Chromium(III) Modification of the First Metal Binding Site of Phosphoenolpyruvate Carboxykinase†

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ABSTRACT: Chicken liver phosphoenolpyruvate carboxykinase (PEPCK) is activated by Cr^{2+} as the sole activator under anaerobic conditions. PEPCK was modified with Cr^{3+}, starting with either Cr^{2+} or Cr^{3+}. Cr^{3+} has the distinct advantage of being a paramagnetic cation that could serve as a paramagnetic probe. Activators Mn^{2+}, Mg^{2+}, and Co^{2+} protect against Cr^{3+} incorporation. EPR, CD, and fluorescence studies indicate that Cr^{3+} was incorporated into the cation binding site of PEPCK. The water proton relaxation rate (PRR) and fluorescence binding studies showed that $Cr^{3+}(n_1)$−PEPCK forms enzyme−substrate complexes similar to those observed for the $Mn^{2+}(n_1)$−PEPCK complex (n_1 represents the metal "enzyme binding site" as opposed to the metal "nucleotide binding site"). $Cr^{3+}(n_1)$−PEPCK requires an additional divalent cation for activity, an indication of two metal sites on PEPCK. $Cr^{3+}(n_1)$−PEPCK retains 15% residual activity as compared to unmodified PEPCK and demonstrates normal Michaelis−Menten kinetics. This is the first report of an active Cr^{3+}-modified enzyme complex.

Chicken liver mitochondrial phosphoenolpyruvate carboxykinase (PEPCK,[1] EC 4.1.1.32) is a 67 kDa monomeric enzyme that catalyzes the reversible GTP (ITP)-dependent conversion of OAA to PEP, GDP, and CO_2 (*1*). PEPCK shows an absolute requirement for divalent cations for activity. Mn^{2+} is the best activator for chicken mitochondrial PEPCK, with Co^{2+} and Mg^{2+} activating to a lesser extent. On the basis of ^1H and ^{31}P NMR relaxation rate studies, the substrates form Michaelis complexes in the outer sphere of the PEPCK-bound Mn^{2+}. The catalytic reaction has been proposed to occur in the second sphere of the enzyme-bound Mn^{2+} (*2*−*4*). Frequency-dependent PRR studies suggest that two water ligands are associated with the enzyme-bound Mn^{2+}, indicating that the enzyme provides four ligands to the hexacoordinate metal (*2*). It is believed that at least one of these water molecules on the Mn^{2+} serves as a bridge between the substrate and enzyme-bound metal.

Substitution-inert metals, such as Cr^{3+}, have been used to investigate the role that the metal plays in enzyme catalysis. Ligand complexes with Cr^{3+} can form exchange-inert complexes where the lifetime of the ligand in the coordination sphere of the cation is long compared to the experimental time for such complexes. Cr^{2+} can be readily oxidized to Cr^{3+} in air. Cr^{3+} has a rate constant of approximately 10^{-5} s^{-1} for substitution of inner-sphere water molecules (*5*). This rate constant is 13 orders of magnitude smaller than that for the reduced form of chromium, Cr^{2+}. When the chromous ion binds to an enzyme and is further subjected to oxidation, the resulting chromic ion can form a ligand exchange-inert complex with the residues on the enzyme. The modified enzyme can be used to obtain information about the nature of the metal binding site(s) (*6*, *7*).

Since many ligands of proteins that act to bind metal ions involve nitrogen or oxygen, it is likely that Cr^{3+} would form stable complexes in such environments. Recently, the in situ oxidation of Cr^{2+} has been used to study the metal binding sites of several proteins. Kowalsky (*8*) first reported the reduction of ferricytochrome *c* with Cr^{2+}. A total of 0.5 mol of chromium atoms was tightly associated per 1 mol of cytochrome *c*. Balakrishnan and Villafranca (*7*) reported the preparation of Cr^{3+}−glutamine synthetase. EPR studies performed on the modified enzymes were used to determine metal−metal distances within the enzyme. Miziorko et al. (*9*) reported the incorporation of stoichiometric amounts of Cr^{3+} into the enzyme ribulosebisphosphate carboxylase under anaerobic conditions. The modified enzyme was inactive. When Mg^{2+} was added to the incubation mixture prior to the addition of Cr^{2+}, a significant reduction in the amount of Cr^{3+} incorporation was observed. This indicated that Mg^{2+} protected against enzyme modification by Cr^{3+} and that Cr^{3+} was specifically incorporated into the Mg^{2+} binding site on the enzyme. Nonspecific Cr^{3+} binding to ribulosebisphosphate carboxylase was observed under aerobic conditions. Similar experiments were performed with enolase using chromium. Cr^{2+} binds to yeast enolase (*10*) and causes

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[1] Abbreviations: AA, atomic absorption; β-met, 2-mercaptoethanol; CD, circular dichroism; Cr^{3+}−PEPCK, PEPCK modified with Cr^{3+}; DEAE, diethylaminoethyl; EPR, electron paramagnetic resonance; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; Hepes, *N*-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IDP, inosine 5′-diphosphate; ITP, inosine 5′-triphosphate; OAA, oxaloacetate; MES, 2-(*N*-morpholino)-ethanesulfonic acid; NADH, reduced nicotinamide adenine dinucleotide; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PRR, water proton longitudinal relaxation rate; Tris, tris-(hydroxymethyl)aminoethane.

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irreversible inhibition of enolase activity upon oxidation to Cr^{3+}. The loss of enolase activity is linearly correlated with the incorporation of Cr^{3+} to enolase (*11*). Stoichiometry experiments revealed that 6.5 ± 0.5 mol of Cr^{3+} was incorporated per mole of enolase monomer in the inactive enzyme. This high level of Cr^{3+} incorporation is believed to be due to nonspecific binding of Cr^{3+} on the surface of enolase. High levels of Cr^{3+} incorporation into proteins due to nonspecific binding have also been observed with the tetrameric protein, yeast pyruvate kinase. Aerobic incubation of Cr^{2+} with pyruvate kinase resulted in approximately six atoms of chromium incorporated per monomer of the inactive enzyme (*12*). It was observed that a stoichiometric Cr^{3+}−yeast pyruvate kinase complex (one chromium atom per pyruvate kinase monomer) could be obtained by preincubating the enzyme with $CrCl_3$ (*12*). PRR studies showed that over a period of 24 h the normalized water proton relaxation rates with the Cr^{3+}−yeast pyruvate kinase complex vary by 25%, an indication of a change of the environment and exchange of water molecules at the chromium ion. This apparent slow exchange of ligands associated with the chromium ion may explain why in situ oxidation was not required for Cr^{3+} modification of the enzyme.

The success of the modification of PEPCK with Co^{3+} (*4*) led to an investigation of the modification of PEPCK with Cr^{3+}. Previous studies carried out in this laboratory showed that Cr^{3+} modification of PEPCK was nonspecific and nonstoichiometric (A. M. Jabalquinto and T. Nowak, unpublished observations). With alteration of the conditions of the modification process, a catalytically active, stoichiometric Cr^{3+}−PEPCK complex can be formed. This paper deals with the modification of the metal binding site of avian liver mitochondrial PEPCK by Cr^{3+}. As shown by CD, fluorescence, and EPR studies, Cr^{3+} is located at the Mn^{2+} binding site of PEPCK. The kinetic and binding properties of the Cr^{3+}−PEPCK complex were determined and compared with those of native PEPCK. Since chromium modification has the additional potential advantage of Cr^{3+} being paramagnetic, PRR studies examining substrate binding to the Cr^{3+}−PEPCK complex were also conducted.

