A physiological role of Mn^{2+} in the regulation of cytosolic phosphoenolpyruvate carboxykinase from rat liver is unlikely

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A cytosolic cell-free system prepared from rat liver was used to study the effect of bivalent cations on the activity of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK). Steady-state concentrations of oxaloacetate in the range 5–50 μM were generated from increasing concentrations of malate + fumarate (10:1); 2 mM ITP and 3 mM Mg^{2+} were added as cofactors. Micromolar concentrations of Mn^{2+} , Fe^{2+} and, to a lesser extent, of Zn^{2+} and Co^{2+} were shown to stimulate PEPCK activity. V_{max} ($\mu mol/min$ per g of liver) increased from 0.67 to 1.68 on addition of 5 μM Fe^{2+} and to 2.34 with 2 μM Mn^{2+} , whereas no significant effect on the K_m for oxaloacetate was observed. The apparent K_a values (total) were 0.62 μM for Mn^{2+} , 1.48 μM for Zn^{2+} , 1.92 μM for Co^{2+} and 3.37 μM for Fe^{2+} , being 2–8-fold lower than the corresponding published values. Variations of the free Mn^{2+} concentration were obtained (a) by

increasing the Mn^{2+} concentration (i.e. activation curve) and (b) by simultaneous addition of Mn^{2+} and increasing concentrations of the chelating agent EGTA (i.e. inactivation curve). Different results were obtained for the activation and inactivation curves. The inactivation curve showed that PEPCK activity was almost unaffected by variations of the free Mn^{2+} concentration over the range 0.05–0.15 μM . Under comparable experimental conditions, rat liver arginase (another Mn^{2+} -dependent enzyme) was completely inactivated. From kinetic evidence, the existence of two distinct molecular forms of cytosolic rat liver PEPCK with different Mn^{2+} affinities is postulated. Considering the high affinity of PEPCK for Mn^{2+} and its relative insensitivity to changes in the free Mn^{2+} concentration, it seems rather unlikely that changes in the free cation concentration play a major role in regulating PEPCK activity *in vivo*.

INTRODUCTION

Phosphoenolpyruvate carboxykinase (GTP) (PEPCK; EC 4.1.1.32) catalyses the following reversible reaction:

The primary role of this enzyme is the catalysis of phosphoenolpyruvate formation as the first committed step in gluconeogenesis.

In the rat liver, PEPCK is located almost exclusively in the cytosol (Nordlie and Lardy, 1963) and the enzyme is a monomer of M_r 69000 (Colombo et al., 1978; Beale et al., 1985). PEPCK contains 13 cysteine residues all in the reduced state (Colombo et al., 1978; Beale et al., 1985) and the purified enzyme requires thiol compounds such as dithiothreitol and GSH for maximal activity (Ballard and Hanson, 1969; Bentle and Lardy, 1976).

Several studies have shown that the activity of rat liver PEPCK is regulated predominantly at the level of transcription in different hormonal and nutritional states (for a review see Nowak, 1986). However, in view of the key position of PEPCK in the regulation of gluconeogenesis, mechanisms of rapid activation, independent from the synthesis and/or degradation of new enzyme, have been postulated. The most likely candidates for such a regulation are transition-metal ions. Indeed, it is well established that PEPCK has a dual cation requirement: millimolar concentrations of Mg^{2+} are associated with the nucleotide substrate, whereas micromolar concentrations of a second cation (e.g. Mn^{2+} or Fe^{2+}) bind to a specific enzymic site as activator (Snoke et al., 1971; Colombo and Lardy, 1981; Schramm et al., 1981; Lee et al., 1981; Schramm, 1986; Nowak, 1986). The high selectivity for

Mn^{2+} and Fe^{2+} in the presence of physiological concentrations of Mg^{2+} has led both Schramm (1986) and Nowak (1986) to propose a mechanism for the regulation of gluconeogenesis via regulation of PEPCK. The regulation depends on adequate availability of free cations in the appropriate tissues and subcellular compartments. However, so far, most of the studies with PEPCK have been carried out under optimized assay conditions that differ from the physiological milieu with respect to pH, substrate concentration or concentrations of the activating cations. Furthermore, it was observed that many features of the activation process changed or were lost during the isolation procedure because of changes in the oxidation state of the enzyme's thiol groups necessary for cation-induced activation (Bentle and Lardy, 1976; Bentle et al., 1976; Brinkworth et al., 1981; Schramm et al., 1981; Puneekar and Lardy, 1987; Höppner et al., 1991). On the basis of studies with the purified enzyme, it has been postulated (Merryfield et al., 1982) that a protein, the so-called ferroactivator, played a significant role in the regulation of PEPCK by Fe^{2+} . However, a specific physiological relevance of this protein on PEPCK *in vivo* remains to be elucidated and has been recently questioned by Höppner et al. (1991).

