Affinity Cleavage at the Metal-Binding Site of Phosphoenolpyruvate Carboxykinase†

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Received March 13, 1997; Revised Manuscript Received August 20, 1997⊗

ABSTRACT: Chicken liver phosphoenolpyruvate carboxykinase (PEPCK) was rapidly inactivated by micromolar concentrations of ferrous sulfate in the presence of ascorbate at pH 7.4. Omitting ascorbate or replacing the Fe^{2+} with Mn^{2+} or Mg^{2+} gives no inactivation. Mn^{2+}, Mg^{2+}, or Co^{2+} at 100-fold molar excess over Fe^{2+} offered complete protection from Fe^{2+}/ascorbate-induced inactivation. The substrates PEP and GTP, but not OAA, GDP, or CO_2, offered full protection from inactivation. The addition of 5 mM EDTA stopped further inactivation of the enzyme. Thermodynamic studies indicate that the inactive enzyme no longer binds Mn^{2+} but still had high affinity for GTP indicating that the inactivation process was specific for the metal site. A decrease in cysteine content was observed over time following PEPCK treatment with Fe^{2+} and ascorbate. The apparent first-order rate constant for free sulfhydryl loss (0.085 ± 0.005 min^{-1}) is similar to the apparent first-order rate constant for inactivation (0.067 ± 0.005 min^{-1}). Amino acid composition analysis revealed that cysteic acid was generated upon Fe^{2+}/ascorbate addition to PEPCK. Native chicken liver PEPCK has an M_r of 67 kDa. SDS−PAGE of the inactivated enzyme showed the presence of two new bands at 31.7 and 35.3 kDa indicating that PEPCK was specifically cleaved at a single site. The rate of cleavage was slower than the rate of inactivation and fully inactivated enzyme was only 50% cleaved. The Fe^{2+}/ascorbate-catalyzed inactivation was not solely due to protein cleavage. The protein fragments generated by cleavage were separated by C4 reverse phase HPLC. The cleavage exposed a new N-terminus which was identified to be the 35.3 kDa C-terminal half of PEPCK. Sequencing of the fragments indicated that the site of cleavage was between Asp296 and Ile297. These results indicate that Asp296 is involved in metal chelation. This agrees with previous studies [Hlavaty, J. J., & Nowak, T. (1997) *Biochemistry 36*, 3389−3403] that suggested that Asp295 and Asp296 are involved in metal binding.

Chicken liver mitochondrial phosphoenolpyruvate carboxykinase [GTP/ITP: oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] (PEPCK)[1] 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 67 kDa monomeric enzyme in higher organisms is the catalysis of PEP formation 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 yet been reported due to the inability to crystallize the enzyme. A recent diffraction study of crystals of the *Escherichia coli* PEPCK has been reported (Matte *et al*., 1996).

PEPCK, as with all known kinases, has 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 that 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.

Recently, the formation of an active Co^{3+}-PEPCK complex was reported (Hlavaty & Nowak, 1997). The Co^{3+}-modified enzyme was proteolytically digested, and the cobalt-containing peptide was purified using reverse phase HPLC. The digest produced a single pure peptide that contained cobalt. Mass, amino acid composition, and sequence analyses identified the peptide as amino acids Thr276−Lys301. This highly conserved region is located near the central portion

† Research supported by NIH Grant (DK17049) to T.N. and the Graduate Assistance in Areas of National Needs Program Fellowship (Project Award Number P200A20261) to J.J.H.

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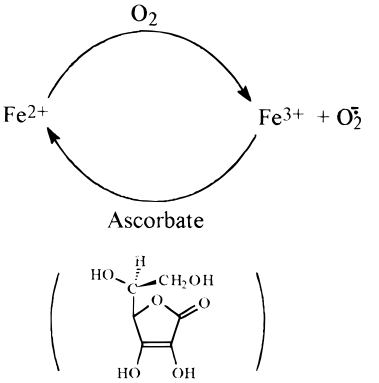
⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1997.

[1] Abbreviations: AA, atomic absorption; β-met, 2-mercaptoethanol; DEAE, diethylaminoethyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; GSH, glutathione; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix assisted laser diode induced−time of flight mass spectroscopy; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PRR, water proton longitudinal relaxation rate; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

S0006-2960(97)00574-6 CCC: $14.00

of PEPCK and is near the putative nucleotide binding region (Weldon *et al*., 1990). Amino acids Asp295 and Asp296 are the only feasible metal ligands in this peptide, discounting backbone carbonyl groups as ligands. Both residues were proposed as ligands to the PEPCK-bound cation (Hlavaty & Nowak, 1997).

Fenton chemistry, using transition metals to catalytically oxidize chelating ligands with deleterious effects, has been employed to explore the reactions of ligand−DNA complexes and more recently, of metal−protein complexes. This class of chemical probes was designed specifically for DNA footprinting studies. Sigman *et al*. (1990) developed these "chemical nucleases" in which 1,10-phenanthroline-ferrous is tethered to a DNA-binding drug to provide nucleotide sequence specificity for the scission of nucleic acids. Schepartz and Cuenoud (1990) and Hoyer *et al*. (1990), have applied these techniques to investigate the active sites of proteins using reagents that specifically cleave enzymes at their binding sites. To be effective, these reagents must have a particular affinity for the enzyme and a redox-capable metal ion that will oxidatively cleave the polypeptide backbone. Recent studies indicate that Fe^{2+} causes oxidative cleavage (Soundar & Colman, 1993; Wei *et al*., 1994) of proteins. The metal-catalyzed oxidation generates reactive species ($O_2^{\bullet -}$ or $OH^{\bullet}$) through Fenton chemistry (eq 2) (Fenton, 1894). The reactive species can interact with nearby susceptible amino acid residues and can specifically cleave the polypeptide backbone at the site where reactive species are generated.



(2)

Wei *et al*. (1994) demonstrated that the metal ligands of malic enzyme could be identified by treating the enzyme with Fe^{2+} and ascorbate. Malic enzyme was inactivated and specifically modified and cleaved. The resulting peptides were separated and sequenced to identify the metal binding site. Similar work was performed on the proteins glutamine synthetase (Farber & Levine, 1986; Liaw *et al*., 1993), staphylococcal nuclease (Ermácora *et al*., 1992), isocitrate dehydrogenase (Soundar & Colman, 1993), and Tet repressor (Ettner *et al*., 1995). In each case, the modification is highly selective for the metal binding site of the protein.

The work presented shows that, in the presence of ascorbate, avian liver PEPCK is very sensitive to ferrous ion oxidation. The modified enzyme was found to be selectively cleaved at the Mn^{2+} binding site. Separation and sequencing of the peptides allowed for the identification of the metal ligands of the protein.

MATERIALS AND METHODS

Materials. Malate dehydrogenase was purchased from Boehringer Mannheim Corporation. GTP, IDP, PEP, NADH, OAA, and tetramethylammonium sulfate were purchased from Sigma. $CoCl_2$, EDTA, $FeSO_4$, and Tris base were purchased from Mallinckrodt. $MnCl_2$, $MnSO_4$, $MgCl_2$, $MgSO_4$, thiourea, glycerol, D-mannitol, and *tert*-butyl alcohol were from Fisher. 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 by a modification of the procedure of Lee and Nowak (1984). Mitochondrial isolation, lysis and ammonium sulfate fractionation were done as previously described. After ammonium sulfate precipitation, the protein was resuspended 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 washing, a 1 L gradient from 1.5 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 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 and was >95% pure.

PEPCK Assay. The PEPCK-catalyzed reaction of PEP carboxylation to OAA 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 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. PEPCK concentration was determined by absorbance at 280 nm [$\epsilon_{1\%}^{280} = 16.5$ $(mg/mL)^{-1}$ (Lee *et al*., 1981)].

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.

