Phosphoenolpyruvate Carboxykinase (Guanosine 5′-Triphosphate) from Rat Liver Cytosol. Dual-Cation Requirement for the Carboxylation Reaction†

Giovanna Colombo,‡ Gerald M. Carlson,§ and Henry A. Lardy*

ABSTRACT: The carboxylation of phosphoenolpyruvate to form oxalacetate catalyzed by highly purified phosphoenolpyruvate carboxykinase isolated from rat liver cytosol has been studied at pH 7.0 and 25 °C. When the enzyme was assayed in the presence of 2 mM Mn^{2+}, the specific activity was approximately 12-fold higher than when assayed with 2 mM Mg^{2+}. Under these conditions, 10 μM ethylenediaminetetraacetic acid (EDTA) caused 96% inhibition of the activity in the presence of Mg^{2+} and a slight increase of the activity in the presence of Mn^{2+}. The activity with Mg^{2+} was also inhibited by ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), *o*-phenanthroline, α,α′-bipyridine, 8-hydroxyquinoline, and 2,3-dimercaptopropanol. In the presence of 10 μM EDTA, enzymatic activity was supported by Mn^{2+} and Co^{2+}, and to a lesser extent by Cd^{2+}; Mg^{2+} was almost completely ineffective. However, large increases in the carboxylation rate were achieved when 2 mM Mg^{2+} was present in combination with micromolar amounts of divalent transition-metal ions. The specific activity with 2 mM Mg^{2+} increased from 0.04 μmol of phosphoenolpyruvate carboxylated min^{-1} mg^{-1} to 8.4, 9.2, 4.2, and 1.1 μmol min^{-1} mg^{-1} when 30 μM Mn^{2+}, Co^{2+}, Cd^{2+}, or Zn^{2+}, respectively, was included in the reaction mixture. The nature of this activation was synergistic rather than additive. Zn^{2+}, at micromolar levels, was a potent antagonist of the carboxylation reaction in the presence of Mg^{2+} plus Mn^{2+}, Mg^{2+} plus Co^{2+}, or Mg^{2+} plus Cd^{2+}. In turn, micromolar levels of Cd^{2+} depressed the rate of carboxylation stimulated by Mg^{2+} plus Mn^{2+} or Mg^{2+} plus Co^{2+} to the level achieved in the presence of Mg^{2+} plus Cd^{2+}. The results suggest a dual-cation requirement in the carboxylation of phosphoenolpyruvate catalyzed by rat liver cytosolic phosphoenolpyruvate carboxykinase. An activating cation, in addition to that chelated by the nucleotide, is required to obtain significant carboxylation of phosphoenolpyruvate. Mn^{2+}, Co^{2+}, and Cd^{2+} can fulfill this role, whereas Mg^{2+} is ineffective. Zn^{2+} appears to bind tightly at this free metal site, but activates the carboxylaction only weakly.

Phosphoenolpyruvate carboxykinase (PEPCK)[1] (EC 4.1.1.32) from a great variety of species catalyzes the reversible decarboxylation of OAA to PEP (eq 1) [see Utter & Kolenbrander (1972) for a review]. Either in the forward (decarboxylation) or in the reverse (carboxylation) direction, reaction 1 requires the presence of both a nucleotide and divalent metal ion(s). With inosine 5′-triphosphate (ITP) or guanosine 5′-triphosphate (GTP) in the forward direction, or with IDP or GDP in the reverse direction, Mn^{2+} has been used as the supporting cation with mitochondrial PEPCK from many different species (Holten & Nordlie, 1965; Chang & Lane, 1966; Noce & Utter, 1975; Hammond & Balinsky, 1978; Barns & Keech, 1972), and with cytosolic PEPCK from the livers of guinea pig, monkey (Holten & Nordlie, 1965; Hammond & Balinsky, 1978), and rat (Foster et al., 1967; Ballard & Hanson, 1969; Bentle & Lardy, 1976; Jomain-Baum et al., 1976). With the enzymes from guinea pig, Mg^{2+}, and to a lesser extent Fe^{2+} and Co^{2+}, replaced Mn^{2+} in supporting PEP synthesis whereas Mg^{2+} could not replace Mn^{2+} in supporting OAA synthesis (Nordlie & Lardy, 1963; Holten & Nordlie, 1965). Mg^{2+} or Mn^{2+} was equally effective in supporting PEP carboxylation with sheep kidney PEPCK (Barns & Keech, 1972).

$$\text{OAA} + \text{NTP} \underset{}{\overset{Me^{2+}}{\rightleftharpoons}} \text{PEP} + \text{NDP} + CO_2 \qquad (1)$$

Foster et al. (1967) reported that PEPCK from the cytosolic fraction of rat liver showed maximum activity when a high ratio of Mg^{2+} to Mn^{2+} was used in the forward direction assay. The synergism was interpreted on the basis of a dual-cation role in PEPCK-catalyzed PEP synthesis: Mg^{2+} forms a MgITP complex which acts as the substrate of the reaction, and a second metal ion (Mn^{2+}) acts as an activator by interacting with the enzyme at a site different from the MgITP site. PEP formation was also greatly enhanced if the rat liver supernatant fraction was incubated with low levels of Fe^{2+}, Co^{2+}, Cd^{2+}, or Mn^{2+} prior to assay of PEPCK activity in a reaction mixture containing only Mg^{2+} (Snoke et al., 1971). When the enzyme was partially purified, only the Mn^{2+} response was retained when assaying in the direction of PEP synthesis with Mg^{2+} (Bentle & Lardy, 1976). The loss of Fe^{2+} stimulation was restored by the addition of a protein purified from rat liver cytosol and termed ferroactivator (Bentle et al., 1976). PEP carboxylation was enhanced by incubating the enzyme with low concentrations of Co^{2+} but not with Mn^{2+} (Bentle & Lardy, 1977). Since modification of enzyme activity cannot alter the ultimate equilibrium position, the question arises as to why there are differences in the kinetic behavior depending on the direction in which PEPCK activity has been determined (Holten & Nordlie, 1965; Utter & Kolenbrander, 1972; Bentle & Lardy, 1976, 1977).

At present, little is known about the details of the carboxykinase reaction. It appears that with PEPCK from verteb-

† From the Department of Biochemistry and the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706. *Received August 6, 1980; revised manuscript received January 6, 1981.* This study was supported in part by Grants AM10334 and AM20678 from the National Institutes of Health.

‡ Recipient of the Evelyn Steenbock Predoctoral Fellowship. Present address: Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

§ Present address: Department of Chemistry, University of South Florida, Tampa, FL 33620.

[1] Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase; OAA, oxalacetic acid; PEP, phosphoenolpyruvate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; BAL, 2,3-dimercapto-1-propanol; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid); Nbs, 5-thio-2-nitrobenzoate; Me^{2+}, divalent metal ion; $NaDodSO_4$, sodium dodecyl sulfate; MDH, malate dehydrogenase.

0006-2960/81/0420-2749$01.25/0

rates two divalent metals (one as a Me^{2+}NTP complex and one as a free metal ion) are necessary to achieve maximal activity in the direction of PEP synthesis, whereas no generalizations can be made for the carboxylation of PEP. To understand the chemistry of the reaction, or the mechanism of effectors such as ferroactivator protein (Bentle & Lardy, 1977) or the metal-chelating picolinate derivatives (Snoke et al., 1971; Jomain-Baum et al., 1976; MacDonald & Lardy, 1978) on PEPCK activity, it is important to understand fully the two metal sites and to know which metals can function at each site. Our data indicate that under steady-state conditions the presence of a divalent transition-metal ion in addition to the metal–nucleotide substrate is an absolute requirement for highly purified rat PEPCK to catalyze the formation of OAA at an appreciable rate. A preliminary report has been presented (Colombo & Carlson, 1978).

Materials and Methods

Reagents were from the following sources: sodium salts of ITP, IDP, ADP, and NADH, P-L Biochemicals, Inc.; PEP, Hepes, and Nbs_2, Sigma Chemical Co.; OAA and Tes, Calbiochem; glycerol solutions of malate and lactate dehydrogenases and pyruvate kinase, Boehringer Mannheim Biochemicals; EDTA and BAL, Aldrich; EGTA, α,α'-bipyridine, and *o*-phenanthroline, Eastman Organic Chemicals; 8-hydroxyquinoline and $NaHCO_3$, Fisher Scientific Co.; dithizone, Matheson Coleman and Bell; chlorides of Mg^{2+}, Mn^{2+}, Cd^{2+}, and Fe^{2+}, J. T. Baker Chemical Co.; $ZnCl_2$ (ultra pure), Alfa Inorganics; $CoCl_2$, Mallinckrodt Chemical Works; $CaCl_2$, Orion Research.