The aim of this work is to investigate the effect of different transition-metal ions on the PEPCK reaction under near-physiological conditions. A cytosolic cell-free system, derived from the gluconeogenic cell-free system set up by Stoecklin et al. (1986), generating low steady-state concentrations of oxaloacetate, is used. The activation constants for Mn^{2+} , Fe^{2+} , Zn^{2+} and Co^{2+} were determined. In order to decrease the concentration of the free cations that are thought to be the allosteric activators of PEPCK *in vivo*, the chelating agent EGTA was added. Parallel experiments were performed with rat liver arginase, another

Mn²⁺-dependent hepatic enzyme, to verify the Mn²⁺-sensitivity of the system. The results indicate that a physiological role of Mn²⁺ in the regulation of PEPCK *in vivo* is very unlikely.

EXPERIMENTAL

Materials

Pyruvate, dithiothreitol, malate dehydrogenase, lactate dehydrogenase, glutamate dehydrogenase and urease S were obtained from Boehringer (Mannheim, Germany). Other enzymes, coenzymes and GSH were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-Malate, L-lactate (sodium salt), ITP (sodium salt) and sucrose were from Serva (Heidelberg, Germany), and L-[U-¹⁴C]malic acid was from The Radiochemical Centre (Amersham, Bucks., U.K.). Chelex 100 was purchased from Bio-Rad (Richmond, CA, U.S.A.) and Sephadex G-25 medium from Pharmacia (Uppsala, Sweden). All other reagents were of the highest purity commercially obtainable. Solutions were prepared with double-quartz-distilled water. Contamination of the assay mixtures with bivalent cations was avoided by taking the precautions described by Mörikofer-Zwez (1983).

Male albino Wistar rats weighing 180–230 g from Madörin (Füllinsdorf, Switzerland) were either fed on a chow diet *ad libitum* (arginase experiments) or deprived of food for 48 h before use (PEPCK experiments).

Cytosol preparation

For the preparation of cytosols, 3 g wet wt. of liver from rats killed by decapitation was immediately homogenized (Potter–Elvehjem homogenizer) at 4 °C in 15 ml of buffer containing 0.14 M sucrose, 50 mM potassium phosphate buffer, pH 7.2, 2 mM GSH and 0.1 mM EDTA, to bind bivalent cations. The homogenate was centrifuged, and the cytosol was freed from EDTA and other low-*M_r* compounds by gel filtration and was further treated as described previously (Stoecklin et al., 1986).

PEPCK assay

The activity of PEPCK was determined in an incubation assay in the direction of phosphoenolpyruvate formation by using the sum of phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate formed as a measure for enzyme activity. Steady-state concentrations of oxaloacetate were generated from malate + fumarate (10:1) and NAD⁺ through the action of malate dehydrogenase and fumarase present in the cytosol. NADH was buffered at a low and constant concentration by lactate dehydrogenase acting on lactate and pyruvate added at a ratio of 30:1. The formation of phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate was determined in a standard incubation mixture containing 33 mM Tris/HCl buffer, pH 7.4, 0.66 mM GSH, 0.8 mM dithiothreitol, 3 mM MgSO₄, 1.63 mM NAD⁺, 20 mM lactate, 0.7 mM pyruvate, 80 mM KCl and 1.7 mM P_i, the last originating from the added cytosol. The concentration of malate + fumarate (10:1) was varied between 1.1 mM and 13.2 mM, generating oxaloacetate concentrations between 5 and 50 µM.

Incubations were carried out in plastic tubes at 30 °C in a final volume of 1.5 ml. Cytosol (0.05 ml) containing 0.8 mg of protein was added, and the samples were preincubated for 3 min. Then the PEPCK reaction was started by addition of 2 mM ITP. Reactions were stopped after 10 min with ice-cold HClO₄ (final

concn. 0.4 M). The samples were treated further as described previously (Mörikofer-Zwez et al., 1981).

Arginase assay

The activity of arginase was assayed as described previously (Maggini et al., 1992).

Analysis of metabolites

Metabolites were determined in the neutralized extracts as described previously (Stoecklin et al., 1986; Maggini et al., 1992). Formation of ¹⁴CO₂ from L-[U-¹⁴C]malate (sp. radioactivity 0.08 µCi/µmol) was measured as described by Mehlmann et al. (1967).

Protein was determined by the biuret method with BSA as standard. Protein concentration in the cytosol was 16.7 ± 1.8 mg/ml (mean ± S.D., *n* = 40) for PEPCK and 14.8 ± 1.3 (mean ± S.D., *n* = 5) for arginase experiments.