MATERIALS AND METHODS

Materials. Malate dehydrogenase was purchased from Boehringer Mannheim Corp. GTP, ITP, GDP, IDP, PEP, NADH, $CaCl_2$, and tetramethylammonium sulfate were purchased from Sigma. $CoCl_2$, $CrCl_2$, $CrCl_3$, $ZnCl_2$, $CuCl_2$, $FeCl_2$, MES, and Tris base were purchased from Mallinckrodt. $CdCl_2$, $MnCl_2$, and $MgCl_2$ were from Baker. Chelex-100, DEAE Sepharose, and hydroxyapatite A resin 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.

PEPCK Purification. Chicken liver mitochondrial PEPCK was purified as previously reported (*4*, *13*). This procedure is a modification of the procedure of Lee and Nowak (*2*). The enzyme used for all studies typically had a specific activity between 4.5 and 7 units/mg and was >95% pure.

PEPCK Assay. The PEPCK-catalyzed reaction of PEP carboxylation to OAA was assayed by the method of Noce and Utter (*14*) as modified by Hebda and Nowak (*15*). 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 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. The PEPCK concentration was determined by absorbance at 280 nm ($\epsilon_{1\%}^{280} = 16.5$ mg^{-1} mL^{-1}) (*16*). 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, such determinations were performed enzymatically. The concentration of substrate was determined as the limiting reagent in the PEPCK assay 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.

Chromium(II) Activation of PEPCK. A stock 40 mM Cr^{2+} solution was prepared by bubbling N_2 through a 10^{-4} N HCl solution for approximately 60 min. This was done to displace O_2 from the solution. $CrCl_2$, which had been stored under a vacuum in a desiccator, was quickly weighed and added to the solution. After the $CrCl_2$ dissolved, bubbling of N_2 through the solution was continued in an attempt to keep the chromium in its reduced state. PEPCK was added to an assay mixture that contained everything but metal. The system was in 5 mM MES buffer (pH 6.8). The MES buffer system was used because it did not present the chromium solubility problems observed with Tris or phosphate buffer systems. N_2 was bubbled through the assay mixture for approximately 30 min to ensure the displacement of O_2. After this time, various amounts of Cr^{2+} were added to the assay mix and activity was immediately measured.

Chromium(III) Modification of PEPCK. PEPCK could be labeled with chromium starting with either Cr^{2+} or Cr^{3+}. Incubation of PEPCK with high concentrations (>1 mM) of either $CrCl_2$ or $CrCl_3$ results in a high stoichiometry of bound chromium, possibly due to nonspecific labeling.

PEPCK could be modified with Cr^{2+} using the following procedure. A stock (1 mM) Cr^{2+} solution was prepared by bubbling N_2 through a 10^{-4} N HCl solution for approximately 60 min. $CrCl_2$ salt, which had been stored under vacuum in a desiccator, was quickly weighed and added to the solution. After the $CrCl_2$ dissolved, bubbling of N_2 through the solution was continued to keep the chromium in its reduced state. Approximately 115 μM PEPCK, in 5 mM MES (pH 6.8) and 100 mM KCl, was treated with bubbling N_2 for approximately 30 min to remove any O_2. Approximately 115−230 μM $CrCl_2$ was added to the enzyme solution. The solution was allowed to sit on ice for 15 min with N_2 bubbling through it. After this time, the N_2 was removed and the solution was allowed to sit for 30 min, exposed to air.

PEPCK could be modified with $CrCl_3$ using the following procedure. Approximately 115 μM PEPCK, in 50 mM Tris-HCl (pH 7.4) and 100 mM KCl, was incubated on ice with 115−230 μM $CrCl_3$ for 0.5−6 h with periodic gentle shaking of the solution. The duration of incubation varied for each preparation. Incubation was halted after a 1:1 Cr^{3+}:PEPCK ratio was observed (stoichiometry measurements are discussed below).

In each preparation, after incubation, excess chromium was removed by placing the solution on a (1 × 10 cm) Chelex-100 column equilibrated in 50 mM Tris-HCl buffer (pH 7.4). The modified enzyme was collected from the column and concentrated using a mini-Amicon concentrator with a PM30 membrane. The enzyme concentration was determined by absorbance at 280 nm or by the Bradford Protein Assay Kit. All absorbance measurements were performed on a Beckman 210 spectrophotometer. Cr^{3+} content was determined by atomic absorption with a Varian AA-775 spectrometer at 357.9 nm using the Varian CRA-90 Carbon Rod atomizer. Both of these procedures consistently gave 1:1 Cr^{3+}:PEPCK ratios. The Cr^{3+}−PEPCK complexes are stable for at least 3 days with no loss of the Cr^{3+} label or alteration of activity.

Substrate Protection Studies. Substrate and metal protection against Cr^{3+} incorporation into PEPCK was determined. Approximately 115 μM PEPCK, in 50 mM Tris-HCl (pH 7.4) and 100 mM KCl, was incubated on ice with 115−230 μM $CrCl_2$ (prepared as described above) or 115−230 μM $CrCl_3$ and known amounts of substrates or metals for 30 min with periodic gentle shaking of the solution. 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 and chromium content were determined from the supernatant. The amount of Cr^{3+} incorporated into PEPCK and remaining activity were measured against time.

It was observed that the addition of β-met to Cr^{3+}−PEPCK followed by gel filtration would restore activity and remove the Cr^{3+} label. Substrate protection against Cr^{3+} removal from the modified enzyme was performed in a manner analogous to that of the Cr^{3+} incorporation studies. The Cr^{3+}-modified 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 chromium content and activity were determined. PEPCK inactivation and reactivation rate constants were calculated from the plots of log(percentage of activity) versus time. The data were fit using Cricket Graph, V.1.2.

Mn^{2+} Binding to Cr^{3+}−PEPCK. The binding of Mn^{2+} to apoPEPCK and Cr^{3+}−PEPCK in the presence and absence of PEP was determined by EPR following the method of Hebda and Nowak (*17*). Samples were drawn into 1 mm (inside diameter) 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 number of binding sites and the binding constants were obtained from a Scatchard plot (*18*) of the EPR data. No EPR signal was observed for 50 μM free Cr^{3+} under these conditions. The observed EPR signal was solely due to free Mn^{2+}.

Circular Dichroism Measurements. Circular dichroism measurements were obtained with a Cary model 60 recording spectropolarimeter that has been upgraded to a computer-assisted AVIV model 62DS unit. Samples of 0.26 μM apoPEPCK, Mn^{2+}−PEPCK, and Cr^{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 and to prepare enzyme solutions. For Mn^{2+}-containing samples, 50 μM $MnCl_2$ was used. 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 the baseline 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 ($\Delta\epsilon_L$)- and right ($\Delta\epsilon_R$)-handed components of circularly polarized light. The observed ellipticity (θ) was converted to ellipticity per mean residue weight. Secondary structure estimations were determined by fitting the mean residual weight ellipticity data to the AVIV program PROSEC, V.3.1, on the basis of an algorithm and reference spectra of J. T. Young and D. Weber that accompanies this instrument.