Phosphoenolpyruvate carboxykinase from rat liver cytosol, purified to homogeneity as previously described (Colombo et al., 1978), was desalted in a Sephadex G-25 column equilibrated with 10 mM Tes–NaOH buffer, pH 7.2, containing 10% glycerol and 0.1 mM EDTA. The purified enzyme showed a single protein band in polyacrylamide disc gel electrophoresis in the presence of $NaDodSO_4$.

PEPCK protein concentration was determined spectrophotometrically at 280 nm by using the extinction coefficient, $E^{1\%,1cm}_{280nm}$, of 16.6 and a molecular weight of 72 000 (Colombo et al., 1978).

Assays of Phosphoenolpyruvate Carboxykinase. The enzymatic activity was determined spectrophotometrically at 25 °C by the decrease of the absorbance at 340 nm. The reactions always were started by the addition of PEPCK to the otherwise complete reaction mixture, except for the experiment described in Figure 6.

The carboxylation of phosphoenolpyruvate to oxalacetate (reverse direction) was measured at pH 6.9–7.0 in a 1-mL reaction mixture containing 56 mM Hepes, 1 mM IDP, 2 mM PEP, 45 mM $NaHCO_3$, 0.25 mM NADH, 22 units of malate dehydrogenase, and divalent metal as indicated for each experiment. The nature and concentration of chelating agents added are indicated for each experimental condition. When PEPCK activity was measured in the direction of PEP formation (forward direction), the reaction was coupled to pyruvate kinase and lactate dehydrogenase catalyzed reactions. The reaction mixture contained in a total volume of 1 mL 55 mM Hepes–KOH (pH 7.5), 1 or 2 mM ITP, 2 or 4 mM $MgCl_2$, 1 mM ADP, OAA as indicated, 0.25 mM NADH, 10 μM EDTA, and 30 μg each of pyruvate kinase and lactate dehydrogenase. The nature and concentration of other added divalent cations are indicated in each experiment. The rate of spontaneous decarboxylation of oxalacetate was determined in the absence of PEPCK and subtracted from the measured rates to give the enzyme-catalyzed rate of PEP formation. Controls without pyruvate kinase also were included.

A unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of product/min under each assay condition. Specific activity is expressed as micromoles of product formed per minute per milligram of PEPCK.

Modification of PEPCK with 5,5′-Dithiobis(2-nitrobenzoic acid). Titrations of enzyme sulfhydryl groups with Nbs_2 were carried out at 23 °C, and the rate of formation of the thionitrobenzoate anion was followed spectrophotometrically at 412 nm. Reactions were carried out in 100 mM Tes–NaOH buffer, pH 7.5, containing 0.1 mM EDTA to avoid any effect of metal impurities. Other additions were as indicated in the figure legends. Reactions were always started by the addition of Nbs_2. The absorbance of a blank containing all the components of the reaction system except PEPCK was continuously subtracted from that of the sample. A molar extinction coefficient of 13 600 M^{-1} cm^{-1} (Ellman, 1959) for the thionitrobenzoate anion liberated was used for all calculations. At various time intervals, aliquots (10 μL) were removed and assayed for enzymatic activity in the reverse direction with 2 mM Mn^{2+}. Glass-distilled, deionized H_2O that had been degassed and saturated with N_2 was used to prepare all the solutions.

Results

Effect of Metal Chelators on PEPCK-Catalyzed Carboxylation of PEP. When rat liver cytosol PEPCK is assayed in the direction of PEP carboxylation, activity has been routinely determined with 2 mM Mn^{2+} in the reaction mixture (Holten & Nordlie, 1965; Ballard & Hanson, 1969; Pogson & Smith, 1975). Because Mg^{2+} supports enzymatic activity in the forward reaction (Foster et al., 1967; Bentle & Lardy, 1976, 1977), it was used as a substitute for Mn^{2+} in the carboxylation reaction. In the presence of 0.2 μM EDTA (carry–over with the PEPCK), 2 mM Mg^{2+} was only 10% as effective as 2 mM Mn^{2+} in supporting PEP carboxylation. The activity with Mg as the sole divalent cation was not reproducible, and the time course was not linear. An increase of the EDTA concentration to 10.2 μM resulted in linear assays and 96% inhibition of the carboxylation rate. Furthermore, EDTA, EGTA, BAL, *o*-phenanthroline, α,α'-bipyridine, and 8-hydroxyquinoline greatly suppressed PEP carboxylation with the effectiveness decreasing in the order listed (Figure 1). Qualitatively, their inhibition can be related to their binding capacity for transition- and heavy-metal ions (O'Sullivan, 1969; Sillén & Martell, 1964). The specific activity with 10 μM EDTA and 2 mM Mg^{2+} ranged from 0.03 to 0.07 μmol of PEP carboxylated min^{-1} mg^{-1} in a great number of experiments.

The effect of the chelators suggests that in the reverse direction with Mg^{2+}, traces of other divalent ions must also be present for activity to be expressed. This is shown in Figure 2. At a fixed concentration of Mg^{2+}, increasing concentrations of EDTA, up to 3 or 4 μM, sharply reduced OAA formation, and from there on the Mg^{2+} activity leveled off (Figure 2, open triangles and left ordinate). The same range of EDTA concentration did not decrease the activity of the enzyme when 2 mM Mn^{2+} was the cation used in the carboxylation reaction (Figure 2, filled triangles and right ordinate). In the presence of 2 mM Mg^{2+} plus 20 μM Mn^{2+}, only 2-fold stimulation occurred when EDTA concentration in the reaction mixture was less than 1 μM, indicating that Mn^{2+} was not able to stimulate completely. However, when the activity with Mg^{2+} as the only added divalent cation was reduced to a low constant level by EDTA, the stimulation by micromolar Mn^{2+} levels was maximal (Figure 2, open triangles, left ordinate vs. filled circles, right ordinate). Note the different activity scales; i.e.,

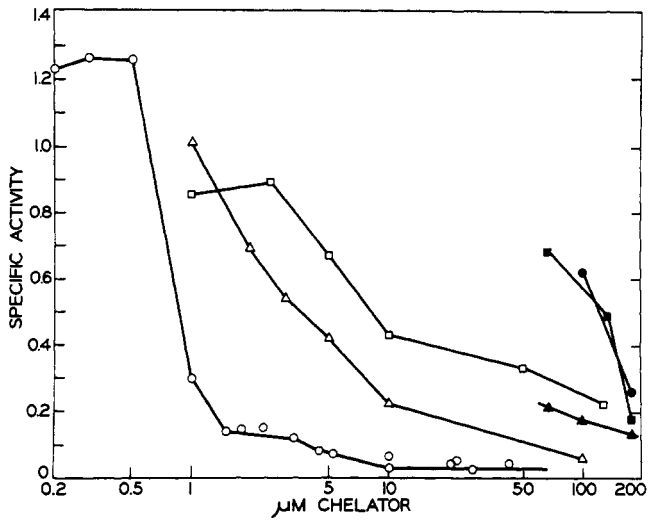

FIGURE 1: Effect of several metal chelators on the carboxylation of PEP catalyzed by PEPCK in the presence of 2 mM Mg^{2+}. The reaction mixture described under Materials and Methods for the reverse direction assay also contained 2 mM $MgCl_2$ and varying concentrations of the following chelators: EDTA (○); EGTA (△); BAL (□); *o*-phenanthroline (▲); α,α'-bipyridine (■); 8-hydroxyquinoline (●). Since PEPCK is stored in an EDTA-containing buffer, the carry-over of this chelator into the assay mixture was 0.3 μM into the EGTA curve, 0.2 μM into the BAL curve, and 0.5 μM into the α,α'-bipyridine, 8-hydroxyquinoline, and *o*-phenanthroline curves. Specific activity is expressed as μmol of PEP carboxylated min^{-1} mg^{-1}.