Calculations

The activity of PEPCK was calculated by using the difference of net formation of phosphoenolpyruvate + 2-phosphoglycerate + 3-phosphoglycerate in samples incubated with and without ITP. The steady-state concentrations of oxaloacetate were calculated by using the equilibrium constants for lactate dehydrogenase and malate dehydrogenase reported by Hohorst et al. (1959) and the measured values for malate, lactate and pyruvate.

To ensure that, in our system, only the gluconeogenic reaction sequence from malate to 3-phosphoglycerate is operative with no interfering side reactions, parallel incubations were carried out with and without ¹⁴C-labelled substrate. ¹⁴CO₂ production as a measure of the direct decarboxylation of oxaloacetate by PEPCK and the formation rate of phosphoenolpyruvate + 2-phosphoglycerate + 3-phosphoglycerate was identical under different conditions of incubation time and/or addition of Mn²⁺ (results not shown). These results exclude a recycling of phosphoenolpyruvate to pyruvate via pyruvate kinase, as well as formation of other trioses beyond the phosphoglycerate step in the gluconeogenic pathway under the incubation conditions used. They furthermore exclude interference by the pyruvate-forming activity of PEPCK through irreversible decarboxylation of oxaloacetate to pyruvate as proposed by Colombo and Lardy (1981). The activity of arginase was calculated using the net formation of urea between 10 and 20 min incubation time as described by Maggini et al. (1992).

Kinetic parameters were obtained by fitting the experimental data to the Hill equation. The calculation was based on a procedure choosing values of *h* (the Hill coefficient) in a restricted range (Atkins, 1973) and fitting the data by non-linear regression (Gampp et al., 1985). Model comparison (Michaelis–Menten versus Hill model) was performed by the F-test to determine the significance of calculated *h* values versus 1.0 (Michaelis–Menten model). The results presented are those giving the best fit with the experimental data.

The concentrations of the free Me²⁺ were calculated from simultaneous equations expressing the stability constants for the EGTA–Me²⁺, ITP–Me²⁺ or ATP–Me²⁺ complexes and the total concentrations of the components, by an iterative algorithm based on the program of Zuberbühler and Kaden (1982). The following stability constants (reported for Tris/HCl buffer, pH 7.4, by Bartfai, 1979) were used: $K^{\text{Mn-EGTA}} = 5.64 \times 10^7 \text{ M}^{-1}$, $K^{\text{Mg-EGTA}} = 7.07 \text{ M}^{-1}$, $K^{\text{Mn-ITP}} = 1.60 \times 10^5 \text{ M}^{-1}$, $K^{\text{Mg-ITP}} = 2.04 \times 10^4 \text{ M}^{-1}$, $K^{\text{Mn-ATP}} = 3.30 \times 10^4 \text{ M}^{-1}$, $K^{\text{Mg-ATP}} = 1.74 \times 10^4 \text{ M}^{-1}$.

Statistics

Statistical significance of differences was assessed by Student's *t* test.

RESULTS

On the basis of previous work carried out in our laboratory (Stoecklin et al., 1986), a cell-free gluconeogenic system was set up that allows the determination of the activity of PEPCK in the direction of oxaloacetate decarboxylation. Variations in the concentration of malate + fumarate generated low steady-state concentrations of oxaloacetate between 5 and 50 μ M, corresponding to the hepatic physiological concentration (6–40 μ M;

Figure 1 Effect of Mn²⁺ and Fe²⁺ on the kinetic constants of PEPCK

PEPCK activity was assayed in the standard incubation mixture described in the Experimental section (i.e. in the presence of 3 mM MgSO₄). The concentration of the substrate oxaloacetate was varied by varying the malate + fumarate (10:1) concentration from 1.1 to 13.2 mM. Key: ○, control without further additions; ●, 2 μ M Mn²⁺; ▲, 5 μ M Fe²⁺; —, curves fitted to the Hill equation (*h* = 1) by the method of Atkins (1973). Results are expressed as means $\pm$ S.E.M. from 4–10 independent experiments.

Table 1 Effect of different bivalent cations on PEPCK activity

PEPCK activity was assayed in the standard incubation mixture described in the Experimental section (i.e. in the presence of 3 mM MgSO₄). Basal activity in the absence of Me²⁺ at 12.7 $\pm$ 1.7 μ M (mean $\pm$ S.D., *n* = 4–6) oxaloacetate was 0.12 $\pm$ 0.01 μ mol/min per g of liver (mean $\pm$ S.E.M., *n* = 19). The concentrations of the added cations were 0.1, 0.25, 0.5, 1.0, 2.0, 5.0, 10.0 μ M for Zn²⁺, Co²⁺ and Fe²⁺, and 0.1, 0.25, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 30.0 μ M for Mn²⁺. Kinetic parameters were determined by fitting the data to the Hill equation (*h* = 1) by the method of Atkins (1973). Results are expressed as means $\pm$ S.E.M. (except as indicated), and the number of independent experiments (*n*) is indicated in the Table.

