γ-phosphoryl group of GTP (ITP) interacts at the catalytic site of PEPCK and hinders β-met accessibility to Co^{3+} at the metal binding site of PEPCK. These protection studies suggest that the PEPCK-bound metal interacts primarily with the phosphoryl group that undergoes transfer during catalysis. These results agree with the previously proposed model that suggests that the role of the enzyme-bound metal may be to polarize the γ-phosphoryl group of GTP through an intervening water molecule to facilitate catalysis (Lee & Nowak, 1984; Duffy & Nowak, 1985).

Since Co^{3+}−PEPCK is catalytically active, this provides the unique opportunity to explore the catalytic and mechanistic role of the bound metal. The kinetic properties and the metal ion and substrate binding properties of the Co^{3+}-modified enzyme were examined. While the metal binding site of a native protein may meet the criteria for stabilization of the trivalent or the divalent oxidation state of cobalt, it is difficult to be certain that Co^{2+} oxidation has occurred at the original metal binding site. Prior studies with other enzymes only confirmed the oxidation state of the enzyme-bound cobalt (Kang & Storm, 1972; Shinar & Navon, 1974; Anderson & Vallee, 1975). The specificity and location of the modification were not investigated in those studies. In the work presented here, no Mn^{2+} binding to Co^{3+}−PEPCK in the presence or absence of PEP was observed by either EPR or PRR measurements (Table 4), indicating that Co^{3+} is incorporated at the Mn^{2+} binding site of PEPCK. The binding of Mn^{2+} to PEPCK is tighter in the presence of PEP (Hebda & Nowak, 1982b). CD and fluorescence studies with Co^{3+}−PEPCK gave spectral properties identical to those for Mn^{2+}−PEPCK and unlike those of apoPEPCK, further indicating that the Co^{3+} label is specific.

Co^{3+}−PEPCK requires an additional metal for activity and follows Michaelis−Menten kinetics. The kinetic constants for all the substrates and the metals, Mn^{2+}, Co^{2+}, and Mg^{2+}, were altered for Co^{3+}−PEPCK as compared to those for apoenzyme (Tables 5 and 6). With the exception of HCO_3^-, the substrates all show decreased $K_{m,app}$ values, indicating alterations in the kinetic parameters for these substrates and cofactors to Co^{3+}−PEPCK. This is anticipated if ionic interactions play an important role in ligand binding in the vicinity of the cation center. The decreased values of k_{cat}/K_m with Co^{3+}−PEPCK suggest that the rates of substrate interaction with this enzyme complex have also decreased.

The requirement for additional metal for catalytic activity with Co^{3+}−PEPCK suggests that free nucleotide is not a substrate for Co^{3+}−PEPCK. This agrees with kinetic results (Lee *et al*., 1981) that demonstrated two roles for divalent cations for PEPCK activity. The enzyme-bound Co^{3+} serves to activate the enzyme. The second cation forms the cation·nucleotide complex that serves as a substrate. The additional metal is required for Co^{3+}−PEPCK activity because of the metal·nucleotide form of the substrate that is necessary for catalysis. Mn^{2+} elicits the highest activity for Co^{3+}−PEPCK, Co^{2+} gives approximately 30−40% of the activity relative to Mn^{2+} activation, and Mg^{2+} gives 6−11% of the relative activity. These results suggest that Mn^{2+}·IDP is a better substrate for Co^{3+}−PEPCK than either Co^{2+}·IDP or Mg^{2+}·IDP. Co^{3+}−PEPCK was also activated by the cations, Zn^{2+} and Cu^{2+}. These cations alone do not activate apoPEPCK. The presence of Co^{3+} at the metal site of the protein prevents the interaction of these nonactivating or inhibiting metals with PEPCK directly, allowing for activation. The nonactivating or inhibiting metals were only able to associate with the nucleotide at the second metal site. Lee and Nowak (1985) proposed that the second metal is in a β,γ-bidentate coordination with GTP. This proposal was based on the lack of any significant dependence of the stereospecificity of thiophosphate derivatives of GTP on the second metal ion. The role of the second metal may activate the γ-phosphate moiety of GTP (ITP), facilitating the nucleophilic attack by the substrate OAA. In the reverse direction, the second cation may stabilize the product GTP (ITP), making the phosphoryl group from the substrate PEP a better leaving group. The role of the second metal may also be to elicit the proper conformation of GDP and of GTP necessary for catalysis. Co^{3+}−PEPCK provides an excellent tool for examining the kinetic, mechanistic, and binding properties of the second metal without the concern of metal binding to the enzyme.