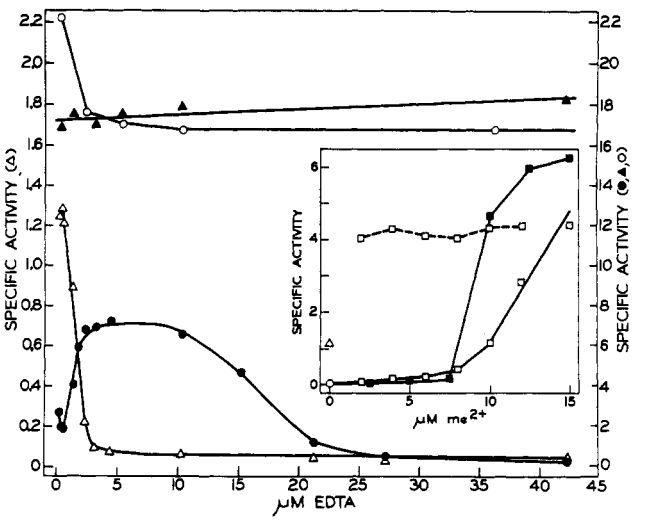

FIGURE 2: Effect of varying EDTA concentrations on PEPCK-catalyzed PEP carboxylation in the presence of 2 mM Mg^{2+}, 2 mM Mn^{2+}, and 2 mM Mg^{2+} plus 20 μM Mn^{2+} or on PEP formation with 2 mM Mg^{2+} added. The reaction mixture described under Materials and Methods for the reverse direction assay also contained EDTA as indicated on the abscissa and divalent metals as follows: (left ordinate, △) 2 mM Mg^{2+}; (right ordinate, ▲ or ●) 2 mM Mn^{2+} or 2 mM Mg^{2+} plus 20 μM Mn^{2+}, respectively. The reaction mixture for the forward direction assay contained EDTA as indicated on the abscissa, 1 mM ITP, 300 μM OAA, and 2 mM Mg^{2+} (right ordinate, ○). Inset: Effect of varying Mn^{2+} (□) or Co^{2+} (■) concentration on PEPCK activity with 2 mM Mg^{2+} and 10 μM EDTA when assayed in the reverse direction. The reaction mixtures contained (△) 2 mM Mg^{2+} plus 0.5 μM EDTA carried over with PEPCK, (○) 2 mM Mg^{2+} plus 10 μM EDTA, and (□ or ■) 2 mM Mg^{2+} plus 10 μM EDTA plus increasing concentrations of Mn^{2+} or Co^{2+}, respectively. For the open squares linked by the dashed line, the reaction was allowed to proceed for 13 min with 2, 4, and 6 μM Mn^{2+}, for 10 min with 8 μM Mn^{2+}, for 6 min with 10 μM Mn^{2+}, and for 3 min with 12 μM Mn^{2+}, and then extra Mn^{2+} was added in a small volume to give a final concentration of 15 μM. Specific activity is expressed as μmol of PEP carboxylated min^{-1} mg^{-1} (△, ▲, ●, □, ■) or μmol of PEP formed min^{-1} mg^{-1} (○).

at 4.5 μM EDTA, the specific activities with 2 mM Mg^{2+} and with 2 mM Mg^{2+} plus 20 μM Mn^{2+} are 0.07 and 7.1 μmol of PEP carboxylated min^{-1} mg^{-1}, respectively.

In the presence of 2 mM Mg^{2+} plus 20 μM Mn^{2+}, concentrations of EDTA higher than 20 μM greatly decrease Mn^{2+} stimulation of the Mg^{2+} activity (Figure 2, filled circles). This result indicates that free Mn^{2+} is probably the activating species. Further indication of the activation by free metal is presented in the inset of Figure 2. In this case, the concentration of EDTA was held constant at 10 μM, and the activity was determined in the presence of 2 mM Mg^{2+} in combination with increasing concentrations of Mn^{2+} or Co^{2+} up to 15 μM. Considerable activation was obtained only when the concentration of Mn^{2+} (open squares and solid line) or Co^{2+} (filled squares) exceeded that of EDTA. Co^{2+} appeared to be more effective than Mn^{2+}. Constant activity was obtained upon raising the concentration of Mn^{2+} to 15 μM by sequential addition of the appropriate amounts of Mn^{2+} after the carboxylation reaction had proceeded for different periods of time in the mixtures containing Mn^{2+} at the level indicated on the abscissa (Figure 2, inset open squares and dashed line).

The same levels of added EDTA that almost completely inhibited the enzyme activity assayed in the reverse direction with 2 mM Mg^{2+} (Figure 2, open triangles and left ordinate) decreased PEPCK activity 25% when assayed in the forward direction with 2 mM Mg^{2+} (Figure 2, open circles and right ordinate). In this case, the activity remained constant when EDTA concentration was varied from 10 to 70 μM.

Activating Effects of Divalent Metal Ions on the Carboxylation Reaction. The results in Figure 2 indicate that divalent metals have a dual role in the carboxylation reaction: first, formation of a metal–nucleotide substrate complex; second, free metal ion activation of the enzyme at a site different from the metal–nucleotide substrate site. With 10 μM EDTA present in the assay mixture acting as a scavenger of trace-metal contaminants (activators and/or inhibitors), Mg^{2+} at millimolar concentration appears to form a Mg^{2+}–nucleoside diphosphate substrate complex. However, either free Mg^{2+} does not interact at the free metal site or, if it does interact, it is not effective. Therefore, enzymatic activity is very low, and for effective catalysis to occur a divalent transition-metal ion has to assume the role of free metal activator. With 10 μM EDTA, PEPCK carboxylating activity with 2 mM Mg^{2+} or with 30 μM Mn^{2+}, Co^{2+}, Cd^{2+}, or Zn^{2+} was very low (Table IA). However, when any one of these latter ions at 30 μM concentration was present in the reaction mixture together with 2 mM Mg^{2+}, the carboxylation of PEP was strikingly increased; the effects were synergistic rather than additive. PEP carboxylation was stimulated 38-, 21-, and 15-fold, respectively, by 30 μM Co^{2+}, Mn^{2+}, or Cd^{2+}. Zn^{2+} was less effective (Table IA). Thus, it is evident that Mg^{2+} alone does not effectively support carboxylation of PEP, but most likely it forms a MgIDP complex, the actual substrate which is necessary for the expression of the activation by micromolar levels of other divalent metals. The very low activity with Mg^{2+} alone in the reverse direction is in contrast with the decarboxylation of OAA to PEP. Mg^{2+} alone is able to support enzymatic activity in the forward direction (Table IB). For the purpose of comparison, the activities obtained with 2 mM Mg^{2+} and 30 μM Mn^{2+}, Co^{2+}, Cd^{2+}, or Zn^{2+} are also reported. Low levels of Mn^{2+} act synergistically with Mg^{2+} present at concentrations equivalent to that of ITP (Foster et al., 1967). Co^{2+} and Cd^{2+} probably act similarly. Zn^{2+} at 30 μM was slightly inhibitory.

Effect of Mn^{2+} and Co^{2+} in the Carboxylation Reaction. The activity as a function of variable concentrations of Mn^{2+} or Co^{2+}, in the presence of fixed concentrations of substrates,

Table I: Synergistic Effects of Divalent Metal Ions on PEPCK Activity in the Reverse (A) or Forward (B) Direction

divalent cation(s)	sp act.	fold activation[c] $(Mg^{2+} + Me^{2+})/(Mg^{2+})$	fold activation[c] $(Mg^{2+} + Me^{2+})/[(Mg^{2+}) + (Me^{2+})]$
(A) Carboxylation of PEP[a]			
2 mM Mg^{2+}	0.04		
30 μM Mn^{2+}	0.37		
2 mM Mg^{2+} + 30 μM Mn^{2+}	8.44	211	20.6
30 μM Co^{2+}	0.20		
2 mM Mg^{2+} + 30 μM Co^{2+}	9.15	229	38.1
30 μM Cd^{2+}	0.24		
2 mM Mg^{2+} + 30 μM Cd^{2+}	4.19	105	15.0
30 μM Zn^{2+}	0.29		
2 mM Mg^{2+} + 30 μM Zn^{2+}	1.16	29	3.5
(B) Formation of PEP[b]			
2 mM Mg^{2+}	15.8		
2 mM Mg^{2+} + 30 μM Mn^{2+}	76.8	4.9	
2 mM Mg^{2+} + 30 μM Co^{2+}	45.7	2.9	
2 mM Mg^{2+} + 30 μM Cd^{2+}	46.6	2.9	
2 mM Mg^{2+} + 30 μM Zn^{2+}	10.8	0.7	

[a] The carboxylation of PEP catalyzed by purified rat liver cytosol PEPCK was determined as described under Materials and Methods for the reverse direction assay with 10 μM EDTA and divalent cation(s) alone or in combination at the indicated concentrations. Specific activity is expressed as μmol of PEP carboxylated min^{-1} mg^{-1}. [b] The formation of PEP from OAA catalyzed by PEPCK was determined at pH 7.5 and 25 °C as described under Materials and Methods for the forward direction assay. The reaction mixture contained 55 mM Hepes-KOH, 1 mM ITP, 300 μM OAA, 10 μM EDTA, and divalent cation(s) at the indicated concentrations. Specific activity is expressed as μmol of PEP formed min^{-1} mg^{-1}. All the reactions were started by the addition of the enzyme to the complete reaction mixture. [c] (Mg^{2+}) represents the activity obtained with 2 mM Mg^{2+} alone. (Me^{2+}) represents the activity obtained with 30 μM Mn^{2+}, Co^{2+}, Cd^{2+}, or Zn^{2+} alone. $(Mg^{2+} + Me^{2+})$ represents the activity obtained when 2 mM Mg^{2+} was present in the reaction mixture in combination with 30 μM Mn^{2+}, Co^{2+}, Cd^{2+}, or Zn^{2+}.