Fe^{2+}/Ascorbate Modification. Ferrous sulfate (20 μM) was added to 2 μM PEPCK, in 50 mM Tris-HCl buffer, pH 7.4. The solution was incubated for 15 min on ice. Ascorbate (4−400 μM) was then added into the solution. Enzyme inactivation was monitored by removing enzyme at selected time intervals and assaying for enzymatic activity. Protection against inactivation was determined by incubating the enzyme with various metals before the addition of iron and ascorbate.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. 15% SDS−PAGE was performed on the inactive or partially inactive enzyme samples. Electrophoresis was carried out at 20 mA at room temperature for 30−60 min.

MALDI-TOF Measurements. Samples of untreated and Fe^{2+}/ascorbate-treated PEPCK were subjected to MALDI-TOF analyses. Before MALDI-TOF measurements could be made on the Fe^{2+}/ascorbate-treated PEPCK sample, it was necessary to remove Fe^{2+} from the samples by using a Filtron 10K Microsep centrifugal concentrator. The concentrator was centrifuged at 6000 rpm for 30 min. After this time, 2 mL of Tris-HCl, pH 7.4, was added and the concentrator was spun for an additional 30 min. This process was repeated three times. The removal of Fe^{2+} from the sample was verified by AA measurements.

Cysteine Determinations. Cysteine determinations were done using the modification of the method described by Habeeb (1972). Approximately 1 nmol of apoPEPCK or Fe^{2+}/ascorbate-treated PEPCK, in a volume of 10 μL, was added to a cuvette containing 1.0 mM DTNB in 4 M guanidine-HCl, 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 with PEPCK was calculated. Absorbance values for controls, containing all reagents except enzyme, were subtracted from the observed experimental values.

Mn^{2+} Binding to Fe^{2+}/Ascorbate-Treated PEPCK. The binding of Mn^{2+} to *Fe^{2+}*/ascorbate-treated 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), and an outline of the method was recently presented (Hlavaty & Nowak, 1997).

Mn^{2+} binding to Fe^{2+}/ascorbate-treated 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 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.

Prior to all PRR and EPR measurements, all Fe^{2+} and ascorbate were removed from the enzyme samples by using a Filtron 10K Microsep centrifugal concentrator. The concentrator was centrifuged at 6000 rpm for 30 min. After this time, 2 mL of Tris-HCl was added and the concentrator was spun for an additional 30 min. This process was repeated three times. The final concentration of enzyme was 50 μM. PRR and EPR measurements on the inactive or partially inactive enzyme samples were taken at room temperature and the enzyme samples were kept at pH 7.4.

Substrate Binding As Determined from Fluorescence. The 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. Fluorescence scans of apoPEPCK or Fe^{2+}/ascorbate-treated PEPCK were run at various time intervals. An excitation wavelength of 297 nm was used to excite the tryptophan residues in PEPCK, and the samples were scanned from 300 to 400 nm.

To determine the GTP binding constants, samples of 10 μM apoPEPCK and Fe^{2+}/ascorbate-treated PEPCK were prepared in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM KCl. Using a 1 mL quartz cell, the samples were separately 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 (accounting for dilution). The recorded digital fluorescence data was converted to % 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). Titrations were performed in duplicate.

HPLC Separation of the Cleavage Fragments. Urea (6 M) was added to 1 mg of the Fe^{2+}/ascorbate inactive PEPCK solution. The mixture was incubated at 90 °C for 30 min. After heating, enough 25 mM Tris-HCl, pH 7.4, was added to the tube to dilute the urea concentration to 2 M. This process was also performed using unmodified enzyme as a control. The peptides were separated using a C4 reverse phase Vydac (4.6 × 250 mm) column with a 100 Å pore size. The peptides were eluted with the following gradient: 99.9% water with 0.1% trifluoroacetic acid for 10 min; 0%−99.9% acetonitrile in 0.1% trifluoroacetic acid wash for an additional 50 min followed by a 99.9% acetonitrile in 0.1% trifluoroacetic acid wash for 10 min. The flow rate was maintained at 0.8 mL/min throughout the entire run. Peptides were detected at 215 nm for all runs. Collected peptides were lyophilized and sequenced.

RESULTS

Fe^{2+}/Ascorbate Inactivation of PEPCK. The addition of $FeSO_4$ (40 μM) in the presence of ascorbate (400 μM) to PEPCK (2 μM) causes a rapid and irreversible inactivation within 30 min at 25 °C in 50 mM Tris-HCl, pH 7.4 (Figure

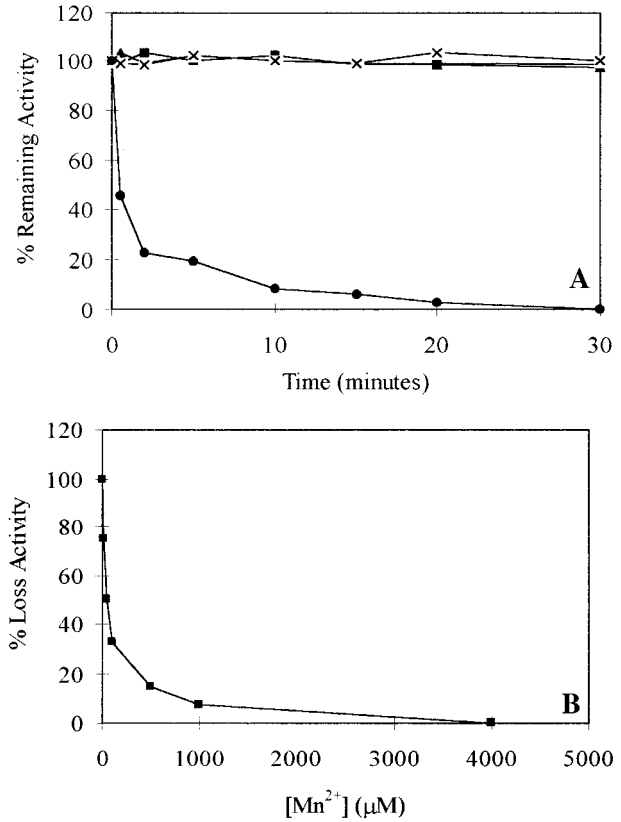


FIGURE 1: Fe^{2+}/Ascorbate treatment of PEPCK. (A) Fe^{2+}/ascorbate inactivation of PEPCK. PEPCK (2 μM) was incubated with 40 μM Fe^{2+} and 400 μM ascorbate, and samples were removed at the indicated time periods and assayed for activity (●). The same treatment was performed with 4 mM Mn^{2+} (■), Mg^{2+} (▲), or Co^{2+} (×) prior to addition of Fe^{2+} and ascorbate. (B) Protection of PEPCK by Mn^{2+} against Fe^{2+}/ascorbate-catalyzed inactivation. PEPCK (2 μM) was incubated with the indicated amount of Mn^{2+}, 40 μM Fe^{2+}, and 400 μM ascorbate for 30 min, and samples were assayed for activity.

1A), with an apparent first-order inactivation rate constant of 0.067 ± 0.005 min^{-1}. Replacing ferrous sulfate with manganese sulfate or magnesium sulfate or removing ferrous sulfate did not lead to inactivation. In the absence of ascorbate, even at high concentrations (2 mM) of Fe^{2+}, no inactivation was observed. The addition of 20 mM H_2O_2 to the inactivation mixture increased the apparent first-order inactivation rate constant to 0.162 ± 0.033 min^{-1} and led to nonspecific deleterious damage to PEPCK as determined by gel electrophoreses (data not shown). Additional peptide fragments were observed when H_2O_2 was present. H_2O_2 was not used in further inactivation. Inactivation was also examined under anaerobic conditions. N_2 was bubbled through a solution of PEPCK (2 μM) in 50 mM Tris-HCl and 100 mM KCl, pH 7.4 for approximately 30 min. This was done to displace O_2 from the solution. $FeSO_4$ (40 μM) and ascorbate (800 μM) were quickly added to the enzyme solution. After mixing, N_2 continued to bubble through the enzyme solution for an additional 30 min. Activity was subsequently measured. No loss of PEPCK activity following Fe^{2+}/ascorbate treatment was observed as compared to untreated controls under aerobic conditions. The endogenous oxygen dissolved in solution is not only sufficient but required for inactivation under experimental conditions.