Substrate Binding As Determined from Fluorescence. A computer-assisted SLM 8100 fluorescence spectrometer was used for all measurements. The sample cell holder was maintained at 24 °C using a circulating water bath. The apoPEPCK and Cr^{3+}−PEPCK solutions were prepared in 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl. The samples were individually measured in a 1 mL quartz cell at an excitation wavelength of 297 nm and an emission wavelength of 334 nm. At recorded time intervals, a known concentration of substrate (GTP, GDP, ITP, or IDP) was added directly into the quartz cell containing the enzyme solution. The amount of fluorescence quenching due to each addition of substrate was recorded. After correction for dilution, the recorded digital fluorescence data were converted to the percentage of quenching at each substrate concentration using the Microsoft EXCEL 5.0 spreadsheet program. Binding constants were determined using the EZ-Fit program, version 2.02, by Perella Scientific Inc. (1989). Each K_D value is an average of two titrations.

Substrate Binding to Cr^{3+}−PEPCK As Determined from PRR Measurements. The binding of substrates to the Cr^{3+}−PEPCK complex was determined by measuring the water proton relaxation rates (PRR) using a Seimco-pulsed NMR spectrometer at 24.3 MHz using the Carr−Purcell (*19*) 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 (*20*), and an outline of the method was recently presented (*4*). The enhancement values were measured as substrates were titrated into separate solutions of 75 μM Cr^{3+}−PEPCK in 50 mM Tris-HCl (pH 7.4) with 100 mM KCl. A concentration range of 0−100 μM was used for the substrates GTP, PEP, GDP, and OAA. For CO_2 (treated as HCO_3^-), a concentration range of 0−160 mM was used. PRR measurements were taken at room temperature, and the enzyme samples were kept at pH 7.4.

When the observed enhancements, or ϵ_{obs}, are plotted versus substrate concentration, values for K_3 and ϵ_t were determined. The term ϵ_{obs} is defined as follows:

$$\epsilon_{obs} = \frac{[EM]_f}{[M]_t}\epsilon_b + \frac{[EMS]}{[M]_t}\epsilon_t \quad (1)$$

where [EM] and [EMS] represent the concentrations of Cr^{3+}−PEPCK and Cr^{3+}−PEPCK−substrate complexes, respectively. $[M]_t$ is the total Cr^{3+} concentration which, by definition in this case, is the same as the starting Cr^{3+}−PEPCK concentration. In treatment of the data, $\epsilon_b = 0.49$ for the Cr^{3+}−PEPCK complex as determined in this study. The term ϵ_t is defined as the enhancement value of the Cr^{3+}−PEPCK−substrate complex at saturating substrate concentrations. K_3 represents the binding constant for the interaction of the substrate with the enzyme−metal complex as follows:

$$K_3 = \frac{[S]_f[EM]}{[EMS]} \quad (2)$$

where $[S]_f$ is the free substrate concentration, [EM] represents the concentration of the binary enzyme−metal complex not associated with substrate, and [EMS] represents the concentration of the enzyme−metal−substrate complex. The titration curves were fit on the basis of eqs 1 and 2 using the computer program PRRFIT2, written in Fortran code. The program is designed using the least-squares method. The program generates values for K_3 and ϵ_t.

RESULTS

Chromium(II) Activation of PEPCK. A kinetic profile of Cr^{2+} activation of PEPCK under anaerobic conditions was performed. Measurements could not be taken above 4 mM Cr^{2+} due to solubility problems. The IDP concentration was kept constant at 2 mM. Cr^{2+} activated PEPCK with a K'_a of 1.30 ± 0.32 mM and a V_{max} of 1.18 ± 0.12 units/mg. The Cr^{2+}-activated enzyme had 15% of the activity of PEPCK−Mn^{2+}, which was used as a control. This places Cr^{2+} as the third highest activating cation, behind Mn^{2+} (100%) and Co^{2+} (30%), but before Ca^{2+} (4%) and Mg^{2+} (2%). The high K'_a value suggests that Cr^{2+} binds to IDP and also causes activation by binding to PEPCK.

Chromium Modification of PEPCK. PEPCK was inhibited in the presence of either $CrCl_2$ (and O_2) or $CrCl_3$ at pH 7.4. When 230 μM Cr^{2+} was incubated with 115 μM PEPCK and the mixture exposed to air, rapid inactivation occurred (assay mixture contained Mn^{2+}). Figure 1 shows a profile of Cr^{2+} inactivation. By 110 s, no further inactivation occurs. Note that approximately 15% of the residual activity remains. After 5 min, this incubation mixture was treated with Chelex-100 to remove any excess chromium. The stoichiometry was determined to be 1 mol of Cr^{3+} bound to 1 mol of PEPCK. The incubation of PEPCK with Cr^{2+}, while exposed to air, causes rapid oxidation and concomitant labeling of the enzyme. The residual activity is due to the nature of this modified enzyme. It was observed that, when the concentration of Cr^{2+} was increased to greater than 400 μM, apparent nonspecific labeling occurs. While no change in the inactivation rate profile was observed, stoichiometry ratios increased considerably to values as high as 7 mol of Cr^{3+} bound to 1 mol of PEPCK.

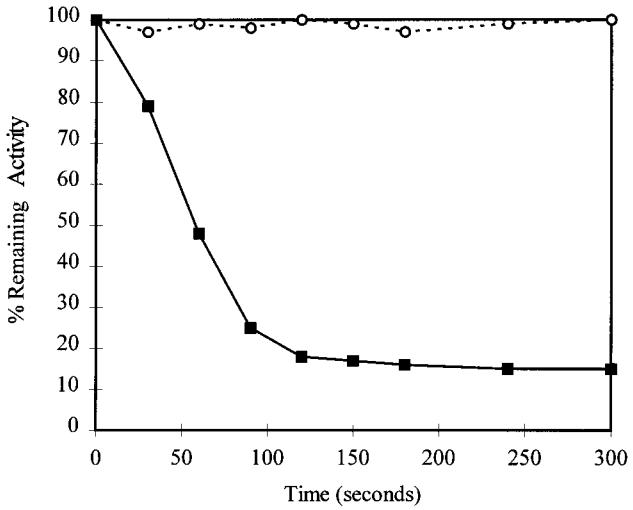

FIGURE 1: Cr^{2+} inactivation of PEPCK as a function of time. When 230 μM Cr^{2+} is added to 115 μM PEPCK and the solution is exposed to air, a rapid inactivation is observed. By 110 s, the inactivation appeared to be complete. No further inactivation was observed. The control using Mn^{2+} rather than Cr^{2+} is represented by ○, and the sample with Cr^{2+} is represented by ■.

Previous studies indicated that incubating pyruvate kinase with $CrCl_3$ (Cr^{3+}) led to stoichiometric incorporation of Cr^{3+} into pyruvate kinase (*13*). This process was attempted with PEPCK as described in Materials and Methods. On average, it was found that incubating PEPCK with $CrCl_3$ for approximately 3 h was sufficient for stoichiometric incorporation of Cr^{3+} into PEPCK. For some preparations, stoichiometric incorporation of Cr^{3+} into PEPCK was observed in as little as 30 min and sometimes in as much as 6 h. If a less than 1:1 Cr^{3+}:PEPCK ratio was obtained after a given incubation time, the partially modified PEPCK complex was incubated with 115−230 μM $CrCl_3$ for a second time for several additional hours. Increasing the concentration of $CrCl_3$ (>500 μM) in the incubation mixture resulted in a high stoichiometry of incorporation due to apparent nonspecific labeling. The modification process is sensitive to both time and $CrCl_3$ concentration. The 1:1 Cr^{3+}−PEPCK complex prepared with $CrCl_3$ was assayed for catalytic activity and was found to be 15% as active as native PEPCK. This is the same value observed for the Cr^{3+}−PEPCK complex that was modified with Cr^{2+} (vide supra). As with Co^{3+}−PEPCK, additional metal was required for activity. These results indicate that an active, stoichiometric Cr^{3+}−PEPCK complex can be created under proper conditions, starting with either Cr^{2+} or Cr^{3+}. Modification of PEPCK by Cr^{2+} or Cr^{3+} is sensitive to buffer and pH. Hepes and phosphate buffers resulted in the precipitation of chromium−buffer complexes.