is shown in parts A and B of Figure 3, respectively (open circles). These saturation curves for either Mn^{2+} or Co^{2+} are sigmoidal. Double-reciprocal plots for the variation of added metal ion are not linear but show an upward curvature at the lower concentrations of either Mn^{2+} or Co^{2+} (results not shown). The deviations from normal Michaelis-Menten kinetics were confirmed when the data were plotted according to Hill (Atkinson, 1966). The n_H slopes obtained for either total Mn^{2+} or total Co^{2+} were 1.7 and 2.1, respectively (Figure 3A,B insets, open circles). A possible interpretation for the sigmoidal kinetics with respect to either Mn^{2+} or Co^{2+}, at a fixed level of IDP, is that at least two metal ion requiring sites are present in the enzyme. It is suggested that the enzyme binds free Mn^{2+} or free Co^{2+} in addition to MnIDP or CoIDP, respectively. However, enzyme inhibition by free IDP also could explain these results.

When the carboxylation reaction was studied as a function of varying concentrations of Mn^{2+} or Co^{2+} in the presence of 2 mM Mg^{2+}, biphasic saturation curves were obtained (Figure 3A,B, filled circles). These complex saturation curves contained an intermediary plateau region that becomes even more apparent when the data are presented in the form of Hill plots (insets, Figure 3A,B, filled circles). The n_H values at low and

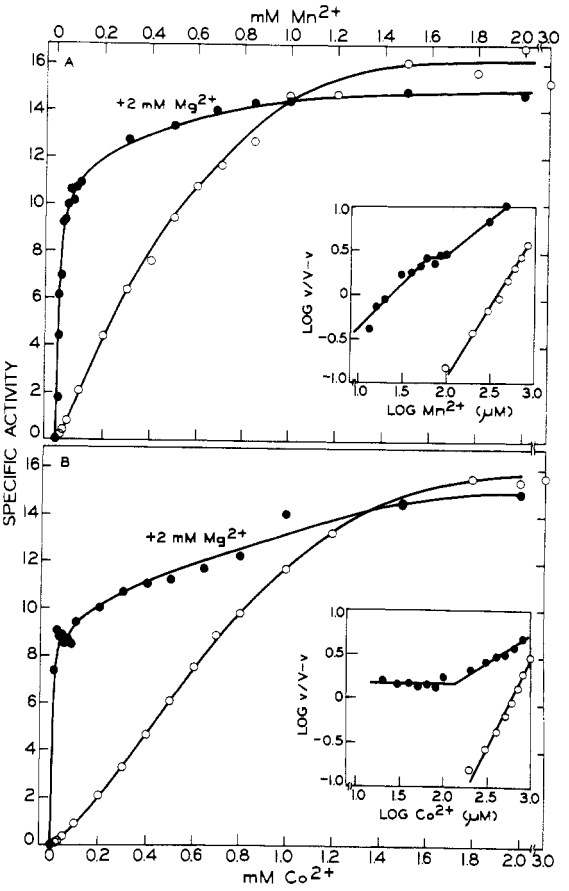

FIGURE 3: PEP carboxylating activity of purified rat liver cytosol PEPCK as a function of the concentration of added Mn^{2+} or Co^{2+} in the absence or presence of 2 mM Mg^{2+}. The reaction mixture described under Materials and Methods for the reverse direction assay also contained 10 μM EDTA. (A) μmol of PEP carboxylated min^{-1} mg^{-1} with Mn^{2+} (○) or with 2 mM Mg^{2+} plus Mn^{2+} (●). (B) μmol of PEP carboxylated min^{-1} mg^{-1} with Co^{2+} (○) or with 2 mM Mg^{2+} plus Co^{2+} (●). Replots of these data according to the Hill equation are presented in the insets in (A) and (B) respectively.

high Mn^{2p} concentrations were 1.0 and 0.8, respectively. The intermediate plateau was approximately between 60 and 100 μM Mn^{2+} ($K_{0.5}$ = 15 μM added Mn^{2+}). With Co^{2+}, the plateau region appeared with concentrations up to 100 μM, and at higher Co^{2+} levels the n_H value was 0.64. In the presence of 2 mM Mg^{2+}, Lineweaver-Burk plots for Mn^{2+} or Co^{2+} were not linear and showed a downward curvature at the higher concentrations of either metal (results not shown).

It is evident from Figure 3A,B that in the flat region of the sigmoids (up to 100 μM Mn^{2+} or Co^{2+}, open circles) PEPCK is synergistically activated by 2 mM Mg^{2+} (filled circles). The effect of micromolar concentrations of Mn^{2+} or Co^{2+} was then studied as a function of the Mg^{2+} concentration. In the absence of Mn^{2+} or Co^{2+}, PEPCK carboxylating activity was extremely low (Figure 4, open circles and right ordinate). In contrast, when 14, 20, 30, or 72 μM Mn^{2+} was present together with Mg^{2+} at the concentrations indicated on the abscissa, PEPCK carboxylating activity was greatly increased (Figure 4, filled circles and left ordinate). Lineweaver-Burk plots of $1/(v - v_0)$ vs. $1/[Mg^{2+}]$ (not shown; v is the activity with millimolar Mg^{2+} plus micromolar Mn^{2+} and v_0 the activity with micromolar Mn^{2+} levels alone) were linear, and the apparent K_a values for Mg^{2+} were 9.1, 5, 3.7, and 3.2 mM as the concentration of Mn^{2+} increased from 14 to 72 μM, respectively. The carboxylating activity of PEPCK as a function of the concentration of Mg^{2+} in the presence of 14 μM Co^{2+} is also shown in Figure 4 (open squares and dashed line).

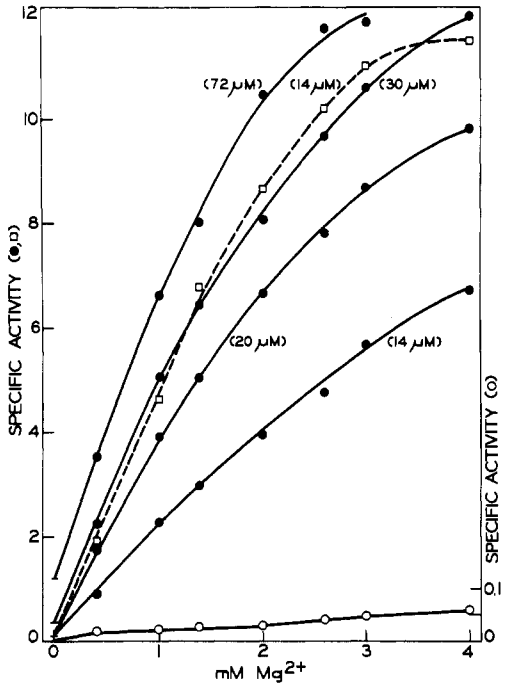

FIGURE 4: Effect of Mn^{2+} or Co^{2+} on the PEP carboxylating activity of rat liver cytosol PEPCK as a function of the concentration of Mg^{2+}. The reaction mixtures, as described under Materials and Methods for the reverse direction, also contained 10 μM EDTA. $MgCl_2$ concentration was varied as indicated on the abscissa. Specific activity is expressed as μmol of PEP carboxylated min^{-1} mg^{-1}. Right ordinate, Mg^{2+} alone (○). Left ordinate, variable Mg^{2+} concentration plus fixed low levels of Mn^{2+} (●) or Co^{2+} (□---□) at the concentrations indicated near each curve.

Identical activity was obtained with either 20 or 34 μM Co^{2+} (not shown). This is in agreement with the results in Figure 3B (filled circles) which showed an elevated constant PEPCK activity with these levels of Co^{2+}. The effect of combining millimolar levels of Mg^{2+} with either Mn^{2+} or Co^{2+} at micromolar levels was to increase greatly the maximal velocity and also to increase the apparent affinity of the enzyme for Mg^{2+}.

In the presence of 1 mM Mg^{2+}, the addition of Co^{2+} and Mn^{2+} together, each at concentrations of either 15 or 25 μM, was no more effective than Co^{2+} alone, indicating that the two metal ions act at the same site.