The binding constants for GTP, ITP, GDP, and IDP (as free nucleotide or as the metal−nucleotide complex) were similar for apoPEPCK, Mn^{2+}−PEPCK, and Co^{3+}−PEPCK, as determined from fluorescence. The K_D values were between 6 and 14 μM for all nucleotides (Table 7). These values agree with previously performed PRR studies that gave a dissociation constant of 7.5 μM for IDP to the enzyme−Mn^{2+} complex (Hebda & Nowak, 1982b) and direct binding studies that gave a binding constant of <2 μM for GTP to the enzyme−Mn^{2+} complex (Lee & Nowak, 1984). The similarity of the K_D values of apoPEPCK, Mn^{2+}−PEPCK, and Co^{3+}−PEPCK with the nucleotide suggests that these substrates interact with Co^{3+}−PEPCK in a fashion similar to the interaction of substrates with apoPEPCK and Mn^{2+}−PEPCK.

Since the cobaltic ion has a substitution-inert coordination sphere, the possibility of directly determining the amino acid residues of the protein as metal ligands is offered. This was accomplished by proteolytically digesting the Co^{3+}-modified enzyme followed by isolation and sequencing of the cobalt−peptide. Presently, only three reported cases of the isolation of a cobalt(III)- or chromium(III)-modified peptide have been reported (Kowalsky, 1969; Miziorko *et al*., 1982; Lee, 1988). In none of these cases were the metal ligands successfully

identified. Lee (1988) reported the amino acid composition analysis of the cobalt−peptide from yeast enolase; however, amino acid sequencing was not successfully performed.

In an effort to determine the location of the cation site in PEPCK, Co^{3+}-modified enzyme was digested by LysC, and the cobalt-containing peptides were purified using reverse phase HPLC. The LysC digest produced a single pure peptide that contained cobalt. Mass, amino acid composition, and sequence analyses identified the peptide as the segment Thr276−Lys301. This highly conserved region is located near the central portion of PEPCK and is near the putative nucleotide binding region (Weldon *et al*., 1990). Amino acids Asp295 and Asp296 are the most feasible metal ligands in this peptide, discounting backbone carbonyl groups as ligands. Asp296 was also identified as the metal ligand using Fenton chemistry to generate specific protein cleavage.[2] His291 and Cys292 may also serve as potential metal ligands, but these residues are not well conserved in other PEPCKs (Table 9). It should be noted that this lack of conservation does not rule out His291 and Cys292 as potential metal ligands for the avian liver mitochondrial PEPCK. It is also of interest to note that Co^{3+} is normally redox-stable only with several nitrogen ligands in the coordination sphere. Since Co^{3+}−PEPCK is stable for over a week at 4 °C with no significant loss of the cobalt label (data not shown), perhaps one or more of the ligands to Co^{3+} may contain nitrogen. The isolated peptide contains several nitrogen-containing amino acids that may bind the metal. It is possible that histidine or tryptophan residues or even amide nitrogens may play a role in metal binding. At this time, only Asp295 and Asp296 have been identified as possible metal ligands. Crystal structure analysis of the avian liver mitochondrial PEPCK may identify the remaining metal ligands, if any.

The recent crystal structure analysis of PEPCK from *E. coli* identified Asp269 as a metal ligand (Matte *et al*., 1996). Asp269 from *E. coli* PEPCK corresponds to Asp296 of the avian liver PEPCK. Considering the numerous differences between the *E. coli* and avian liver enzymes, it is interesting to note that the metal consensus site of X-Gly-Asp-Asp, where "X" is either valine or isoleucine, is conserved. A search through the on-line enzyme database revealed that PEPCK from 20 different sources (both GTP- and ATP-utilizing enzymes) contains this highly conserved metal consensus site. The sequence of Gly-Asp-Asp-Ile is conserved throughout all GTP-utilizing PEPCKs, whereas the conserved sequence of Gly-Asp-Asp-Glu is found in all ATP-utilizing PEPCKs. Several other mitochondrial enzymes, isocitrate dehydrogenase (Soundar & Colman, 1993) and malic enzyme (Wei *et al*., 1994), also contain the metal binding site consensus sequence of X-X-Asp-Asp-X-X, where "X" is an uncharged and often hydrophobic amino acid. This conserved sequence of two adjacent aspartic acid residues often surrounded by uncharged and hydrophobic amino acid residues may indicate the potential metal binding site in other metal-requiring mitochondrial enzymes.