Substrate Protection. Potential substrate protection against the incorporation of Cr^{3+} into PEPCK was investigated. All the substrates have been shown to bind to apoEPECK. No protection was observed against Cr^{3+} inactivation of or incorporation into PEPCK with any of the substrates. This indicates that substrates do not protect against Cr^{3+} modification of PEPCK. These results contrast those observed for Co^{3+}−PEPCK, where GTP, ITP, and PEP all protected against Co^{3+} modification (*4*). The protection experiments were repeated starting with $CrCl_2$ (Cr^{2+}) under anaerobic conditions followed by exposure of the PEPCK−substrate−

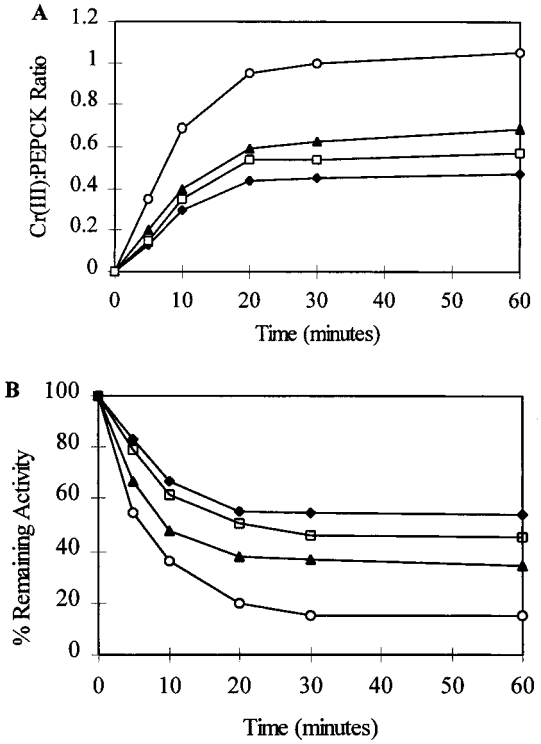

FIGURE 2: Metal protection against Cr^{3+} incorporation into PEPCK. PEPCK in 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl was incubated in the presence of 230 μM $CrCl_3$ at pH 7.4 alone (○) or in the presence of 2 mM $MnCl_2$ (Mn^{2+}) (◆), $MgCl_2$ (Mg^{2+}) (▲), or $CoCl_2$ (Co^{2+}) (□). At recorded time intervals, aliquots were withdrawn and the Cr^{3+} content and PEPCK activity were determined as discussed in the text. (A) The plot of Cr^{3+} incorporation into PEPCK as a function of time is shown. (B) The PEPCK percentage of remaining activity as a function of time is plotted for each of the sets of experiments shown in panel A.

metal solution to air. Again, no protection against Cr^{3+} modification was observed for any of the substrates. This indicates that the substrates do not protect against O_2 access at the metal site on PEPCK.

Previous studies (*21*) showed that Co^{2+} binds tightly to PEPCK and is not readily displaced by high concentrations of Mn^{2+} or other metals; therefore, protection by Mn^{2+} or Mg^{2+} against Co^{3+} incorporation into PEPCK could not be determined. Because of the method of Cr^{3+} modification of PEPCK (i.e., starting with Cr^{3+}), the effects of Mn^{2+} or Mg^{2+} protection against Cr^{3+} incorporation into PEPCK could be studied. Figure 2A shows the level of Cr^{3+} incorporated into PEPCK in the absence and presence of additional metal as a function of incubation time. Enzyme incubated in the presence of Cr^{3+} but in the absence of any metal showed stoichiometric incorporation of Cr^{3+} into PEPCK within 20 min. Mn^{2+}, Mg^{2+}, and Co^{2+} all show substantial protection against the incorporation of Cr^{3+} into PEPCK, with Mn^{2+} offering the most protection. After 10 min, enzyme preincubated with Mn^{2+}, Mg^{2+}, or Co^{2+} had 60−70% less Cr^{3+} incorporated into PEPCK than the enzyme in the absence of additional metal.

Figure 2B demonstrates the residual activity from Cr^{3+} incorporation into PEPCK in the absence and presence of metal as a function of incubation time. The initial measurements were made prior to the addition Cr^{3+}. Enzyme preincubated in the presence of Cr^{3+} but in the absence of any metal had a 65% decrease in enzymatic activity after 10 min with a k_{inact} of 1.85×10^{-1} min^{-1}. The enzyme preincubated in the presence of Mn^{2+}, Mg^{2+}, and Co^{2+} showed substantially smaller decreases in enzymatic activity after 10 min. As shown in Table 1, the k_{inact} values for PEPCK in the presence of the metals were 1.2−1.7 times lower than the k_{inact} value for PEPCK in the absence of any additional metal. These results also agree with chromium incorporation data and indicate that Mn^{2+}, Mg^{2+}, and Co^{2+} protect against Cr^{3+} incorporation into PEPCK. These results suggest that the Cr^{3+} modification is specific for the metal site on PEPCK.

Table 1: Rate Constants[a] for Inactivation and Reactivation of PEPCK by Cr^{3+}

substrate or metal	k_{inact} (min^{-1})	k_{react} (min^{-1})
none (control)	1.85×10^{-1}	2.10×10^{-1}
GDP	NP[b]	1.98×10^{-1}
OAA	NP	1.83×10^{-1}
CO_2 (HCO_3^-)	NP	2.08×10^{-1}
PEP	NP	0.37×10^{-1}
GTP	NP	0.23×10^{-1}
Mn^{2+}	1.08×10^{-1}	−
Mg^{2+}	1.57×10^{-1}	−
Co^{2+}	1.19×10^{-1}	−

[a] PEPCK inactivation and reactivation rate constants were calculated from the slopes as determined from the plots of log(percentage of activity) vs time. The data were fit using Cricket Graph, V.1.2. Standard deviations were not greater than 3% for all fits. [b] NP means no protection was observed for Cr^{3+} incorporation into PEPCK.

When Cr^{3+}−PEPCK was treated with β-met, activity increased. When Cr^{3+}−PEPCK was treated with 143 mM β-met for 30 min, a 64% reduction in the amount of Cr^{3+} label was seen with a concomitant restoration in PEPCK activity. β-met may reduce the exchange-inert Cr^{3+} label to the labile Cr^{2+} oxidation state, thus removing the label and restoring PEPCK activity. The standard reduction potential for the one-electron reduction of Cr^{3+} to Cr^{2+} is −0.407 V (*22*) which makes this process unfavorable. In contrast, the standard reduction potential for the one-electron reduction of Co^{3+} to Co^{2+} is 1.83 V (*22*) which makes this process very favorable. This may explain why the Cr^{3+} reduction occurs at a slower rate than the Co^{3+}−PEPCK reduction [when the Co^{3+}−PEPCK was treated with 143 mM β-met for 30 min, a 97% reduction in the amount of Co^{3+} label was seen with a concomitant restoration of PEPCK activity (*4*)].