Protection of PEPCK against Fe^{2+}/Ascorbate-Induced Inactivation. Incubating PEPCK with either 4 mM Mn^{2+}, Mg^{2+}, or Co^{2+} prior to treatment with Fe^{2+} and ascorbate

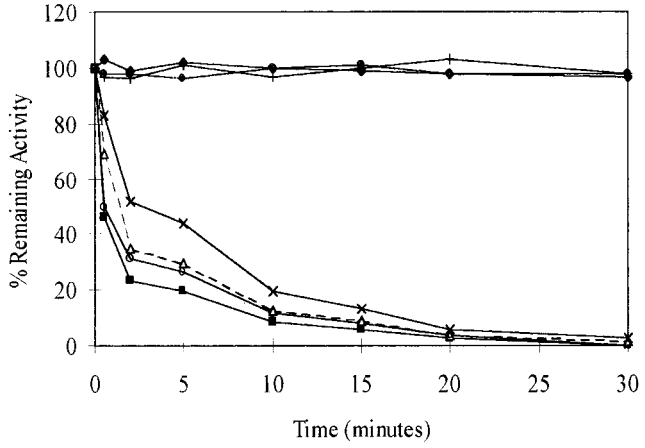


FIGURE 2: Substrate protection against Fe^{2+}/ascorbate inactivation of PEPCK. PEPCK incubated in the presence of 400 μM ascorbate (◆) has no activity loss over the indicated time intervals. PEPCK in the presence of 40 μM Fe^{2+} and 400 μM ascorbate (■) was inactivated within 30 min. PEPCK was treated as in Figure 1, but the incubation mixtures also contained 25 μM PEP (●), 25 μM GTP (+), 2 mM OAA (△), 2 mM GDP (×), or 200 mM HCO_3^- (○). Samples were assayed as previously described.

offered protection (Figure 1A). This suggests that the inactivation process specifically occurs at the metal binding site on PEPCK. Lower concentrations of Mn^{2+} also provided substantial, but reduced, protection (Figure 1B). Half-maximal protection was observed at 50 μM Mn^{2+}. This is in excellent agreement with the K_D value (35−50 μM) for Mn^{2+} to PEPCK (Hebda & Nowak, 1982). Addition of 5 mM EDTA (pH 7.4) also afforded 100% protection. The addition of 5 mM EDTA to a partially modified enzyme also stopped further inactivation, providing the opportunity to quench the reaction at various time intervals and allowing for the exploration of time dependent inactivation and cleavage rates.

Protection from Fe^{2+}/ascorbate-induced inactivation was also observed by the substrates PEP and GTP (Figure 2). For protection in the presence of GTP, Fe^{2+} concentration was increased to 200 μM to offset the effect of any GTP−Fe^{2+} complex formation and to ensure the presence of free Fe^{2+}. No protection from inactivation was observed by GDP, OAA, or CO_2 (treated as HCO_3^-). 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 ascorbate accessibility to Fe^{2+} at the metal binding site of PEPCK. The substrates PEP and GTP interact with the enzyme-bound metal but in second sphere complexes (Lee & Nowak, 1984; Duffy & Nowak, 1985; Hlavaty & Nowak, 1997).

Selective Protein Cleavage of Fe^{2+}/Ascorbate-Inactivated PEPCK. PEPCK inactivated by Fe^{2+}/ascorbate was subjected to 15% polyacrylamide gel electrophoresis in the presence of SDS to examine the possible oxidative cleavage of the polypeptide chain. Native PEPCK has a M_r of 67 kDa (Weldon *et al*., 1990). The inactivated enzyme clearly shows the presence of three bands, one at 67 kDa indicating undigested protein and two new bands at 31.7 and 35.3 kDa (Figure 3). This suggests that PEPCK was partially but specifically cleaved at a single site. Densitometry scanning results on this gel indicate that 50% of the fully inactive PEPCK was cleaved. Both the 31.7 and 35.3 kDa fragments equally account for the remaining 50% of the enzyme. The

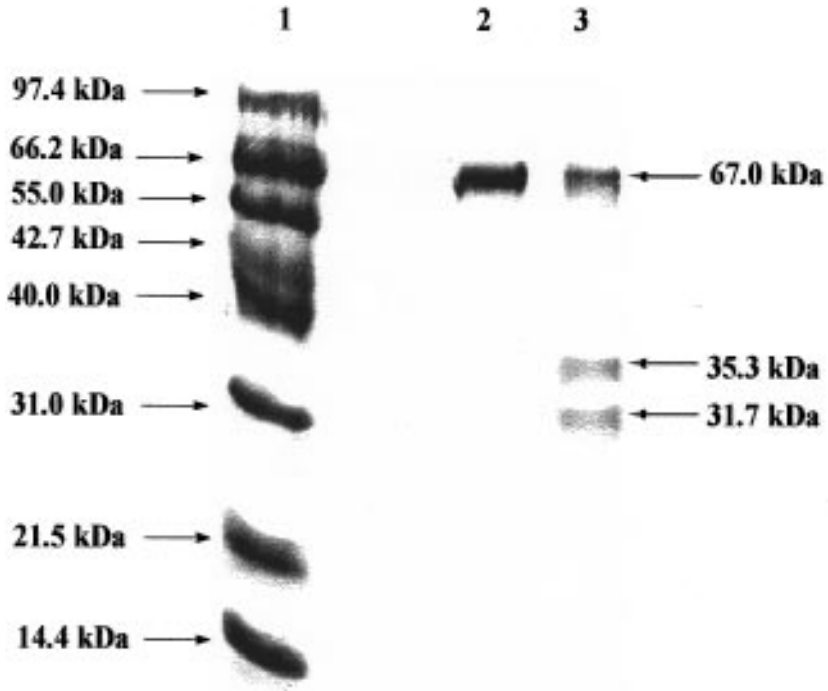


FIGURE 3: 15% SDS−PAGE gel pattern of Fe^{2+}/ascorbate-inactivated PEPCK. Lane 1, molecular weight markers; lane 2, untreated PEPCK; lane 3, modified PEPCK (0% residual activity) following treatment with Fe^{2+}/ascorbate. The presence of two new bands at 31.7 and 35.3 kDa are observed.

presence of undigested enzyme suggests that other oxidation processes besides cleavage must account for enzyme inactivation.

MALDI-TOF mass spectroscopy analyses were performed on Fe^{2+}/ascorbate-treated PEPCK after 30 min. The reaction was stopped by the addition of EDTA. Fe^{2+}, ascorbate, and EDTA were removed from the sample as described under Materials and Methods before analysis. Discounting the doubly charged species (at lower masses), three main peaks are observed at 32 008, 35 023, and 67 050 Da. The 67 kDa peak corresponds to uncleaved PEPCK. The 32 and 35 kDa peaks correspond to the peptides generated from cleavage. These results are in excellent agreement with the SDS−PAGE results shown in Figure 3 and verify that PEPCK is specifically cleaved at a single location following treatment with Fe^{2+} and ascorbate.

Effects of Ascorbate Concentration on Cleavage and Rate of Cleavage. The effect of ascorbate concentration on protein cleavage was investigated by incubating 2 μM PEPCK with 40 μM Fe^{2+} and various concentrations of ascorbate (Figure 4A). The reactions were quenched after 30 min by the addition of 5 mM EDTA and the cleavage products were analyzed by SDS−PAGE. The densitometric analysis was performed by scanning the gel that is shown as an insert in Figure 4A. Maximal cleavage was observed when 400 μM ascorbate was used in the incubation mixture. These results indicate that ascorbate is required for both inactivation and protein cleavage. Ascorbate concentrations higher than 800 μM, but in the absence of Fe^{2+}, caused inactivation of the enzyme (data not shown). This inactivation could be reversed by gel filtration. The inactivation of PEPCK at high concentrations of ascorbate was not investigated further.

The time dependent cleavage of PEPCK by Fe^{2+}/ascorbate was determined by quenching the reaction at various time intervals by the addition of 5 mM EDTA followed by SDS−PAGE separation. Figure 4B demonstrates that the addition of EDTA to an incubation mixture of the enzyme with Fe^{2+} and ascorbate rapidly quenches the metal-catalyzed oxidative inactivation.