Initial sequencing attempts on the cobalt−peptide failed due to N-terminal alteration of the peptide at Thr276. Successful sequencing of the peptide was accomplished only after treatment with ArgC that generated an additional fragmentation. N-Terminal blockage with threonine is rare. Perhaps the conditions required for the isolation of the peptide or the conditions used to remove the cation from the peptide caused the modification to the N-terminus. If the subsequent Co^{3+} reduction and purification results in oxidative deamination of the α-amino group, then subsequent Edman chemistry is not feasible. The difficulty experienced with Co^{3+}−PEPCK may explain why identification of the metal ligands using this technique has not been previously accomplished.

These studies suggest that the metal plays an ionic role in enzyme activity as opposed to a direct ligand binding function. The role of the enzyme-bound metal in PEPCK may be to activate the enzyme by an ion dipole interaction via a bound water with the substrates. The observed activity of Co^{3+}−PEPCK demonstrates that substrate binding in the first coordination sphere of the metal ion does not occur during enzymatic catalysis. This supports the previously proposed model based on results of NMR studies (Lee & Nowak, 1984; Duffy & Nowak, 1985). It may be argued that if the enzyme-bound metal ionically interacts with substrates; then the increased oxidation state of Co^{3+} should facilitate that interaction, conceivably increasing V_{max} values. One possible explanation for the decreased V_{max} values (see Tables 5 and 6) observed for the Co^{3+}−PEPCK complex as compared to unmodified enzyme may be due to the decreased ionic radius of Co^{3+} as compared to the Mn^{2+}. Mn^{2+} exists in a high-spin d^5 state with an ionic radius of 0.97 Å. The exchange-inert low-spin Co^{3+} ion has an ionic radius of 0.69 Å. The decreased ionic radius of Co^{3+} may not allow the optimal interaction of substrates with the enzyme and might account for the decreased k_{cat} values for Co^{3+}−PEPCK. Co^{3+} decreases the k_{cat}/K_m values 3−5-fold due to decreased V_{max} values. This is consistent if the ionic character plays an important role in ligand binding.

It has become apparent that several different categories of metal atoms may be present in metal-utilizing enzymes. The function of these metals may be in catalysis, structural stabilization, or protein regulation. The metals involved in catalysis appear to have ligand sites that differ considerably from those that function solely in a structural role (Simpson *et al*., 1968). For example, a zinc atom plays a role in the catalytic mechanism of thermolysin from *B. thermoproteolyticus*, but in addition, calcium atoms stabilize the secondary and tertiary structure (Latt *et al*., 1969). These two distinct metal sites are more than 10 Å from each other. Not much is known about the effects of metals on protein stabilization. The *in situ* conversion of exchange-labile Co^{2+} to exchange-inert Co^{3+} by oxidation with H_2O_2 offers a potential method of selectively "freezing" cobalt atoms at the metal binding site which would stabilize a given conformation. This process was employed with alkaline phosphatase (Anderson & Vallee, 1975) where Co^{3+} modification clarified the roles of the four metal atoms involved in both catalysis and structure.

In summary, the results of this study have demonstrated that cobalt(III) has been selectively introduced into the cation site in avian liver mitochondrial PEPCK. The Co^{3+}−PEPCK complex is active with structural, binding, and catalytic properties similar to unmodified PEPCK. The binding site for the cation has been located using solution chemistry and

[2] When PEPCK is incubated with Fe^{2+} and ascorbate (Fenton chemistry), complete loss of activity is observed and approximately 50% of the protein is cleaved at a single site. This site of cleavage was identified from peptide sequencing results as occurring between Asp296 and Ile297 (J. Hlavaty and T. Nowak, 1997, manuscript in preparation).

peptide analysis. This methodology may be extendible to other metal-requiring proteins. The Co^{3+}−PEPCK complex will serve as an experimental tool to investigate the properties of the second cation and its binding site.

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