As the substrates bind to the active site, their presence should alter the rate of removal of the Cr^{3+} label. Protection studies with GTP, GDP, PEP, OAA, and CO_2 (treated as HCO_3^-) against Cr^{3+} removal from the modified enzyme were performed. Figure 3A contains plots of Cr^{3+} removed from PEPCK in the absence and presence of substrate as a function of incubation time. The initial measurements were made before the addition of β-met. Cr^{3+}−PEPCK preincubated with 143 mM β-met but in the absence of any substrate showed a 35% loss of the Cr^{3+} from PEPCK after 10 min. Cr^{3+}−PEPCK preincubated with 143 mM β-met in the presence of either GDP, OAA, or HCO_3^- showed similar losses of the Cr^{3+} label, an indication of only small amounts of protection against Cr^{3+} removal by these substrates. Almost no protection from Cr^{3+} removal was observed for HCO_3^-. GTP and PEP showed substantial protection against the removal of Cr^{3+}. After 10 min, Cr^{3+}−PEPCK preincu-

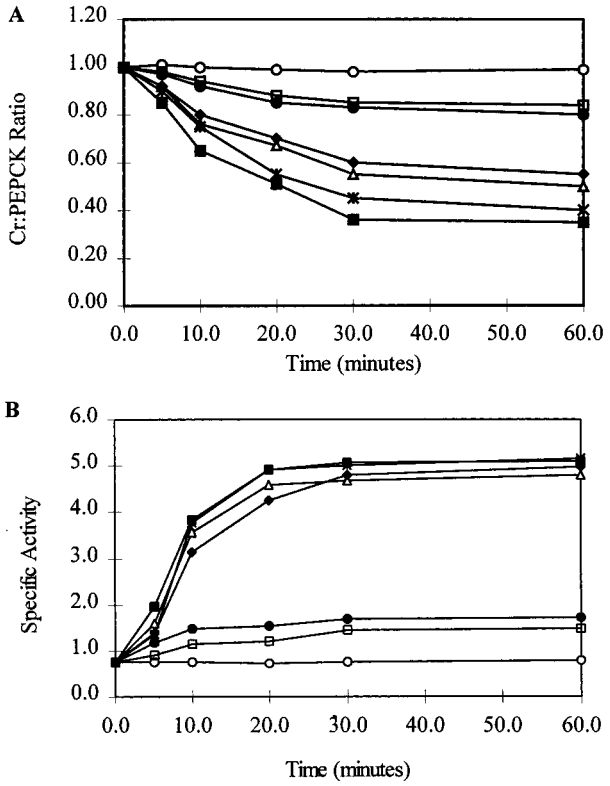

FIGURE 3: Substrate protection against removal of Cr^{3+} from Cr^{3+}−PEPCK. Treatment of Cr^{3+}−PEPCK in 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl with 143 mM β-met restores activity with a concomitant loss of chromium after 30 min. Incubation in the presence of known concentrations of substrates as indicated and 143 mM β-met was studied. At recorded time intervals, aliquots were withdrawn and the Cr^{3+} content and PEPCK activity were determined. The initial (time zero) measurements were made prior to the addition of β-met. (A) A plot of the Cr^{3+} content of PEPCK as a function of time. Cr^{3+}−PEPCK in the absence of substrate or β-met (○) was assayed over time as a control. Cr^{3+}−PEPCK incubated with either 143 mM β-met only (■), 2 mM GDP and 143 mM β-met (△), 2 mM OAA and 143 mM β-met (◆), 200 mM HCO_3^- and 143 mM β-met (*), 2 mM GTP and 143 mM β-met (□), or 2 mM PEP and 143 mM β-met (●). Aliquots of enzyme were removed at various periods of time and assayed for activity. (B) A plot of PEPCK activity as a function of time for the complexes shown in panel A.

bated with 143 mM β-met and either GTP or PEP showed <10% reduction in the amount of Cr^{3+} label.

Figure 3B shows the recovery of catalytic activity upon reduction with β-met in the absence and presence of substrate as a function of incubation time. The initial measurements were made before the addition of β-met. Cr^{3+}−PEPCK preincubated with 143 mM β-met but in the absence of any substrate showed an increase in activity from 0.75 to 3.82 units/mg after 10 min with a k_{react} of 2.10×10^{-1} (see Table 1). Cr^{3+}−PEPCK preincubated with 143 mM β-met but in the presence of either GDP, OAA, or HCO_3^- also showed similar increases in activity. The k_{react} values for PEPCK in the presence of these substrates were only slightly smaller than the k_{react} values in the absence of any substrates (see Table 1), an indication of little protection against Cr^{3+} reduction. In contrast, Cr^{3+}−PEPCK preincubated with 143 mM β-met in the presence of either GTP or PEP showed only a slight increase in activity from 0.75 to 1.1 and 1.5 units/mg, respectively, after 10 min. The k_{react} values for PEPCK in the presence of PEP or GTP are 6−10 times

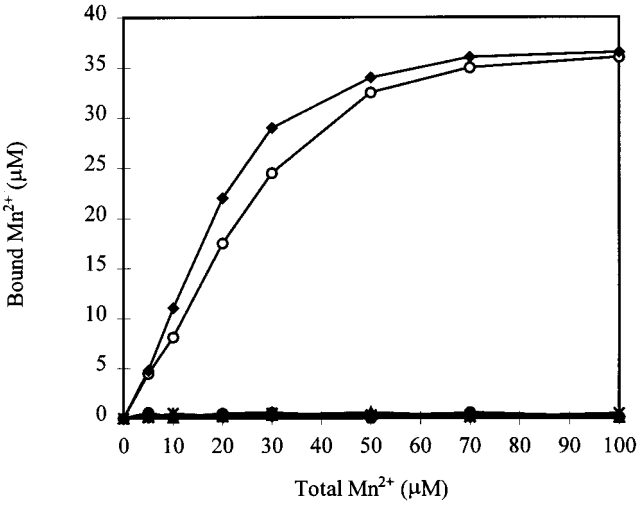

FIGURE 4: EPR titration of Mn^{2+} into native and Cr^{3+}-modified PEPCK solutions. The Mn^{2+} binding curve is determined from separate EPR titrations of Mn^{2+} into 50 μM apo- and Cr^{3+}-modified PEPCK in the presence and absence of PEP. Cr^{3+}−PEPCK samples identified as 1 were prepared using Cr^{2+} and samples identified as 2 were prepared using Cr^{3+} as described in Experimental Procedures. Bound Mn^{2+} is plotted as a function of total Mn^{2+} added. One mole of Mn^{2+} binds to 1 mol of apoPEPCK in the presence or absence of PEP with a K_D of 14 μM (as determined from Scatchard analysis; data not shown). No Mn^{2+} binding to either of the Cr^{3+}−PEPCK solutions was observed. These results indicate that Cr^{3+} is incorporated at the metal binding site. Samples are indicated as follows: apoPEPCK (○), Cr^{3+}−PEPCK-1 (▲), Cr^{3+}−PEPCK-2 (*), PEPCK−PEP (◆), Cr^{3+}−PEPCK−PEP-1 (◇), and Cr^{3+}−PEPCK−PEP-2 (●).

smaller than the k_{react} value for PEPCK in the absence of any substrates, an indication of considerable protection against Cr^{3+} removal from PEPCK. The protection experiments indicate that the substrates GTP and PEP offer significant protection against either the oxidation or reduction of the PEPCK-bound chromium. The protection offered by the other substrates was minimal.