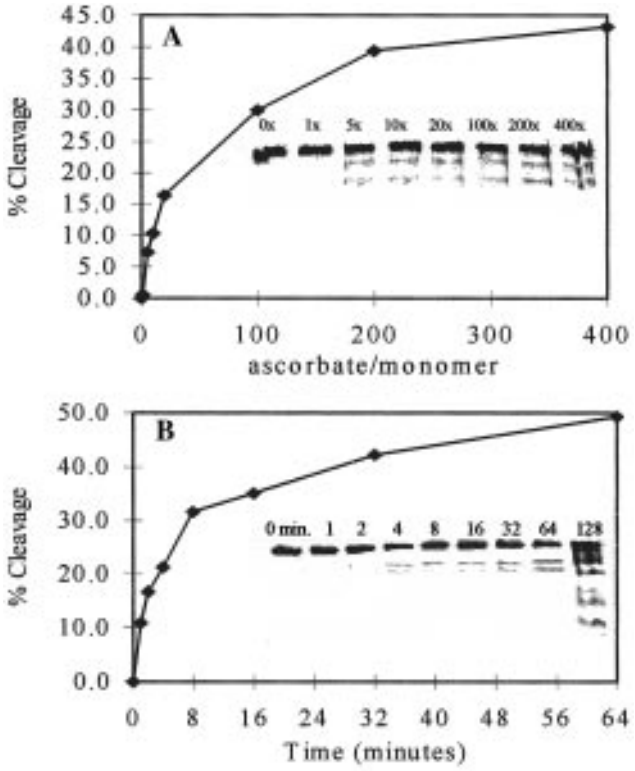


FIGURE 4: (A) Effects of ascorbate concentration on cleavage. PEPCK (2 μM) in the presence of Fe^{2+} (40 μM) was incubated with various concentrations of ascorbate, as indicated by the molar ratios of ascorbate to PEPCK. After 30 min, the reactions were quenched by the addition of 5 mM EDTA and the samples were subjected to SDS−PAGE. (B) Rate of cleavage. PEPCK (2 μM) was incubated in the presence of Fe^{2+} (40 μM) and ascorbate (400 μM). EDTA (5 mM) was added to the samples at the times indicated, and the samples were then subjected to electrophoresis. The gels, which appear as insets in the respective figures, were scanned to quantitate the cleavage.

Table 1: Correlation of Fe^{2+}/Ascorbate-Induced Inactivation of PEPCK and Cleavage of the Metal-Binding Site[a]

time (min)	% remaining activity	relative % amount of uncleaved enzyme	fragment I (35.3 kDa)	fragment II (31.7 kDa)
0	100.0	100	0	0
0.5	46.0	89	5	6
2	23.1	83	9	8
4	19.6	78	11	11
8	8.6	69	16	15
16	5.9	65	18	17
32	0.0	59	21	20
64	0.0	50	25	25

[a] Experimental conditions as described within text. The enzyme samples were incubated for the times indicated. The samples were quenched by addition of 5 mM EDTA, assayed for activity, and subjected to SDS−PAGE. Densitometry scans determined the extent of protein cleavage. Untreated PEPCK was taken as 100%.

The apparent first-order rate constant for cleavage was determined to be 0.019 ± 0.004 min^{-1}. This is nearly three times slower than the apparent first-order rate constant for enzyme inactivation under identical conditions. As shown in Table 1, there is a correlation between enzyme inactivation and peptide bond cleavage, but the rate of cleavage lags behind the rate of activity loss. Furthermore, since only 50% of the fully inactivated enzyme is cleaved (Table 1), this suggests that cleavage is not solely responsible for inactivation and other oxidative damage is occurring to PEPCK. Table 1 also indicates that the relative rates of formation of the two fragments are equal and correspond to the amount of total cleaved enzyme. All inactivation and cleavage rates are first order.

As shown in the inset Figure 4B, by 128 min nonspecific cleavage begins to occur. This suggests that further polypeptide cleavage can only occur after the specific PEPCK cleavage at the metal site. The additional fragments generated by the prolonged exposure of PEPCK to Fe^{2+} and ascorbate were not investigated.

Effects of Substrates and Metal on Protein Cleavage: Similar to the inactivation studies discussed above, the substrates PEP, GTP, OAA, GDP, and CO_2 (treated as HCO_3^-) were examined for their ability to inhibit protein cleavage when PEPCK was exposed to Fe^{2+}/ascorbate. Figure 5A shows that even minimal concentrations (15 μM) of either PEP or GTP fully protect PEPCK from Fe^{2+}/ascorbate-induced cleavage. As with the inactivation studies, Fe^{2+} concentration was increased to 200 μM when GTP was present to offset the effect of any GTP-Fe^{2+} complex formation and to ensure the presence of free Fe^{2+}. The full protection against polypeptide cleavage afforded by PEP and GTP agrees with the full protection these substrates offered against inactivation. OAA, GDP, and HCO_3^- (Figure 5B) offer no protection against Fe^{2+}/ascorbate-induced protein cleavage.

The effect of Mn^{2+} concentration (Figure 5C) on cleavage was also examined. As Mn^{2+} concentration increased, enhanced protection against cleavage was observed. At 4 mM Mn^{2+} (100 molar excess over Fe^{2+}, lane 7 in Figure 5C) complete protection against Fe^{2+}/ascorbate-induced cleavage was observed.

These results concur with the inactivation studies discussed above. The addition of Mn^{2+} to the reaction offers protection against the Fe^{2+}/ascorbate effects. The protection that the substrates PEP and GTP, which are known to specifically affect the environment of enzyme-bound metal, offer against both Fe^{2+}/ascorbate-induced protein inactivation and cleavage indicates that the site of inactivation is specific for the metal binding site of PEPCK.

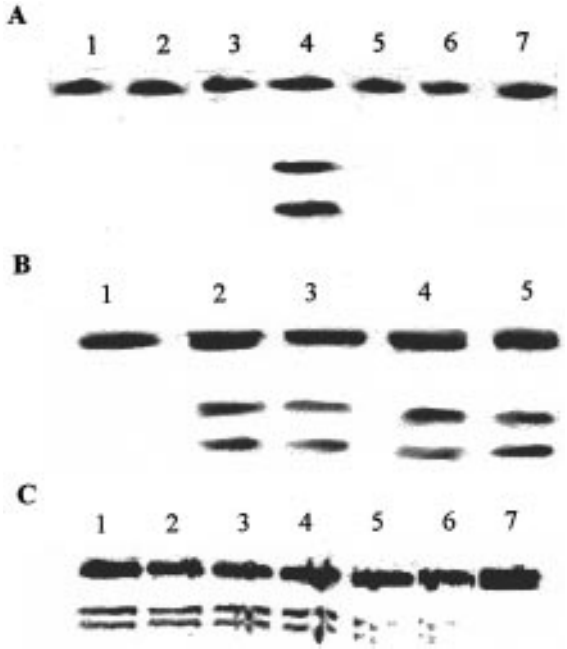


FIGURE 5: Ligand protection studies of Fe^{2+}/ascorbate cleavage. In all cases except for the PEPCK control, PEPCK (2 μM) was incubated in the presence of Fe^{2+} (40 μM) and ascorbate (400 μM). The control was performed in the absence of Fe^{2+}/ascorbate. Additional ligands were added as indicated. After 30 min of incubation, the reactions were quenched by the addition of 5 mM EDTA and the samples were subjected to SDS−PAGE. (A) PEP and GTP effects on cleavage. Lane 1, 15 μM GTP; lane 2, 25 μM GTP; lane 3, 50 μM GTP; lane 4, no added ligands; lane 5, 15 μM PEP; lane 6, 25 μM PEP; lane 7, 50 μM PEP. (B) Effects of OAA, GDP, and CO_2 (treated as HCO_3^-) on cleavage. Lane 1, PEPCK control; lane 2, no added ligands; lane 3, 2 mM OAA; lane 4, 2 mM GDP; lane 5, 200 mM HCO_3^-. (C) Effects of [Mn^{2+}] on cleavage. Lane 1, No Mn^{2+}; lane 2, 10 μM Mn^{2+}; lane 3, 50 μM Mn^{2+}; lane 4, 100 μM Mn^{2+}; lane 5, 1000 μM Mn^{2+}; lane 6, 2000 μM Mn^{2+}; lane 7, 4000 μM Mn^{2+}.