Effect of Cd^{2+} in the Carboxylating Reaction. Cd^{2+} supported enzymatic activity in the absence of Mg^{2+}, though rather poorly (Figure 5A, open circles). With 1 mM IDP, maximal activity was obtained at a Cd/IDP ratio of approximately 1, and higher concentrations of Cd^{2+} were inhibitory. When 2 mM Mg^{2+} was included in combination with a range of Cd^{2+} concentrations, synergistic activation of PEP carboxylation was obtained (Figure 5A, filled circles). Maximal activation was less than that obtained with Mn^{2+} or Co^{2+} under similar experimental conditions (Figure 3A,B, filled circles), and the activation pattern was also different. With 2 mM Mg^{2+}, the activity was enhanced when added Cd^{2+} was 10–25 μM; its stimulatory effect diminished at higher concentrations, and the rate of OAA formation with Mg^{2+} plus Cd^{2+} reached a plateau at the same velocity that was obtained in the presence of Cd^{2+} alone (Figure 5A). Furthermore, Cd^{2+} decreased the activity obtained in the presence of 2 mM Mg^{2+} plus either 40 μM Mn^{2+} (Figure 5B, filled squares) or 16 μM Co^{2+} (Figure 5B, open circles) to approximately the level of activity obtained with 2 mM Mg^{2+} plus the respective Cd^{2+} concentrations (Figure 5A, filled circles). This reversal by Cd^{2+} of the Mn^{2+} or Co^{2+} activation indicates that these metals

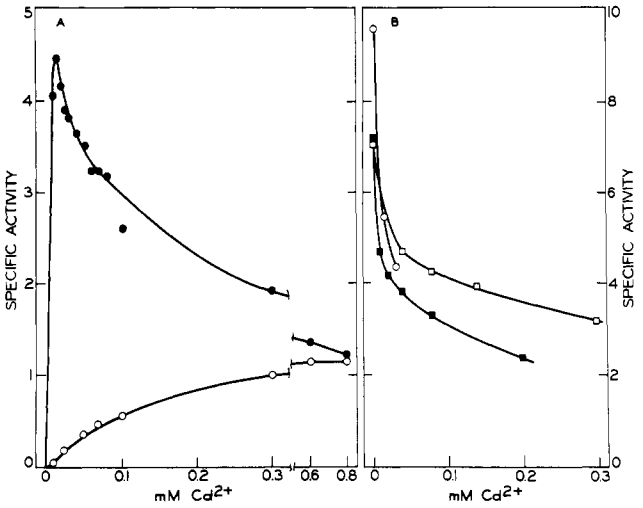

FIGURE 5: PEP carboxylating activity of purified PEPCK as a function of the concentration of $CdCl_2$. The reaction mixtures, as described under Materials and Methods for the reverse direction assay, also contained 10 μM EDTA. Specific activity is expressed as μmol of PEP carboxylated min^{-1} mg^{-1}. (A) OAA formed with varied concentrations of Cd^{2+} in the absence (○) or the presence (●) of 2 mM Mg^{2+}. (B) Inhibition of OAA formation in the presence of 2 mM Mg^{2+} plus 40 μM Mn^{2+} (■), 2 mM Mg^{2+} plus 16 μM Co^{2+} (○), or 0.5 mM Mn^{2+} (□) as a function of varied concentrations of Cd^{2+} indicated on the abscissa.

probably interact at a common site on the enzyme and that the affinity for Cd^{2+} is greater than that for Mn^{2+} or Co^{2+}. However, Cd^{2+} is a less effective activator than Mn^{2+} or Co^{2+} inasmuch as the activities with 2 mM Mg^{2+} plus 20 μM of either Cd^{2+} (Figure 5A), Mn^{2+} (Figure 3A), or Co^{2+} (Figure 3B) were 4.5, 7, and 9.1, respectively. The same range of Cd^{2+} concentrations that reversed the Mn^{2+} and Co^{2+} activation of OAA synthesis also decreased the activity obtained with 0.5 mM Mn^{2+} as the only cation present in the reaction mixture (Figure 5B, open squares).

We have previously reported that Cd^{2+} at low concentration is a potent time-dependent inhibitor of PEPCK, consistent with the presence of vicinal dithiols on the enzyme (Carlson et al., 1978). Snoke et al. (1971) had found that incubating liver supernatant fractions with 1.6 and 16 μM Cd^{2+} prior to the assay resulted in 40 and 60% stimulation, respectively, of PEP formation when Mg^{2+} was present in the assay mixture. From Table I and Figure 5A (filled circles), it is evident that with purified PEPCK Cd^{2+} stimulates activity when included together with Mg^{2+} either in the forward or in the reverse direction assay. In the latter case, Cd^{2+} was stimulatory over a narrow range of low concentrations. Cd^{2+} stimulation was less than maximal at concentrations greater than 25 μM (Figure 5A). Thus, it was reasonable to assume that Cd^{2+} could bind to purified PEPCK at the free metal site and activate the enzyme. PEPCK (0.35 μM) was incubated for 2 min with the increasing concentrations of Cd^{2+} shown in Figure 6 together with 25 μM EDTA. The activity was subsequently determined in the forward direction in the presence of Mg^{2+} and 10 μM EDTA. Thus, the point designated as 30 μM Cd^{2+} in Figure 6 represents 5 μM free Cd^{2+} in the incubation mixture prior to the assay, and so on. The dilution from the incubation into the assay was 20-fold. As shown in Figure 6, PEP formation was not affected when Cd^{2+} concentration (0–18 μM) was less than EDTA concentration. Stimulation of 76% occurred with 24 μM Cd^{2+}–25 μM EDTA. Maximal stimulation after 2 min of incubation was obtained with 27 μM Cd^{2+}–25 μM EDTA. Stimulation, though to a lesser extent, was still evident up to 50 μM Cd^{2+}–25 μM EDTA, but

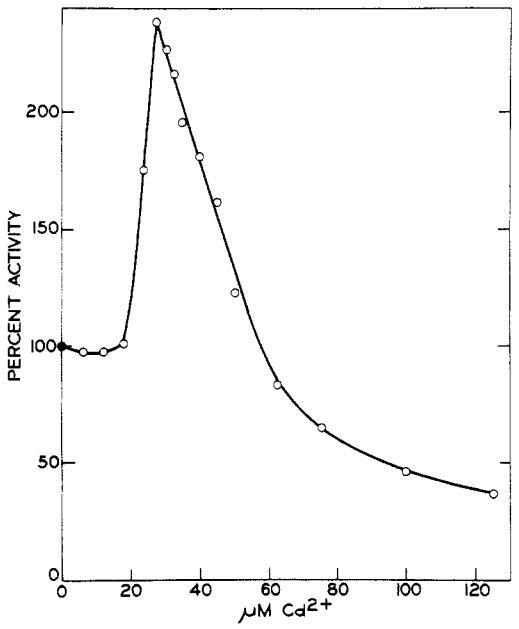

FIGURE 6: Response of PEPCK to preliminary incubation with varying Cd^{2+} concentrations when assayed in the presence of Mg^{2+} in the direction of PEP formation. Prior to assay, incubations of rat liver cytosol PEPCK (0.35 μM) at 22 °C contained 11 mM Tes buffer (pH 7.2), 10% glycerol, and 25 μM EDTA and were initiated by the addition of $Cd(CH_3CO_2)_2$ at the concentrations indicated on the abscissa (6–126 μM). After 2 min of incubation, 50-μL aliquots were removed and assayed for activity in the forward direction in the presence of Mg^{2+}. The assay mixture (pH 7.5), as described under Materials and Methods for the formation of PEP, contained in a final volume of 1 mL 4 mM Mg^{2+}, 2 mM ITP, 1 mM ADP, 150 μM OAA, and 10 μM EDTA. The activity of the enzyme incubated with various concentrations of Cd^{2+} (○) is expressed as the percent of the activity obtained in the absence of Cd^{2+} (●).

higher concentrations inhibited.

Though at first glance the results shown in Figure 6 appear to be contradictory with our previously reported results of inhibition of PEPCK by low Cd^{2+} concentrations (Carlson et al., 1978), this is not the case. Throughout a range of Cd^{2+} concentrations, its effect on enzymatic activity is, as stated above, time dependent. A given concentration of Cd^{2+} is first stimulatory and later inhibitory. For Figure 6, the incubation time and Cd^{2+} concentration range were selected to emphasize the activating action of Cd^{2+} on PEPCK activity.