Circular Dichroism Spectra. The CD spectra of apoPEPCK, Mn^{2+}−PEPCK, and Cr^{3+}−PEPCK solutions were measured from 190 to 300 nm. The two PEPCK−metal complexes gave identical spectra and were slightly different from that of apoPEPCK (spectra not shown). On the basis of secondary structure estimations, both Mn^{2+}−PEPCK and Cr^{3+}−PEPCK show a 2% increase in β-sheet content as well as a 2% increase in random coil content, obtained at the expense of α-helical content in apoPEPCK. These observations agree with the previous studies that showed a 3−5% increase in β-sheet content as well as a 3% increase in random coil content at the expense of α-helical content upon metal binding (*4*). No change was observed in the CD spectra of Cr^{3+}−PEPCK upon addition of Mn^{2+} (spectrum not shown). It is important to stress that both Mn^{2+}−PEPCK and Cr^{3+}−PEPCK showed similar changes in their respective CD spectra, indicating that Cr^{3+} is at the Mn^{2+} binding site on PEPCK.

Mn^{2+} Binding to the Cr^{3+}−PEPCK Complex. To determine if the modification of PEPCK by Cr^{2+} or Cr^{3+} was specific, the binding of Mn^{2+} to Cr^{3+}-modified PEPCK was measured by EPR. Both solutions of Cr^{3+}−PEPCK modified by either Cr^{2+} (identified with a 1 in Figure 4) or Cr^{3+} (identified with a 2 in Figure 4), as described in Materials and Methods, were tested. Mn^{2+} was titrated into apoPEPCK

Table 2: Kinetic Parameters for Various Substrates with Native and Cr^{3+}−PEPCK

sample	variable substrate[a]	K'_m (mM)	V_{max} (units/mg)	k_{cat}/K_m (min^{-1} M^{-1})
apoPEPCK (control)	PEP	0.173 ± 0.043	6.02 ± 0.17	2.58×10^6
	IDP	0.121 ± 0.021	5.95 ± 0.09	3.65×10^6
	HCO_3^-	15.0 ± 1.0	5.74 ± 0.08	0.028×10^6
Cr^{3+}−PEPCK (1:1)	PEP	0.129 ± 0.056	0.903 ± 0.023	0.52×10^6
	IDP	0.103 ± 0.033	1.01 ± 0.03	0.73×10^6
	HCO_3^-	40.3 ± 5.1	0.804 ± 0.042	0.0015×10^6

[a] Kinetic assays were performed as described. Mn^{2+} (2 mM) was added to the assay mix. No measurable velocity was observed if additional metal was not added to the assay mix. No β-met was added to the assay mix.

and into Cr^{3+}-modified PEPCK solutions. A Mn^{2+} binding curve is shown in Figure 4. One mole of Mn^{2+} binds to 1 mol of apoPEPCK with a K_D of 32.1 μM. No significant Mn^{2+} associates with the Cr^{3+}−PEPCK complexes. These results suggest that Cr^{3+} is at the Mn^{2+} binding site on PEPCK and the specific binding occurs regardless of the method of Cr^{2+} incorporation.

EPR titrations of Mn^{2+} into apoPEPCK and Cr^{3+}-modified PEPCK were also performed in the presence of 50 μM PEP. These data are also shown in Figure 4. The presence of PEP enhanced Mn^{2+} binding to the apoenzyme ($K_D = 13.6$ μM). No Mn^{2+} binding to the Cr^{3+}−PEPCK complex was observed in the presence or absence of PEP. These results confirm that Cr^{3+} is at the Mn^{2+} binding site on PEPCK.

Kinetic Properties of Cr^{3+}−PEPCK. The order of cation activation for apoPEPCK was determined to be Mn^{2+} (100%) > Co^{2+} (30%) > Cr^{2+} (15%) > Ca^{2+} (4%) > Mg^{2+} (2%). No activity was observed when Cu^{2+}, Fe^{2+}, Zn^{2+}, or Cd^{2+} was the only metal present. These results agree well with previous data that showed that the order of cation activation for apoPEPCK was Mn^{2+} > Co^{2+} > Mg^{2+} (*17*). The order of cation activation for Cr^{3+}−PEPCK was Mn^{2+} (100%) > Co^{2+} (25%) > Cr^{2+} (10%) > Mg^{2+} (3%) > Ca^{2+} (2%) > Cu^{2+} (1%) > Zn^{2+} (0.6%). No activity was observed when Fe^{2+} or Cd^{2+} was added. These results are similar to those observed for Co^{3+}−PEPCK (*4*). It is interesting to note that a greater diversity of metals activate the Cr^{3+}−PEPCK than apoPEPCK. This is due to the presence of Cr^{3+} at the metal site, which prevents the interaction of nonactivating or inhibiting metals with PEPCK. The observed activity of the modified enzyme with these metals is due to the formation of metal−nucleotide complexes as the active form of the nucleotide substrate.

The kinetic properties of apoPEPCK and of Cr^{3+}−PEPCK were studied at pH 7.4. No β-met was used in the assays. The velocity response for all of the substrates follows Michaelis−Menten kinetics. The kinetic constants for the several substrates are given in Table 2. PEP and IDP showed decreased K'_m values for Cr^{3+}−PEPCK in contrast to those for apoPEPCK, suggesting a tighter interaction of these substrates and cofactors with the modified enzyme. The kinetic efficiency with PEP and Mn^{2+}−IDP is 5 times lower with Cr^{3+}−PEPCK than with native PEPCK. The kinetic efficiency with HCO_3^- is 19 times lower with Cr^{3+}−PEPCK than with native PEPCK. These catalytic constants are very similar to those measured for Co^{3+}−PEPCK (*4*). This indicates that modifying PEPCK with either Co^{3+} or Cr^{3+} gives a modified enzyme that is roughly 15% as active as Mn^{2+}−PEPCK. Since there is a significant difference in V_{max} values but only a comparatively smaller change in K'_m values between native PEPCK and Cr^{3+}−PEPCK, the decreased activity is indeed due to the nature of Cr^{3+}−PEPCK.

Table 3: Binding Constants for Nucleotide Binding to ApoPEPCK 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)
apoPEPCK	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
Cr^{3+}−PEPCK	4.2 ± 0.6	8.2 ± 0.9	12.6 ± 1.7	11.9 ± 1.3

[a] Fluorescence assays 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 correction for dilution, the recorded digital fluorescence data were converted to percentage of 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.

Substrate Binding As Determined from Fluorescence. Fluorescence spectra of PEPCK were measured at an excitation wavelength of 297 nm. This was done to ensure that the fluorescence emission arises from tryptophan residues and that any contributions from tyrosine or phenylalanine were negligible.

Mn^{2+} decreases the fluorescence intensity over the entire emission range (spectrum not shown). The fluorescence intensity for Cr^{3+}−PEPCK was decreased over the entire emission range compared to that of apoPEPCK and was similar to the spectrum of Mn^{2+}−PEPCK (spectra not shown). The addition of 50 μM Mn^{2+} to Cr^{3+}−PEPCK causes no additional change. This demonstrates that Cr^{3+} binding to PEPCK causes the same structural changes as Mn^{2+} binding to PEPCK. This agrees with the CD and EPR data (vide supra), and these results are nearly identical to those seen with Co^{3+}−PEPCK (*4*).

GTP, ITP, GDP, and IDP all quench PEPCK fluorescence. All of these substrates induce an approximately 30% decrease in fluorescence intensity. No change in fluorescence intensity was observed with the addition of PEP, OAA, or HCO_3^- to apoPEPCK, Mn^{2+}−PEPCK, Cr^{3+}−PEPCK, or any PEPCK−nucleotide complex. A fit of percentage of quenching versus substrate concentration gave the K_D values for the substrate−enzyme complexes. These data are presented in Table 3. The binding constants for apoPEPCK and Mn^{2+}−PEPCK were from the work reported by Hlavaty and Nowak (*4*). The observed K_D values are between 4 and 14 μM for all enzyme−nucleotide complexes tested. These K_D values are in good agreement with previously obtained binding constants for native PEPCK determined by either PRR or direct binding studies (*2*, *18*). The binding constants for Cr^{3+}−PEPCK are slightly lower than those measured for Co^{3+}−PEPCK (*4*).