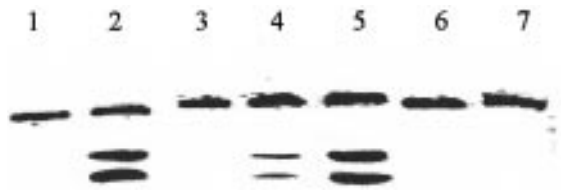


FIGURE 6: Effects of radical scavengers on cleavage. PEPCK (2 μM) was incubated in the presence of Fe^{2+} (40 μM) and ascorbate (400 μM) and 0.5 mM of thiourea, glycerol, D-mannitol, or *tert*-butyl alcohol. After 30 min, the reactions were quenched by the addition of 5 mM EDTA and the samples were subjected to SDS−PAGE. Lane 1, PEPCK control; lane 2, PEPCK + Fe^{2+}/ascorbate; lane 3, 0.5 mM thiourea; lane 4, 0.5 mM *tert*-butyl alcohol; lane 5, 0.5 mM D-mannitol; lane 6, 0.5 mM glycerol; lane 7, 6 M urea-denatured PEPCK.

Denatured PEPCK Is Not Cleaved. Treatment of denatured (with 6 M urea) PEPCK with Fe^{2+}/ascorbate results in no cleavage after 30 min as determined by SDS−PAGE (Figure 6, lane 7). Intact protein is required for the Fe^{2+}/ascorbate-induced inactivation and cleavage processes.

Radical Scavenger Effect on PEPCK Cleavage. The addition of several radical scavengers such as thiourea,

glycerol, D-mannitol, and *tert*-butyl alcohol (at 0.5 mM) to the incubation mixture had varying effects on the inactivation and cleavage rates. Glycerol, D-mannitol, and *tert*-butyl alcohol did not affect normal PEPCK activity. Thiourea causes complete inactivation by 30 min. This suggests that thiourea causes denaturation of the protein within that time frame. Figure 6 shows the effect that these scavengers had on PEPCK cleavage. Thiourea and glycerol afforded full protection from cleavage. The protection against cleavage by thiourea may be explained by the denaturation of PEPCK at this concentration. No Fe^{2+}/ascorbate-induced cleavage occurs with denatured protein (*vide supra*). Reduced but significant protection against cleavage is observed from *tert*-butyl alcohol. D-Mannitol afforded no protection against cleavage. These results suggest that the inactivation and subsequent cleavage processes are due to radical reactions at the active site of PEPCK.

Cysteine Determination. The rate constants of Fe^{2+}/ascorbate-induced PEPCK inactivation and cleavage differ by 3-fold and the fully inactivated enzyme is only 50% cleaved. This suggests that cleavage is not solely responsible for enzyme inactivation. This also suggests that damage or modification to amino acids near or at the active site of PEPCK may lead to the enhanced rate of enzyme inactivation. One amino acid that is susceptible to such modification is cysteine. Native PEPCK contains 13 cysteine residues (Makinen *et al*., 1983; Weldon *et al*., 1990) none of which is in a disulfide bond (Makinen *et al*., 1983). The formation of disulfide bonds or cysteic acid due to a free radical reaction may account for the observed decrease in activity.

To explore this possibility, the determination of free cysteine was performed on apoPEPCK and Fe^{2+}/ascorbate-treated enzyme at various time intervals. Approximately 1 nmol of PEPCK was added to a cuvette containing 1.0 mM DTNB in 4 M guanidine-HCl in a final volume of 0.4 mL. The change in absorbance at 412 nm was measured and the number of moles of thiophenolate anions of 5-thio-2-nitrobenzoic acid released upon the reaction with DTNB with PEPCK was calculated (Habeeb, 1972).

Three separate measurements were made with apoPEPCK and Fe^{2+}/ascorbate-treated enzyme at 16, 32, and 128 min. The absorbance values indicated that 13 free sulfhydryls are present on apoPEPCK. This value remains constant over the time frame examined for untreated PEPCK. In contrast, by 16 min, the number of free cysteine residues determined for the Fe^{2+}/ascorbate-treated PEPCK is reduced to eight. By 30 min, four free cysteine residues are detected on the Fe^{2+}/ascorbate-treated enzyme. After 128 min, almost no free cysteine residues are detected. These results indicate that radical damage is occurring to amino acids on the Fe^{2+}/ascorbate-treated PEPCK other than just at the point of cleavage. This also explains the enhanced rate of protein inactivation over protein cleavage. The apparent first-order rate constant for the free sulfhydryl loss is 0.085 ± 0.005 min^{-1}, which is similar to the apparent first-order rate constant for inactivation (0.067 ± 0.005 min^{-1}). Makinen and Nowak (1989) have located a reactive cysteine in PEPCK, although they suggest that it does not directly participate in catalysis. Perhaps the rapid loss of free sulfhydryl groups in PEPCK accounts for the increased rate of inactivation as compared to the rate of cleavage.

The fate of free sulfhydryl groups in PEPCK upon treatment with Fe^{2+} and ascorbate was examined. It was possible that disulfide bonds formed in PEPCK following treatment with Fe^{2+} and ascorbate. Native PEPCK contains no disulfide bonds. The formation of disulfide bonds could account for not only the loss of free sulfhydryl groups, but the loss of activity as well. Two separate measurements were made with Fe^{2+}/ascorbate-treated enzyme at 16 and 32 min. The reaction was stopped in each case by the addition of EDTA. The samples were then treated with 143 mM β-met, which would reduce any disulfide bonds, for 30 min. The β-met was then removed from the enzyme samples by using a Filtron 10K Microsep centrifugal concentrator. The concentrator was centrifuged at 6000 rpm for 30 min. After this time, 2 mL of Tris-HCl, pH 7.4, was added and the concentrator was spun for an additional 30 min. This process was repeated three times. The samples were tested for free sulfhydryl groups as described above. No change in the amount of free sulfhydryl groups is observed when the Fe^{2+}/ascorbate-treated PEPCK samples were incubated with β-met. This indicates that disulfide bonds are not being formed in the Fe^{2+}/ascorbate-treated PEPCK. The experiments were repeated with DTT with no change in the results.

Another possibility that could account for the loss of free sulfhydryl groups in Fe^{2+}/ascorbate-treated PEPCK is the formation of cysteic acid. The formation of cysteic acid due to a free radical reaction may account for the observed decrease in activity. Cysteic acid can be detected by amino acid composition analysis. Two separate measurements were made with Fe^{2+}/ascorbate-treated PEPCK at 16 and 32 min. The reaction was stopped in each case by the addition of EDTA. Untreated PEPCK was used as a control. The samples were analyzed for amino acid composition. Since the cysteic acid peak elutes early in the HPLC run and often coelutes with aspartic acid, it was difficult to quantitate the amount of cysteic acid formed. Under the experimental conditions tested, the Fe^{2+}/ascorbate-treated PEPCK clearly had a large peak at approximately 1.2 min, indicating the presence of cysteic acid. No such peak was observed in the untreated PEPCK controls. These results show that cysteic acid is formed in PEPCK following treatment with Fe^{2+} and ascorbate. This would account not only for the loss of free sulfhydryl groups but also for the rapid loss of activity.

Tryptophan Fluorescent Measurements. Tryptophan fluorescence was examined for apoPEPCK (control) and Fe^{2+}/ascorbate-treated PEPCK after 16, 30, and 120 min. Tryptophan fluorescence intensity decreases with prolonged exposure to Fe^{2+}/ascorbate. After 16 min, Fe^{2+}/ascorbate-treated PEPCK had 6% remaining activity and a 10% decrease in tryptophan fluorescent intensity at 335 nm. By 30 min, the Fe^{2+}/ascorbate-treated enzyme was completely inactive and had a 40% decrease in tryptophan fluorescent intensity at 335 nm. The apparent first-order rate constant for fluorescent deenhancement is 0.011 ± 0.002 min^{-1}. This apparent first-order rate constant corresponds with the apparent first-order rate constant for cleavage (0.019 ± 0.004 min^{-1}). The decreased tryptophan fluorescence intensity suggests that either radical damage is occurring to tryptophan residues at or near the active site on PEPCK or that the cleavage exposes tryptophan residues to solvent. Radical damage to tryptophan residues may also explain the enhanced rate of inactivation compared to protein cleavage.