Modification of PEPCK with 5,5′-Dithiobis(2-nitrobenzoic acid). Purified rat liver cytosolic PEPCK is reversibly inactivated by treatment with Nbs_2 (Carlson et al., 1978). Chemical modification with a limited amount of this reagent proved to be a suitable alternative method to show the effect of a divalent cation on PEPCK. The results are presented in Figure 7. When PEPCK was reacted with approximately 3-fold molar excess Nbs_2, approximately three cystine disulfide bridges were rapidly formed (Figure 7, open circles) and enzymatic activity was completely abolished. In the presence of 1.44 mM Mg^{2+} plus 1 mM ITP or 0.2 mM Mn^{2+} plus 1 mM ITP (Figure 7, filled circles and open squares, respectively), the rate of disulfide formation was decreased. Upon the release of 1.9 mol of Nbs/mol of enzyme (about one cystine formed), 14% and 20% residual activity remained, respectively, with 1.44 mM Mg^{2+} plus 1 mM ITP and with 0.2 mM Mn^{2+} plus 1 mM ITP. Furthermore, total protection against inactivation occurred with 1.44 mM Mn^{2+} plus 1 mM ITP up to 80 min of reaction (1.4 mol of Nbs released/mol of PEPCK) or 1.2 mM Mg^{2+} plus 0.2 mM Mn^{2+} plus 1 mM ITP up to 50 min of reaction with Nbs_2 (1.5 mol of Nbs released/mol of PEPCK) (Figure 7, open and filled triangles,

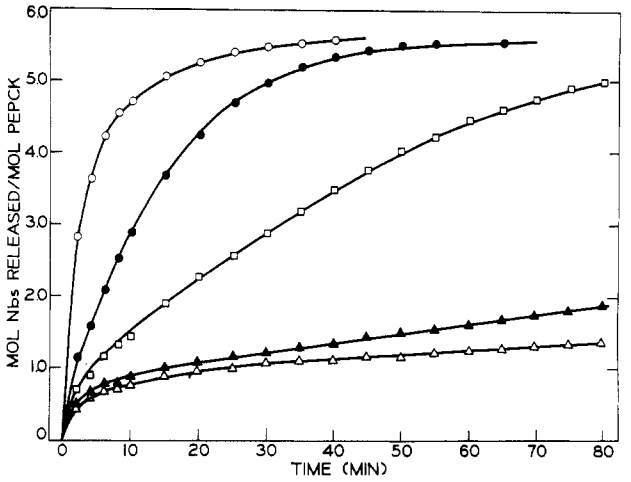

FIGURE 7: Effect of ITP and divalent cations on disulfide formation upon modification of PEPCK with approximately a 3-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid). The reactions contained in a final volume of 1 mL 1.01 μM PEPCK, 100 mM Tes–NaOH buffer (pH 7.5), 1.6% glycerol, 0.11 mM EDTA, and the following additions as indicated: (○) control, no additions; (●) 1.44 mM Mg^{2+} plus 1 mM ITP; (□) 0.2 mM Mn^{2+} plus 1 mM ITP; (▲) 1.2 mM Mg^{2+} plus 0.2 mM Mn^{2+} plus 1 mM ITP; (△) 1.44 mM Mn^{2+} plus 1 mM ITP. All samples were incubated for 15 min at 23 °C, and then the reactions were started by the addition of Nbs_2 so that its final concentration was 2.9 μM. The reactions (Nbs liberation) were followed at 412 nm, as indicated under Materials and Methods, against a blank lacking the enzyme.

respectively). In the latter case, a slight decrease of 9% in activity occurred at 80 min. The most notable feature of this modification pattern is that neither 1.44 mM Mg^{2+} plus 1 mM ITP nor 0.2 mM Mn^{2+} plus 1 mM ITP protected against modification or inactivation. However, with the combination of 1.2 mM Mg^{2+}, 0.2 mM Mn^{2+}, and 1 mM ITP, protection against modification and inactivation was achieved in what appears to be a synergistic manner.

Antagonistic Effect of Zn^{2+} on Divalent Metal Ion Activation of PEPCK. As shown in Table IA, Mn^{2+}, Co^{2+}, or Cd^{2+} at 30 μM were more effective activators than Zn^{2+} at 30 μM. However, Zn^{2+} at micromolar levels was found to be a potent antagonist of the stimulated carboxylation of PEP achieved with 2 mM Mg^{2+} plus 16 μM Mn^{2+}, Co^{2+}, or Cd^{2+} (Figure 8A). Approximately 50% decrease of the activity stimulated by 16 μM Mn^{2+} or Co^{2+} occurred when ~1.5 μM Zn^{2+} was included in the assay (inset, Figure 8A). It appears that Zn^{2+} displaces Mn^{2+} or Co^{2+} from the enzyme without affecting the metal–nucleotide site. When included together with Mg^{2+}, Zn^{2+} at concentrations higher than 8 μM was slightly stimulatory (Figure 8A, open circles and dashed line). A constant specific activity (1.1–1.3 μmol of PEP carboxylated min^{-1} mg^{-1}) was obtained with 2 mM Mg^{2+} plus 10–84 μM added Zn^{2+}.

The effect of Zn^{2+} on the forward reaction was studied because in this direction the basal activity in the presence of Mg^{2+} is sizeable, and micromolar Zn^{2+} levels did not greatly affect this basal activity (Table IB). Therefore, any effect of Zn^{2+} on Mn^{2+}- or Co^{2+}- activated PEPCK might be differentiated from its effect on basal activity. From 1 to 7.5 μM, Zn^{2+} had no apparent effect on PEPCK-catalyzed PEP formation in the presence of Mg^{2+} alone (Figure 8B, open circles and dashed line). However, when the activity was determined in the presence of 2 mM Mg^{2+} plus 16 μM Mn^{2+} or plus 16 μM Co^{2+}, displacement of Mn^{2+} or Co^{2+} by Zn^{2+} is indicated by a decreasing velocity with increasing Zn^{2+} concentrations (Figure 8B); 50% reversal of the activation by 16 μM Mn^{2+} or Co^{2+} was obtained with approximately 2.5 μM Zn^{2+} added

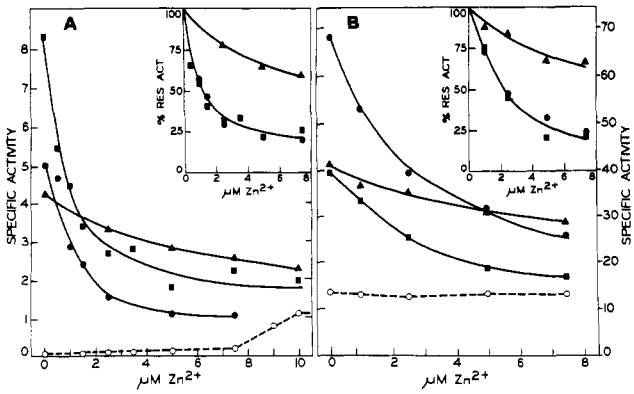

FIGURE 8: Antagonistic effect of Zn^{2+} on the Mn^{2+}, Co^{2+}, or Cd^{2+} activation of purified rat liver cytosol PEPCK. (A) Effect of varying concentrations of added Zn^{2+} on the rate of OAA formation. The assay mixtures as described under Materials and Methods for the reverse direction also contained 10 μM EDTA and 2 mM Mg^{2+} (O---O) or 2 mM Mg^{2+} plus either 16 μM Mn^{2+} (●), 16 μM Co^{2+} (■), or 16 μM Cd^{2+} (▲). (B) Effect of varying levels of added Zn^{2+} on the rate of PEP formation. The assay mixtures for the forward direction also contained 10 μM EDTA, 1 mM IDP, 300 μM OAA, and 2 mM Mg^{2+} (O---O) or 2 mM Mg^{2+} plus 16 μM Mn^{2+} (●), 16 μM Co^{2+} (■), or 16 μM Cd^{2+} (▲). Specific activity is expressed as μmol of OAA formed (A) or μmol of PEP formed (B) min^{-1} mg^{-1}. The insets represent the percent of residual activity vs. the concentration of added Zn^{2+}. An activity of 100% represents in each case the activity obtained with 2 mM Mg^{2+} plus 16 μM of either Mn^{2+}, Co^{2+}, or Cd^{2+} minus the activity obtained in the presence of 2 mM Mg^{2+} alone (no Zn^{2+} added). For the calculation of the percent of residual activity, the activity with 2 mM Mg^{2+} plus Zn^{2+} at the concentrations indicated on the abscissa was subtracted from the activity obtained with 2 mM Mg^{2+} plus 16 μM Mn^{2+}, Co^{2+}, or Cd^{2+} at the respective concentrations of added Zn^{2+}.

to the assay mixture (inset, Figure 8B).

When PEPCK was assayed either in the reverse or in the forward direction, the activation of the Mg^{2+} activity by 16 μM Cd^{2+} was also antagonized when Zn^{2+} was added to the respective reaction mixtures (Figure 8A,B, filled triangles). However, at variance with the Mn^{2+} and Co^{2+} activation, higher concentrations of Zn^{2+} were required to achieve 50% reversal of the activation elicited by 16 μM Cd^{2+} (insets, Figure 8A,B).