PRR Substrate Titrations into Cr^{3+}−PEPCK. No significant change in $1/T_p$ is seen for the Cr^{3+}−PEPCK complex over a period of 24 h, suggesting that there is no conformational change occurring within the Cr^{3+}−PEPCK complex over time. The binary enhancement was determined to be 0.49 for the Cr^{3+}−PEPCK complex.

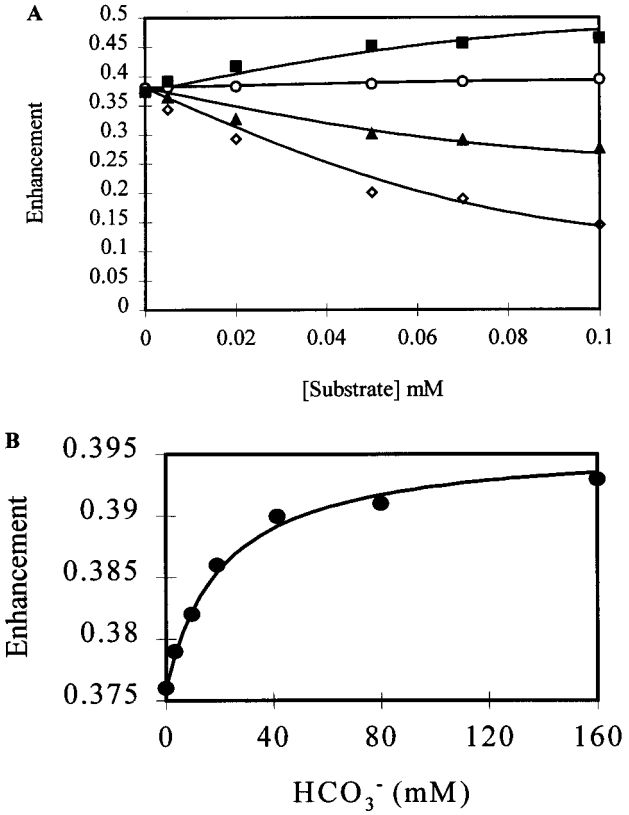

FIGURE 5: PRR substrate titrations into Cr^{3+}−PEPCK. (A) PEP (◇), OAA (○), GTP (▲), and GDP (■) were titrated into 50 μM Cr^{3+}−PEPCK, and the enhancement values were determined from PRR measurements. (B) HCO_3^- (●) was titrated into 50 μM Cr^{3+}−PEPCK in a manner similar to that described for panel A. The curved lines represent the best fits to the experimental data.

Table 4: Binding Constants of Substrates to Cr^{3+}−PEPCK As Determined by PRR[a]

substrate	K_3	ϵ_t
GTP	13.7 ± 0.9 μM	0.230 ± 0.017
PEP	11.5 ± 0.7 μM	0.076 ± 0.009
GDP	14.0 ± 0.9 μM	0.514 ± 0.025
CO_2 (HCO_3^-)	21.3 ± 1.5 mM	0.396 ± 0.021

[a] Substrates were titrated into Cr^{3+}−PEPCK complexes, and enhancement values were measured as described in the text.

Figure 5 shows the PRR titration curves for titration of various substrates into Cr^{3+}−PEPCK. The data were fit using PRRFIT2 as described in Materials and Methods. As shown in Figure 5A, no significant change in enhancement is observed with OAA. A slight increase in enhancement is observed with GDP. A decrease in enhancement is seen with both PEP and GTP. Figure 5B shows the titration profile for CO_2 (treated as HCO_3^-). The profile for CO_2 was exaggerated to show the change in enhancement. For CO_2 (treated as HCO_3^-), an increase in enhancement is seen over the titration range. The overall profile of these curves agrees with the profiles observed for Mn^{2+}−PEPCK (*18*). It should be noted that the change in enhancement values is small but reproducible in each case. This is due to the starting enhancement value of 0.49 for the Cr^{3+}−PEPCK binary complex. The titration results suggest that PEP, GTP, GDP, and CO_2 affect the Cr^{3+} at the PEPCK metal site in a manner analogous to that of Mn^{2+}, further suggesting that Cr^{3+} is at the Mn^{2+} binding site on PEPCK.

The generated curves that fit the PRR data yield K_3 and ϵ_t values. These are presented in Table 4. The K_3 value is the dissociation constant for the dissociation of substrate from the Cr^{3+}−PEPCK complex. The ϵ_t is the final enhancement value of the Cr^{3+}−PEPCK complex with saturating substrate. The K_3 (or K_D) values obtained for dissociation of the substrates from the Cr^{3+}−PEPCK complex agree with the values obtained from the fluorescence studies discussed above. The binding constant values for GTP, PEP, and GDP are between 11 and 14 μM. The binding constant for CO_2 (treated as HCO_3^-) is 21.3 mM.

DISCUSSION

Reports of stable Cr^{3+}−protein complexes (*7*−*9*, *11*, *12*) have suggested the potential utility of Cr^{3+} as a probe of the PEPCK metal activator site. The paramagnetic properties of Cr^{3+} combined with its preference for oxygen and nitrogen ligand donors and its inertness to ligand substitution reactions render Cr^{3+} a potentially valuable probe for exploration of the PEPCK metal site. Since Mn^{2+} is the most activating cation for PEPCK, several assumptions can be made about the metal site on PEPCK. Mn^{2+} is an octahedral, six-coordinate ion that favors oxygen ligands. This selectivity indicates that PEPCK would be suitable for Cr^{3+} modification, since Cr^{3+} prefers an octahedral arrangement and has a high preference for oxygen and nitrogen ligands. Furthermore, the success of creating a stable Co^{3+}−PEPCK complex (*4*) suggested that a stable Cr^{3+}−PEPCK complex could also be formed.

Nothing is known about the activating properties of Cr^{2+} on PEPCK. If Cr^{2+} did not activate PEPCK, then it was possible that chromium may not interact at the Mn^{2+} binding site on PEPCK. Under anaerobic conditions, Cr^{2+} is a good activating cation for PEPCK. The PEPCK activity with Cr^{2+} was approximately 15% of that of Mn^{2+}−PEPCK. This makes it the third highest activating cation for PEPCK, following Mn^{2+} and Co^{2+}.

Incubating PEPCK with low concentrations of Cr^{2+} followed by exposure to atmospheric O_2 at pH 7.4 and subsequent gel filtration to remove excess metal results in the stoichiometric incorporation of Cr^{3+} into PEPCK after 2 min (Figure 1). The enzyme is partially inactivated with a k_{inact} of 7.27×10^{-1} min^{-1}. Similarly, incubating PEPCK with low concentrations of Cr^{3+} for a longer time period also resulted in stoichiometric incorporation of Cr^{3+} into PEPCK. The formation of a catalytically active Cr^{3+}−PEPCK complex is demonstrated by the analytical determination of stoichiometric incorporation of Cr^{3+} into PEPCK with retention of approximately 15−20% of the activity compared to unmodified PEPCK. To the best of the our knowledge, this is the first report of the formation of an active Cr^{3+}−enzyme complex. All previous reports about the formation of a Cr^{3+}−protein complex indicated that the modified protein was inactive (*7*−*9*, *11*, *12*). The formation of a catalytically active Cr^{3+}−PEPCK suggests that the catalytic process occurs in the second coordination sphere of the PEPCK-bound metal. This is consistent with the previous proposal based on ^1H and ^{31}P nuclear relaxation rate studies (*2*, *3*) that substrates bind in an outer-sphere complex to the PEPCK metal. These results are also consistent with the observation of a catalytically active Co^{3+}−PEPCK complex (*4*).