GTP and Mn^{2+} Binding to Fe^{2+}/Ascorbate-Treated PEPCK. To determine the specificity of the Fenton reaction with PEPCK, GTP, and Mn^{2+} binding constants were determined

Table 2: GTP Binding Constants to apoPEPCK and Fe^{2+}/Ascorbate-Treated PEPCK[a]

PEPCK complex	% activity	n^b	K_D (μM)	% Q_{max}[c]
apoPEPCK	100	1.0	5.9 ± 0.2	33.2
PEPCK + Fe^{2+}/ascorbate				
after 16 min	5.3	0.9	7.9 ± 0.4	27.6
after 32 min	0	0.8	10.2 ± 0.5	20.8
after 128 min	0	0.0	−	−

[a] GTP binding constants were determined by fluorescence using 10 μM enzyme. [b] "*n*" represents the stoichiometry of GTP to PEPCK extracted from the binding curve. [c] "Q_{max}" represents the fitted maximal % quenching value as described within the text.

Table 3: Mn^{2+} Binding Constants to apoPEPCK and Fe^{2+}/Ascorbate-Treated PEPCK[a]

		EPR		PRR	
PEPCK complex	% activity	n^b	K_D (μM)	n^b	K_D (μM)
apoPEPCK	100	0.98	49.7 ± 3.3	0.88	59.7 ± 2.3
PEPCK + Fe^{2+}/ascorbate					
after 16 min	5.3	0.28	45.3 ± 8.6	0.33	45.2 ± 9.9
after 32 min	0	0.03	39.3 ± 10.3	0.04	43.5 ± 10.3
after 128 min	0	0.00	−	0.00	−

[a] Mn^{2+} binding constants were determined by EPR and PRR techniques using 50 μM enzyme. [b] "*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).

for apoPEPCK and Fe^{2+}/ascorbate-treated PEPCK after 16, 32, and 128 min. GTP binding was measured by fluorescence. Mn^{2+} binding was measured by EPR and PRR. Table 2 lists the binding constants for GTP. Table 3 lists the binding constants for Mn^{2+}. The binding constants for both GTP and Mn^{2+} to apoPEPCK agree well with previously reported results (Hebda & Nowak, 1982; Lee & Nowak, 1984). After 16 min, the Fe^{2+}/ascorbate-treated enzyme had a significant reduction (63%) in the amount of Mn^{2+} binding but only a 10% decrease in GTP binding. As shown by both the cysteine and tryptophan experiments, not all of the inactivation processes appear to occur at the metal site. The loss of PEPCK activity following treatment with Fe^{2+} and ascorbate is due to a combination of the modification of the metal site and a modification of surrounding amino acids near or at the active site of PEPCK. This accounts for the lack of direct correlation between metal binding and activity loss. By 32 min, Mn^{2+} binding was nearly abolished, while only a 20% reduction in GTP binding was observed. The inactive enzyme no longer binds Mn^{2+} but the GTP site remains fundamentally intact. After 128 min, GTP binding could not be measured by fluorescence since fluorescent intensity was low and no additional change in fluorescent intensity was observed. No Mn^{2+} binding was seen by EPR or PRR after 128 min.

HPLC Analysis of the Cleavage Products. The mixtures containing cleaved fragments were separated on a reverse phase HPLC C-4 column. ApoPEPCK was used as a control. Urea (6 M) denaturation was required to separate the cleaved fragments by HPLC, indicating that the cleaved enzyme remains intact. This also explains why little change in GTP binding (*vide supra*) is seen over time.

A distinct difference is observed between the chromatographic profiles for PEPCK and Fe^{2+}/ascorbate-treated PEPCK (Figure 7). ApoPEPCK (Figure 7A) contains only one undigested PEPCK peak (excluding the void volume peak at 4 min) eluting at 37 min. Fe^{2+}/ascorbate-treated PEPCK (Figure 7B) contains three peaks. The peak at 37 min corresponds to undigested protein. As determined from sequence analysis (see Table 4) and MALDI-TOF (*vide supra*), the peak eluting at 21 min corresponds to the 31.7 kDa fragment and the peak eluting at 27 min corresponds to the 35.3 kDa fragment.

Sequence Analysis of the Cleavage Products. The protein fragments generated by the Fe^{2+}/ascorbate inactivation of PEPCK were isolated by reverse phase HPLC and sequenced. As shown in Table 4, the sequence of the first eleven amino acids from the peak eluting at 21 min corresponds to amino acids 1−11 of PEPCK. The peak at 21 min therefore corresponds to amino acids 1−296 of PEPCK, which has a calculated M_r of 32.1 kDa. The sequence of the first ten amino acids from of the peak eluting at 27 min corresponds to amino acids 297−306 of PEPCK. The peak at 27 min therefore corresponds to amino acids 297−607 of PEPCK, which has a calculated M_r of 35.0 kDa. These results demonstrate that the site of cleavage is between Asp296 and Ile297 and indicate that Asp296 is involved in metal ligation in PEPCK.

The third peak in Figure 7B (elution time of 37 min) was also partially sequenced and confirmed to be that of undigested PEPCK.

Attempts to perform C-terminal sequencing on the peptides by treatment with carboxypeptidase A followed by MALDI-TOF mass spectroscopy difference results to determine the amino acid were unsuccessful. It is possible that the C-terminus of these peptides was modified after treatment with Fe^{2+} and ascorbate. The modified C-terminus would not be recognized by carboxypeptidase A thus not allowing for digestion of the peptide. There is also a 1% error associated with MALDI-TOF mass spectroscopy. The large size of the starting peptides may mask any mass differences observed upon digestion of the C-terminus.

DISCUSSION

Avian liver mitochondrial PEPCK binds one mole of Mn^{2+} per mole of enzyme at neutral pH, as determined by EPR and PRR, to form an enzyme−metal complex which is the active form of the enzyme (Hebda & Nowak, 1982b). Kinetic and binding studies show that enzyme-bound Mn^{2+} facilitates the interaction of PEP and nucleotide to the enzyme (Lee *et al*., 1981). Fenton chemistry (Fenton, 1894) techniques were utilized to determine the protein ligands to the metal.

The results presented in this paper indicate that avian liver mitochondrial PEPCK was inactivated and specifically cleaved with Fe^{2+}/ascorbate (Fenton chemistry) within 30 min. The specific cleavage generates a 31.7 and a 35.3 kDa fragment. Other activating divalent cations, such as Mn^{2+}, Mg^{2+}, or Co^{2+}, provide complete protection against inactivation. EPR and PRR studies indicate a substantial decrease in bound Mn^{2+} in the Fe^{2+}/ascorbate-treated PEPCK after 30 min. These results indicate that the inactivation and cleavage processes are specific for the metal-binding site on PEPCK. Fluorescence titration studies show comparatively little change in GTP stoichiometry over the same time period. GTP binding to PEPCK does not have an absolute requirement for Mn^{2+} (Lee & Nowak, 1984).