Effect of Other Divalent Metal Ions on the Carboxylation of PEP in the Presence of 2 mM Mg^{2+}. As was the case with Mn^{2+}, Co^{2+}, and Cd^{2+}, micromolar concentrations of Fe^{2+} activated PEP carboxylation when included in the assay together with 2 mM Mg^{2+}. The assays were linear only for 2–3 min after initiating the reaction with the enzyme, and then the rate of OAA formation decreased. When the initial velocities were used, 8.5, 11.1, 12.3, and 12.7 μmol of PEP min^{-1} mg^{-1} were carboxylated in the presence of 2 mM Mg^{2+} and 20, 30, 40, and 50 μM Fe^{2+}, respectively. The control activity with 2 mM Mg^{2+} alone was 0.03 μmol min^{-1} mg^{-1}. Higher concentrations of Fe^{2+} could not be tested because a complex absorbing at 340 nm that interferes with the coupled assay was formed.

In the presence of 2 mM Mg^{2+}, 20 μM Cu^{2+} stimulated PEP carboxylation only slightly; the specific activity was 1.2 μmol min^{-1} mg^{-1}. As was reported earlier (Snoke et al., 1971; Bentle & Lardy, 1977), 100 μM Ca^{2+} did not stimulate PEP carboxylation in the presence of 2 mM Mg^{2+}.

Discussion

In the present study, we have compared the effects of a variety of divalent transition-metal ions on the catalytic properties of highly purified rat liver cytosol PEPCK when assayed in the reverse direction (carboxylation of PEP to form OAA). We find that at pH 7.0 the PEP carboxylating activity with Mg^{2+} as the only added divalent cation is probably due to traces of other contaminating metal ions that activate; it was almost completely abolished by several chelators (Figure 1) but could be recovered with micromolar concentrations of divalent transition-metal ions in excess of the chelator (Figure 2 and Table IA). It is not likely that an enzyme-bound metal would account for the "Mg^{2+} activity" in the absence of added divalent transition-metal ions because no metals have been found associated with PEPCK's isolated from yeast (Cannata & de Flombaum, 1974), chicken liver mitochondria (Noce & Utter, 1975), or from rat liver cytosol (Johnston, 1970; Jomain-Baum et al., 1976). Rather, there is a dual-cation requirement for PEPCK activity to be expressed in the reverse direction.

The PEP carboxylating activity obtained when micromolar concentrations of a "second metal" are present in combination with millimolar levels of Mg^{2+} is greater than the sum of the individual activities obtained with either metal alone (Table IA). Synergism also occurs for PEP formation, but in this direction there is a considerable activity with millimolar Mg^{2+} alone (Table IB). The results undoubtedly imply that for rat liver cytosol PEPCK one metal (Mg^{2+} at millimolar levels) forms a metal–nucleotide substrate complex which by interacting at the active site is enough to elicit activity in the forward but not in the reverse direction. The presence of a transition-metal ion at micromolar levels, which is likely to interact at a "free metal" site, is an absolute requirement for the carboxylation of PEP to occur when the metal–nucleoside diphosphate is MgIDP. With chicken liver PEPCK, an interesting dual-cation effect has been reported by Coates & Nowak (1978); Cr(III)–IDP acted as substrate in the carboxylation of PEP, but Mn^{2+} was required for the reaction to occur.

The presence of two binding sites related to the metal requirement of rat liver cytosolic PEPCK is also indicated by the fact that sigmoidal kinetics were obtained when the rate of PEP carboxylation at fixed levels of substrates was plotted against total Mn^{2+} or total Co^{2+} concentration (Figure 3A,B, open circles) when either of these metals was singly present in the assay. In this case, since the reaction is carried out at a fixed concentration of IDP, increasing the concentration of either Mn^{2+} or Co^{2+} results in a simultaneous increase of both the concentrations of MnIDP and Mn^{2+} or CoIDP and Co^{2+}, respectively. The sigmoidal shape of the velocity–metal ion plots can be assumed to be due to an increase in the concentrations of substrate (Me^{2+}IDP) and activator (Me^{2+}). With PEPCK isolated from pig and chicken liver mitochondria, Miller et al. (1968) and Hebda & Nowak (1978) have reported that 1 mol of Mn^{2+} binds per mol of enzyme with dissociation constants of 40 and 30 μM, respectively. With PEPCK from rat liver, the presence of a site for a free divalent cation is confirmed by the fact that, in the presence of 2 mM Mg^{2+}, PEP carboxylation is synergistically increased by Mn^{2+} or Co^{2+} between 10 and 100 μM (Figure 3A,B). Evidently, the formation of the Mg^{2+} complex of the nucleotide substrate leaves the added low levels of Mn^{2+} or Co^{2+} free to interact with the enzyme at the "free metal" site. The effects observed in the presence of 2 mM Mg^{2+} when Mn^{2+} or Co^{2+} concentration was varied over a wider range of concentrations (up to 3 mM) are not easily understood. In both cases, the relationship between rate and concentration of Me^{2+} does not follow Michaelis-Menten kinetics. The biphasic curves might be simply rationalized in terms of multiple sets of Me^{2+} binding sites ("tight" and "loose") with widely different affinities, but the significance of this is obscure at present. Alternatively,

as the concentration of Mn^{2+} or Co^{2+} increases, replacement of MgIDP by MnIDP or CoIDP could occur. At least with ITP, the stability constants with Mn^{2+} or Co^{2+} are greater than with Mg^{2+} (Walaas, 1958). We have no indications that two different forms of PEPCK might exist in our preparation (Colombo et al., 1978).

Cadmium was not as effective as Mn^{2+} or Co^{2+} in supporting PEP carboxylation. Nevertheless, in the presence of 2 mM Mg^{2+}, maximal synergistic activation occurred with 10–25 μM added Cd^{2+}. At higher Cd^{2+} concentration, the activation decreased. This may be a reflection of the formation of a less active CdIDP complex (Figure 5A, open circles). As will be discussed later, an alternative explanation is possible. Zn^{2+} slightly stimulated PEP carboxylation by PEPCK, but maximal activity was less than with the same concentrations of Mn^{2+}, Co^{2+}, or Cd^{2+}.

Regardless of the shape of the Me^{2+}–velocity curves in the presence of fixed, high Mg^{2+} concentrations, micromolar levels of Mn^{2+} or Co^{2+} elicited greater activity than Cd^{2+}, and this in turn was greater than Zn^{2+}. Because not only EDTA (present at 10 μM) but also the substrates for the carboxylation reaction have the ability to bind Me^{2+}, the relative affinity of PEPCK for these metals can be estimated only on a qualitative basis. The data in Figure 3A,B indicate that the affinity of the enzyme is greater for Co^{2+} than for Mn^{2+}. This was also indicated by the fact that the combined effect of micromolar levels of Co^{2+} plus Mn^{2+} in the presence of MgIDP was not greater than that of Co^{2+} alone. Jomain-Baum et al. (1977) have reported that rat liver cytosol PEPCK binds Co^{2+} more avidly than Mn^{2+}. The enzyme appears to bind Cd^{2+} more avidly than Co^{2+} or Mn^{2+} under conditions where the actual substrate is MgIDP, for 10–25 μM Cd^{2+} depressed the activation obtained with Co^{2+} or Mn^{2+} to the lower activity obtained with Cd^{2+} alone (Figure 5B, open circles and filled squares). Cd^{2+} appeared to compete with free Mn^{2+} also when the substrate is MnIDP (Figure 5B, open squares). In the presence of 2 mM Mg^{2+}, added Zn^{2+} (up to 10 μM) in combination with 16 μM of either Mn^{2+}, Co^{2+}, or Cd^{2+} effectively counteracted the activation by the latter cations (Figure 8A). With approximately 1.5 μM added Zn^{2+}, there was a 50% decrease of the PEP carboxylation stimulated by Mg^{2+} plus Co^{2+} or Mg^{2+} plus Mn^{2+} but only a 10% decrease of the activity stimulated by Mg^{2+} plus Cd^{2+} (inset, Figure 8A). This is taken as an indication that the affinity of PEPCK for Zn^{2+} is greater than for Mn^{2+} or Co^{2+} but less than that for Cd^{2+}. The effect of Zn^{2+} on the activation by Co^{2+}, Mn^{2+}, and Cd^{2+} was also elicited when PEPCK activity was determined in the forward direction (Figure 8B). Stickland (1959) reported that with PEPCK from pigeon liver mitochondria Zn^{2+} inhibited OAA synthesis by 90% when added in the presence of $MnCl_2$. The failure of different combinations of micromolar levels of Mn^{2+}, Co^{2+}, Cd^{2+}, and Zn^{2+} to act additively in stimulating enzyme activity in the presence of 2 mM Mg^{2+} suggests that these metals probably interact at the same site on the enzyme. However, no definite conclusion can be made without data regarding the binding of metals to rat liver cytosol PEPCK.