Cr^{3+}, unlike Co^{3+}, does not require a careful regulation of its coordination environment. Cr^{3+} can readily accept both oxygen and nitrogen ligands in its coordination sphere. Since Cr^{2+} is easily oxidized to Cr^{3+} by simple exposure to atmospheric O_2, no harsh oxidizing conditions are required to form a Cr^{3+}−enzyme complex. It would appear that Cr^{3+} modification of proteins has an advantage over Co^{3+} modification. One limitation of in situ Cr^{3+} modification is its nonspecific incorporation into proteins. It was observed here that, with alteration of the conditions of the modification process, with reduction of the starting concentrations of either Cr^{2+} or Cr^{3+}, a stoichiometric Cr^{3+}−PEPCK complex can be formed.

Protection from Cr^{3+} incorporation into PEPCK was not observed by any substrate. This contrasts with the results seen with Co^{3+}−PEPCK, where GTP, ITP, and PEP all protected against cobalt incorporation into PEPCK. Protection from Cr^{3+} modification of PEPCK was observed with Mn^{2+}, Mg^{2+}, and Co^{2+}. This indicates that Cr^{3+} is at the metal site of the enzyme. Protection from Cr^{3+} removal from PEPCK is seen with GTP and PEP. Little protection from Cr^{3+} reduction was seen with GDP, OAA, or CO_2 (treated as HCO_3^-). The substrates that offer protection against Cr^{3+} removal from PEPCK 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 interacts with the enzyme-bound metal in such a way that it protects against β-met accessibility to Cr^{3+} at the metal binding site of PEPCK. These protection studies agree with the results found for Co^{3+}−PEPCK (*4*).

Cr^{3+} modification of PEPCK was determined to be specific as no Mn^{2+} binding to Cr^{3+}−PEPCK in the presence or absence of PEP was observed by EPR measurements. These results were confirmed by CD and fluorescence studies where Cr^{3+}−PEPCK gave spectral properties very similar to those for Mn^{2+}−PEPCK and unlike those of apoPEPCK.

Cr^{3+}−PEPCK has properties similar to those of Co^{3+}−PEPCK. Both enzymes retained approximately 15% of the activity compared to Mn^{2+}−PEPCK, and both modifications can be reversed by treatment with 143 mM β-met. Cr^{3+}−PEPCK has CD and fluorescence spectra similar to those of Co^{3+}−PEPCK, and the kinetic and binding constants were comparable for both modified enzymes. These similar features suggest that both exchange-inert metals "lock" PEPCK into a specific conformation.

Cr^{3+}−PEPCK requires an additional metal for activity and follows Michaelis−Menten kinetics. With the exception of HCO_3^-, the substrates all showed a slight decrease in K'_m values compared to those of Mn^{2+}−PEPCK (Table 2), although all values are within error of those of apoPEPCK. The requirement for additional metal for catalytic activity with Cr^{3+}−PEPCK suggests that free nucleotide is not a substrate for Cr^{3+}−PEPCK. The additional metal requirement for Cr^{3+}−PEPCK confirms that metal−nucleotide is the proper form of the substrate. Lee et al. (*23*) proposed that the second metal is in a β,γ-bidentate coordination with the nucleotide. The role of the second metal may be to 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.

The binding constants for GTP, ITP, GDP, and IDP were similar for both apoPEPCK and Cr^{3+}−PEPCK, as determined from fluorescence. The K_D values were between 4 and 13 μM for all nucleotides. These values agree with the results of previous fluorescence, PRR, and direct binding studies (*2*, *4*, *17*) that gave binding constants of <2 to 14 μM for all nucleotides. No fluorescent quenching by PEP, OAA, or HCO_3^- of the apoPEPCK, Mn^{2+}−PEPCK, Cr^{3+}−PEPCK, or PEPCK−GTP complexes was observed. The lack of PEPCK fluorescence quenching by these substrates agrees with the results performed on the rat liver cytosol, *Escherichia coli* or *Saccharomyces cerevisiae* forms of PEPCK that showed that only nucleotide complexes quench PEPCK fluorescence (*24*). The K_D values measured in this study agree with those measured for similar complexes with other species of PEPCK (*24*). The similarity of the K_D values for Cr^{3+}−PEPCK and Mn^{2+}−PEPCK suggests that substrates interact with Cr^{3+}−PEPCK in a fashion similar to that of Mn^{2+}−PEPCK.

The paramagnetic properties of Cr^{3+} offered the possibility of exploring substrate interactions with Cr^{3+}−PEPCK using PRR techniques. The binary enhancement for Cr^{3+}−PEPCK was determined to be 0.49. This value is considerably lower than that for Mn^{2+}−PEPCK which has a binary enhancement near 12.2 (*17*). The low binary enhancement for Cr^{3+}−PEPCK indicates that τ_s, the electron relaxation time of Cr^{3+}, dominates for this complex as opposed to τ_r, the rotational correlation time. The relaxation is probably due to outer-sphere solvent as the ligated water is expected to be in slow exchange. Free Cr^{3+} gives a much stronger PRR response. The binding constants obtained for the substrates for Cr^{3+}−PEPCK using PRR techniques agree well with those from the fluorescence studies discussed above and with all previous reports (*4*, *17*). The paramagnetic properties of Cr^{3+} also offer the possibility of determining the distance between the enzyme-bound metal and the nucleotide-bound metal at the active site of PEPCK. These studies have been performed (manuscript in preparation).

In general, it appears that PEPCK prefers "hard" metal cations, which are small, have a high charge, and are not readily polarized, as activators. Mn^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}, Co^{3+}, and Cr^{3+} are all hard or "borderline hard" metals that activate PEPCK. "Soft" or "borderline soft" metals, such as Cd^{2+}, Zn^{2+}, Fe^{2+}, Ni^{2+}, Pb^{2+}, and Pt^{2+} do not activate or are poor activators of PEPCK. This may be due to the nature of the metal ligands furnished by PEPCK. Hlavaty and Nowak (*4*, *13*) showed that Asp295 and Asp296 may provide ligands to the enzyme-bound metal. H_2O, OH^-, and ROH are hard ligands. The stability of the Cr^{3+}−PEPCK and Co^{3+}−PEPCK complexes suggests that nitrogen may also be a metal ligand. These ligands may come from lysine, tryptophan, or histidine residues or amide nitrogens from the polypeptide backbone. Correspondingly, both NH_3 and RNH_2 are hard or borderline hard ligands. On the basis of this premise, soft ligands such as RSH, RS^-, CO, and R_2S can be discounted. This would eliminate the proposal for cysteine and methionine residues or carbonyl oxygens from the polypeptide backbone as potential ligands for the enzyme-bound metal.

In conclusion, the Cr^{3+} derivative of PEPCK has been prepared and characterized. The usefulness of the Cr^{3+} probe lies in its exchange-inert, paramagnetic properties. The Cr^{3+} derivative of PEPCK and the Co^{3+} derivative were used for further studies examining the structure and roles of the second metal binding site of PEPCK.

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