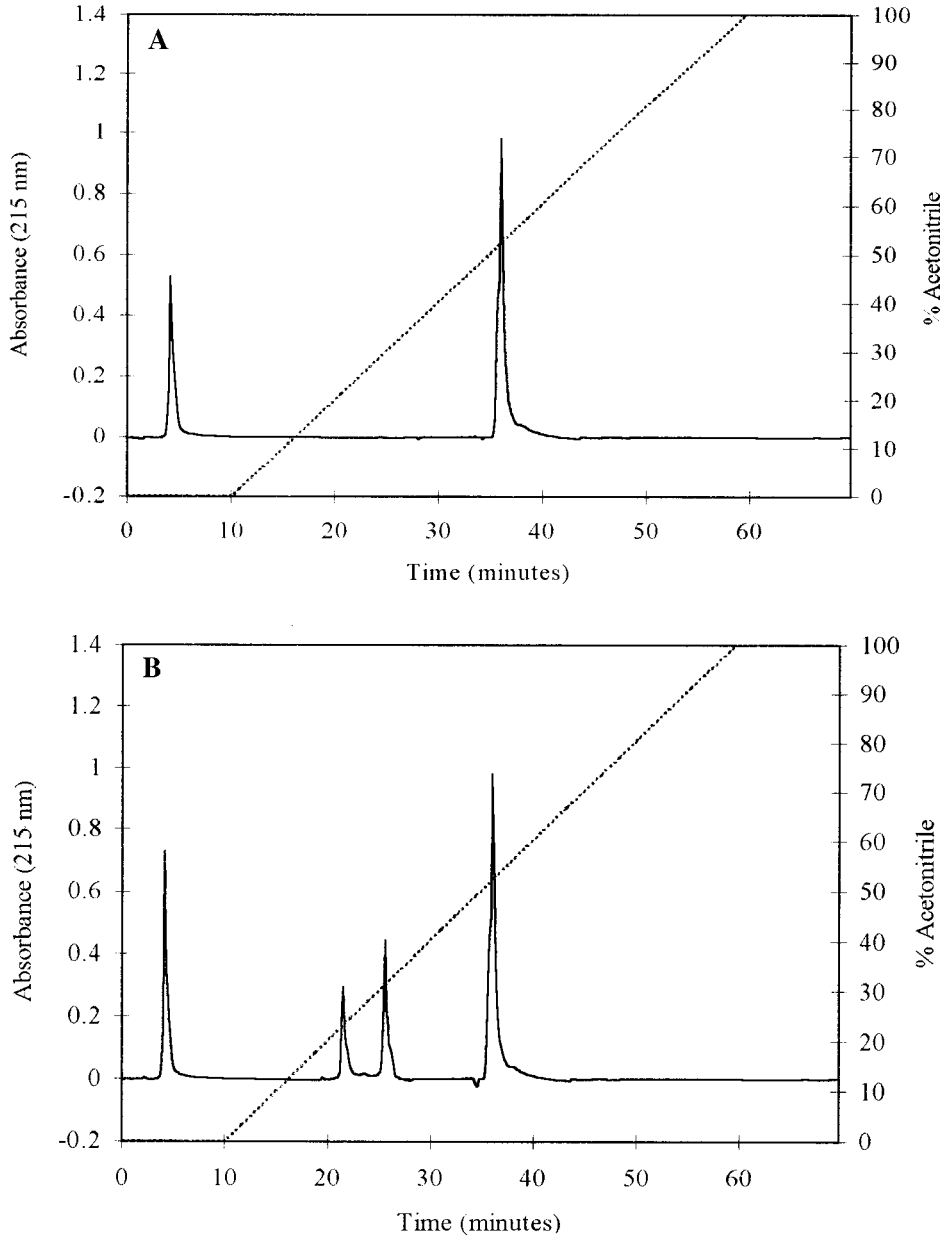


FIGURE 7: HPLC chromatographic profiles of apo- and Fe^{2+}/ascorbate-treated PEPCK. The solid line (—) represents the HPLC chromatographic profile. The dashed line (- - -) represents the acetonitrile gradient. (A) ApoPEPCK was denatured with 6 M urea. A reverse phase C4 HPLC column was used and the protein was eluted with an acetonitrile gradient as indicated. (B) Fe^{2+}/ascorbate-treated PEPCK was eluted in an identical manner to that of apoPEPCK, described above in (A). Twice as much PEPCK was injected here as in A. The apparent peak at 4.5 min is the void volume peak.

Table 4: Amino Acid Sequences of Isolated Peptides from the Fe^{2+}/Ascorbate-Digested PEPCK

	first peak[a]			second peak[b]		
cycle number	amino acid	pmol of amino acid	PEPCK Leu1−Ala11	amino acid	pmol of amino acid	PEPCK Ile297−Gly306
1	Leu	1156.0	Leu	Ile	9091	Ile
2	Ser	179.5	Ser	Ala	5741	Ala
3	Thr	278.7	Thr	Trp	10152	Trp
4	Ser	101.0	Ser	Met	8368	Met
5	Leu	404.1	Leu	Lys	6504	Lys
6	Ser	61.3	Ser	Phe	313	Phe
7	Ala	274.6	Ala	Asp	3336	Asp
8	Leu	211.2	Leu	Asp	2467	Asp
9	Pro	199.0	Pro	Arg	4450	Arg
10	Ala	210.1	Ala	Gly	1028	Gly
11	Ala	88.4	Ala	—	—	—

[a] "First peak" corresponds to the peak that eluted at 21 min as shown in Figure 3B. [b] "Second peak" corresponds to the peak that eluted at 27 min as shown in Figure 3B.

Protection from Fe^{2+}-catalyzed inactivation and cleavage of PEPCK are both generated by GTP and PEP. Little protection was obtained with GDP, OAA, or CO_2 (treated as HCO_3^-). The lack of protection by GDP suggests that the protection by GTP is not due to indirect effects of Fe^{2+}−nucleotide coordination. The substrates (GTP and PEP) that offer protection against Fe^{2+}-catalyzed inactivation and cleavage of PEPCK are those substrates 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−metal such that there is no accessibility of ascorbate to the Fe^{2+} at the metal binding site of PEPCK. The lack of protection by the substrates GDP and CO_2 agrees with PRR studies that showed that GDP and CO_2 do not significantly alter the interaction of solvent water with PEPCK-bound Mn^{2+} (Hebda & Nowak, 1982). These protection studies suggest that the phosphoryl group of PEP or the γ-phosphoryl group of GTP significantly alters the environment about the PEPCK-bound cation. These results agree with previously proposed models that suggest 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; Hlavaty & Nowak, 1997).

Radical scavengers, such as glycerol and *tert*-butyl alcohol, significantly inhibited protein cleavage. This indicates that polypeptide cleavage is the result of radical formation at the metal binding site.

There is only one specific site of cleavage of PEPCK by Fe^{2+}/ascorbate treatment that occurs subsequent to extensive inactivation. This cleavage site was identified as being between Asp296 and Ile297. These results suggest that Asp296 of PEPCK is involved in metal ligation. Aspartate residues are commonly involved in metal binding. Vallee and Auld (1993) found that aspartate predominates in catalytic metal sites where the binding frequency is Asp > His ≫ Glu. The M_r values of the two fragments calculated from amino acid sequence were 32 087 and 35 055 Da, in good agreement with the experimental values estimated from SDS−PAGE and MALDI-TOF mass analyses. The 32 kDa fragment was found to be the N-terminal portion (1−296) of PEPCK and the 35 kDa fragment was the C-terminal end (297−607) of PEPCK.

Nonspecific cleavage was only observed after 128 min of incubation. It appears that the first specific cleavage is required for additional nonspecific cleavage to occur. Non-specific cleavage does not occur with denatured enzyme. The additional cleavage fragments generated after 128 min were not investigated further.