An independent indication that free divalent metal ions interact with PEPCK at a site different from the "metal nucleotide" substrate site was obtained by chemical modification of the enzyme with Nbs_2 (Figure 7), or by incubation of the enzyme with Cd^{2+} prior to its assay in the forward direction with Mg^{2+} (Figure 6). In the latter case, the activity increased to 140% above the control value when PEPCK was incubated for 2 min with 2 μM free Cd^{2+} (25 μM EDTA–27 μM Cd^{2+}). The enzyme must effectively interact with Cd^{2+} since the activation was obtained even though a 20-fold dilution into the assay mixture containing 10 μM EDTA had occurred. We have previously reported that modification of the enzyme with a 1-, 2-, or 3-fold molar excess of Nbs_2 resulted in the formation of cystine disulfide bridges instead of the expected mixed disulfide derivatives (Carlson et al., 1978). This is consistent with the presence of three vicinal dithiol groups in the enzyme. Cadmium has a strong affinity and high selectivity for dithiol ligands (Sanadi et al., 1959; Gaber & Fluharty, 1968). It is possible then that the initial activation is due to the interaction of Cd^{2+} with a specific dithiol in PEPCK (perhaps located in the region of the "free metal" site). The decreased activation at higher Cd^{2+} levels and the eventual inhibition of the "Mg^{2+} activity" could result from the interaction of Cd^{2+} with a different dithiol(s) that may very well include the cysteine residue that is critical for enzyme activity (Carlson et al., 1978). It is also conceivable that Cd^{2+} could disrupt the enzyme structure by the formation of trithiol complexes (Gaber & Fluharty, 1968).

At least two out of the three cystine disulfide bridges obtained upon reaction of PEPCK with a 3 M excess of Nbs_2 were not formed when MgITP plus Mn^{2+} was included in the modification (Figure 7, filled triangles vs. open circles). Over the same time span, the three disulfides were formed either if MgITP or if excess ITP over Mn^{2+} was present. The apparent synergistic protection afforded by the combination of MgITP plus Mn^{2+} could be the result of a significant change of enzyme conformation so that some SH groups are no longer accessible to Nbs_2 modification. Alternatively, protection could arise from a conformational change in conjunction with an effective shielding of some SH groups by Mn^{2+} itself.

The possibility of at least one dithiol located in the region of the free metal site is suggested by the fact that higher concentrations of added Zn^{2+} were necessary to counteract the Cd^{2+} than the Co^{2+}- or Mn^{2+}-activated PEP carboxylation in the presence of MgIDP. Sanadi et al. (1959) proposed Cd^{2+} as a dithiol-specific reagent. Jacobs et al. (1956) noted that the usual order of ligand affinity (zinc > cadmium) is reversed in the case of dithiol ligands. Gaber & Fluharty (1968) indicated that Cd^{2+} was bound 1–2 orders of magnitude more strongly than Zn^{2+} by thiol-substituted dextrans and that it was capable of forming di- or trithiol complexes. The possible location of the metal site at the enzyme domain that contains a vicinal dithiol does not preclude, however, the involvement of other amino acid side chains of the protein in the chelating site.

The results presented here clearly support the view that activation by low levels of divalent transition-metal ions is a fundamental property of PEPCK purified from rat liver cytosol. In the presence of millimolar levels of Mg^{2+}, the activating effect by micromolar levels of Mn^{2+}, Co^{2+}, or Cd^{2+} was not dependent on the direction in which the enzyme was assayed (forward or reverse). Furthermore, these same divalent metals at micromolar levels also stimulated the decarboxylation of OAA to form pyruvate in the presence of IDP (Colombo & Lardy, 1981). PEPCK isolated from baker's yeast is the only other example in which the effect of combinations of two metals has been reported. Micromolar levels of Cd^{2+} activated PEP synthesis and carboxylation in the presence of the Mn–nucleotide substrate complex, and it also stimulated pyruvate formation in the presence of ADP (Cannata & de Flombaum, 1974). The reasons for the differences between the present and previous findings with respect to Co^{2+} and Mn^{2+} activations (Bentle & Lardy, 1976, 1977) are not clear. It should be noted, however, that in this report

the concentration of the metals has been varied over a wider range and that a highly purified enzyme has been used. Furthermore, the activations have been performed under steady-state rather than preincubation conditions.

A detailed study of the effects of divalent transition-metal ions on the catalytic parameters of PEPCK assayed in the direction of PEP synthesis and PEP carboxylation as well as their involvement in the pyruvate-forming activity of this enzyme is presented in the accompanying paper.

References

Atkinson, D. E. (1966) *Annu. Rev. Biochem. 35*, 85.

Ballard, F. J., & Hanson, R. W. (1969) *J. Biol. Chem. 244*, 5625.

Barns, R. J., & Keech, D. B. (1972) *Biochim. Biophys. Acta 276*, 284.

Bentle, L. A., & Lardy, H. A. (1976) *J. Biol. Chem. 251*, 2916.

Bentle, L. A., & Lardy, H. A. (1977) *J. Biol. Chem. 252*, 1431.

Bentle, L. A., Snoke, R. E., & Lardy, H. A. (1976) *J. Biol. Chem. 251*, 2922.

Cannata, J. J. B., & de Flombaum, M. A. C. (1974) *J. Biol. Chem. 249*, 342.

Carlson, G. M., Colombo, G., & Lardy, H. A. (1978) *Biochemistry 17*, 5329.

Chang, H. C., & Lane, M. D. (1966) *J. Biol. Chem. 241*, 2413.

Coates, R. L., & Nowak, T. (1978) *Joint Central-Great Lakes Meeting of the American Chemical Society*, p 66.

Colombo, G., & Carlson, G. M. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1423.

Colombo, G., & Lardy, H. A. (1981) *Biochemistry* (following paper in this issue).

Colombo, G., Carlson, G. M., & Lardy, H. A. (1978) *Biochemistry 17*, 5321.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.

Foster, D. O., Lardy, H. A., Ray, P. D., & Johnston, J. B. (1967) *Biochemistry 6*, 2120.

Gaber, B. P., & Fluharty, A. L. (1968) *Q. Rep. Sulfur Chem. 3*, 317.

Hammond, K. D., & Balinsky, D. (1978) *Int. J. Biochem. 9*, 199.

Hebda, C., & Nowak, T. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1296.

Holten, D. D., & Nordlie, R. C. (1965) *Biochemistry 4*, 723.

Jacobs, E. E., Jacobs, M., Sanadi, D. R., & Bradley, L. B. (1956) *J. Biol. Chem. 3*, 147.

Johnston, J. B. (1970) Ph.D. Thesis, University of Wisconsin, Madison, WI.

Jomain-Baum, M., Schramm, V. L., & Hanson, R. W. (1976) *J. Biol. Chem. 251*, 37.

Jomain-Baum, M., Schramm, V. L., & Hanson, R. W. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 760.

MacDonald, M. J., & Lardy, H. A. (1978) *J. Biol. Chem. 253*, 2300.

Miller, R. S., Mildvan, A. S., Chang, H. C., Easterday, R. L., Maruyana, H., & Lane, M. D. (1968) *J. Biol. Chem. 243*, 6030.

Noce, P. S., & Utter, M. F. (1975) *J. Biol. Chem. 250*, 9099.

Nordlie, R. C., & Lardy, H. A. (1963) *J. Biol. Chem. 238*, 2259.

O'Sullivan, W. J. (1969) in *Data for Biochemical Research* (Dawson, R. M. C., Elliot, D. C., Elliot, W. H., & Jones, K. M., Eds.) Oxford University Press, New York.

Pogson, C. I., & Smith, S. A. (1975) *Biochem. J. 152*, 401.

Sanadi, D. R., Langley, M., & White, F. (1959) *J. Biol. Chem. 234*, 183.

Sillén, L. G., & Martell, A. E. (1964) *Stability Constants*, Special Publication No. 17, The Chemical Society, London.

Snoke, R. E., Johnston, J. B., & Lardy, H. A. (1971) *Eur. J. Biochem. 24*, 342.

Stickland, R. G. (1959) *Biochem. J. 73*, 660.

Utter, M. F., & Kolenbrander, H. M. (1972) *Enzymes, 3rd Ed. 6*, 136.

Walaas, E. (1958) *Acta Chem. Scand. 12*, 528.