The apparent first-order rate constant for cleavage was more than 3-fold slower than the apparent first order rate constant for inactivation. As shown in Figure 3 (lane 3), only 50% of the fully inactive enzyme was cleaved. It appears that enzyme cleavage is only partially responsible for enzyme inactivation. There are distinct steps in inactivation and cleavage. After Fe^{2+} binds to PEPCK, the Fe^{2+}/ascorbate system generates reactive oxygen species such as H_2O_2, $OH^{\bullet}$, or $O_2^{\bullet -}$ radicals (Fenton, 1894). These radicals react with the nearby metal ligand Asp296 as well as with other nearby susceptible amino acids to cause inactivation and subsequent cleavage of the polypeptide backbone. The Fe^{2+}/ascorbate-induced cleavage of PEPCK is specific and generates two peptides, fragment I, which corresponds to the N-terminal half of PEPCK (amino acids 1−296) and fragment II, which corresponds to the C-terminal half of PEPCK (amino acids 297−607). The cleavage process could potentially render a modified N-terminus on fragment II that would prohibit Edman degradation amino acid sequencing. Since sequencing of this peptide was possible, this indicates that the new N-terminus was not modified. There are several proposed mechanisms for cleavage of the polypeptide backbone that would allow for an unmodified N-terminus. Rana and Meares (1991) proposed a hydrolytic mechanism for cleavage of the polypeptide backbone. The proposed mechanism is mediated by an EDTA-Fe-peroxo species and would yield unmodified peptides. On the basis of the protection against PEPCK cleavage observed from radical scavengers (Figure 6), a radical mediated cleavage of the PEPCK polypeptide backbone seems likely. Platis *et al*. (1993) proposed cleavage of the polypeptide backbone mediated by hydroxyl radical abstraction of the $C_\alpha H$ hydride and subsequent recombination with oxygen. The last step of this mechanism is the hydrolysis of the cyano group on the N-terminus of the C-terminal peptide. This process would generate a free N-terminus at the cleavage site allowing for Edman degradation amino acid sequencing. Edman degradation amino acid sequencing has also been performed on the peptides resulting from Fe^{2+}/ascorbate-induced cleavage of staphylococcal nuclease (Ermácora *et al*., 1992), isocitrate dehydrogenase (Soundar & Colman, 1993), and malic enzyme (Wei *et al*., 1994) indicating that a free N-terminus can be formed after oxidative cleavage of a polypeptide backbone.

The mechanism proposed by Platis *et al*. for cleavage of the polypeptide backbone also shows that the C-terminus of the second peptide is modified. There is no free carboxyl group on the C-terminus; instead, a ketone is formed. The lack of C-terminal sequencing by MALDI-TOF techniques on fragment I from PEPCK suggests that the cleavage process rendered a modified C-terminus on fragment I. The results obtained with PEPCK are consistent with the Platis *et al*. proposed mechanism for radical cleavage of the polypeptide backbone.

Since the apparent first-order rate constant for inactivation is faster than the apparent first-order rate constant for cleavage, this suggests that the oxidation of other essential amino acid residues may induce inactivation. Amino acid composition analysis determined that cysteic acid was formed over the incubation period, indicating that free cysteine residues were modified. Intrinsic tryptophan fluorescent spectra also decreased after incubation suggesting that tryptophan amino acid residues may be modified upon treatment of PEPCK with Fe^{2+}/ascorbate. The oxidative damage to PEPCK eliminates the Mn^{2+} binding site but does not eliminate the nucleotide binding site in a parallel fashion.

The identification of Asp296 as a site of metal ligation agrees well with previous studies using Co^{3+}-modified PEPCK which identified Asp295 and Asp296 as the ligands to the enzyme-bound metal (Hlavaty & Nowak, 1997). Two separate and distinct techniques for determining the PEPCK metal ligands identified the same region. Table 5 shows a sequence homology of PEPCK from seven sources between amino acids 290 through 301 (using the avian mitochondrial PEPCK numbering system). Note that 8 out of the 12 amino acids are identical for all species and that 11 out of the 12

Table 5: Amino Acid Sequence of the Putative Mn^{2+} Binding-Site of Various PEPCKs[a]

source	position 290					295		↓			300	301
chicken myto.	**I**	H	**C**	**V**	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**
chicken cyto.	**I**	E	**C**	**V**	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**
A. suum	V	R	V	I	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**
Haemonchus contortus	**I**	R	**C**	**V**	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**
Drosophila	**I**	E	**C**	**V**	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**
rat	**I**	E	**C**	**V**	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**
human	V	E	**C**	**V**	**G**	**D**	**D**	**I**	**A**	**W**	**M**	**K**

[a] Comparison of the amino acid sequences of PEPCK from various species between the region of 290−301 (avian liver mitochondrial PEPCK numbering). 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), *A. suum* (Geary *et al*., 1993), *H. contortus* (Klein *et al*., 1992), *Drosophila melanogaster* (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. Identities between these enzymes and the chicken liver mitochondria PEPCK are indicated in bold. The site of cleavage on chicken liver mitochondrial PEPCK due to treatment with Fe^{2+}/ascorbate is indicated by the arrow.

amino acids shown are highly conserved in this region. In particular, Asp295, Asp296, and Ile297 are completely conserved throughout all species. *Ascaris suum* PEPCK was also shown to be inactivated and specifically cleaved following treatment with Fe^{2+}/ascorbate (Hlavaty, 1997). Sequencing of the fragments determined that *A. suum* PEPCK was cleaved between Asp329 and Ile330. This region corresponds exactly to the cleaved region for the avian liver mitochondrial PEPCK (see Table 5), demonstrating the selectivity of the radical reaction.

Four distinct experiments identified these two adjacent aspartic acids located in the central portion of PEPCK as being important for either catalysis or metal binding. Fenton chemistry identified Asp296 in avian liver PEPCK (and the corresponding Asp329 in *A. suum* PEPCK) as a metal ligand. Co^{3+}-modification of avian liver PEPCK identified Asp295 and Asp296 as metal ligands (Hlavaty & Nowak, 1997). The crystal structure of the *Escherichia coli* PEPCK identified Asp269 as a metal ligand (Matte *et al*., 1996). Asp269 in *E. coli* PEPCK corresponds to Asp296 in avian liver PEPCK. Lastly, when Asp268 of *E. coli* PEPCK was mutated to an asparagine, almost total PEPCK activity was lost (Hou *et al*., 1995). Asp268 in *E. coli* PEPCK corresponds to Asp295 in avian liver PEPCK.

The sequence X-X-Asp-Asp-Ile-X-X, where X represents a hydrophobic or uncharged amino acid, is conserved in avian liver malic enzyme which requires Mn^{2+} as an activating cation (Wei *et al*., 1994). These authors found that treatment of malic enzyme with Fe^{2+}/ascorbate also identified the cleavage site between an aspartic acid and an isoleucine (Asp258 and Ile259). When Asp258 in malic enzyme was mutated to an asparagine, the enzyme was inactive and no metal binding was observed (Wei *et al*., 1995). There are numerous similarities between PEPCK and malic enzyme. Both are liver mitochondrial enzymes requiring Mn^{2+} for activation, and both enzymes perform catalysis in the outer sphere of the enzyme-bound metal (Hsu *et al*., 1976; Lee & Nowak, 1984; Duffy & Nowak, 1985). Furthermore, both enzymes utilize OAA as a substrate. The similarities between the metal-binding sites for both enzymes is intriguing. These results support the observations that Asp295 and Asp296 in avian liver PEPCK play a role in metal binding, catalysis, or both.

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 mitochondrial PEPCK. 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, whereas, avian liver PEPCK is not activated by nor does it bind to ATP. Lastly, no significant sequence homology exists between the *E. coli* and avian liver enzymes. 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 a either valine or isoleucine, is conserved. A search through the on-line Swiss protein database revealed that PEPCK from 20 different sources (both GTP- and ATP-utilizing enzymes) contain this highly conserved metal consensus site.

In summary, the results of this study have demonstrated that the addition of Fe^{2+}/ascorbate (Fenton chemistry) to avian mitochondrial PEPCK completely inactivates and selectively cleaves the enzyme at the cation site. The apparent first order rate constant for inactivation is greater than the apparent first-order rate constant for cleavage indicating that other oxidative damage is occurring to PEPCK. This selective protein modification does not destroy the nucleotide binding site on PEPCK. The binding site for the cation has been located using solution chemistry and peptide analysis. These results were subsequently confirmed when this process was performed on *A. suum* PEPCK. This methodology may be extendible to other metal-requiring proteins.

ACKNOWLEDGMENT

The authors express their appreciation to Don Schifferl for his assistance in optimizing the EPR and PRR spectrometers, to Dr. Bakshy Chibber and Kay Finn in the Bio-Core Facility for obtaining amino acid sequence data, and to Dr. Bill Boggess in the Mass Spectroscopy Facility for obtaining MALDI-TOF data.

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BI970574P