Added cation	<i>V</i> _{max} (μ mol/min per g of liver)	<i>K</i> _a (Me ²⁺) (μ M)	Final [oxaloacetate] (mean $\pm$ S.D.) (μ M)	<i>n</i>
Zn ²⁺	0.79 $\pm$ 0.02	1.48 $\pm$ 0.11	12.6 $\pm$ 1.3	4
Co ²⁺	0.85 $\pm$ 0.03	1.92 $\pm$ 0.18	11.9 $\pm$ 1.7	4
Fe ²⁺	1.40 $\pm$ 0.08	3.37 $\pm$ 0.67	13.6 $\pm$ 1.9	6
Mn ²⁺	1.43 $\pm$ 0.04	0.62 $\pm$ 0.08	12.7 $\pm$ 1.9	5

Siess et al., 1982). The calculated oxaloacetate concentrations (see the Experimental section) decreased maximally by 10% during the whole observation period of 10 min. The concentration of phosphoenolpyruvate + 2-phosphoglycerate + 3-phosphoglycerate, as a measure of the PEPCK activity, increased linearly over this incubation period, and the lactate/pyruvate ratio did not change significantly (results not shown). Figure 1 shows the relation between PEPCK activity and oxaloacetate concentration. In the absence of added cations, *V*_{max} (μ mol/min per g of liver) (mean $\pm$ S.E.M., *n* = 4–10) was 0.67 $\pm$ 0.05; on addition of 5 μ M Fe²⁺ and 2 μ M Mn²⁺, it increased to 1.68 $\pm$ 0.24 and 2.34 $\pm$ 0.36 respectively. No significant variation in the apparent *K*_m (oxaloacetate) (mean $\pm$ S.E.M., *n* = 4–10) was observed (no additions, 17.4 $\pm$ 2.7 μ M; 5 μ M Fe²⁺, 13.7 $\pm$ 4.8 μ M; 2 μ M Mn²⁺, 21.7 $\pm$ 4.6 μ M).

The effect of different bivalent cations on PEPCK activity was further investigated at a fixed substrate concentration. Beside the known and well-characterized effects of Mn²⁺ and Fe²⁺, also Co²⁺ and Zn²⁺ have been studied (Table 1). All four cations were shown to enhance PEPCK, although the activating effects of Co²⁺ and Zn²⁺ (~ 5-fold activation) were not as strong as with Mn²⁺ and Fe²⁺ (~ 10-fold activation). Mn²⁺ was the most effective activator, with a *K*_a (total) of 0.62 μ M. The highest *K*_a (total) was measured for Fe²⁺ (3.37 μ M), whereas for Zn²⁺ and Co²⁺ the *K*_a values (total) were 1.48 μ M and 1.92 μ M respectively. Because of binding of the cations to ITP, the actual activation constants, expressed in terms of free concentration, are considerably lower than the values calculated from the added total cation concentrations. However, only for Mn²⁺ an exact calculation of the free concentration was possible, since stability constants for the other Me²⁺-ITP complexes were not available. Under our experimental conditions, only about 7% of the added Mn²⁺ was free. The *K*_a (Mn²⁺ free) was calculated to be 45 $\pm$ 6 nM. Assuming similar stability constants as for the Mn²⁺-ITP complex, the *K*_a (Me²⁺ free) for the other cations would be approx. 236 $\pm$ 47 nM (Fe²⁺), 104 $\pm$ 8 nM (Zn²⁺) and 134 $\pm$ 13 nM (Co²⁺).

Since the most likely candidates for PEPCK activation *in vivo* appear to be Mn²⁺ and Fe²⁺, their free concentration was lowered by addition of the chelating agent EGTA. Metal ion concentrations in the range 0.5–2.0 μ M were used, together with EGTA concentrations of 0.25–5.0 μ M. The results (Table 2) show that with EGTA concentrations up to 3 μ M, there was no significant effect on PEPCK activity, in spite of a 8–10-fold decrease in the free Me²⁺ concentrations (calculated as described in the Experimental section). In the presence of Mn²⁺, a further increase in the EGTA concentration to 5 μ M caused a marked decrease in PEPCK activity. The free Mn²⁺ concentration under these conditions was 15-fold lower than for the controls without EGTA.

The presence of an impurity (e.g. another bivalent cation) in the cell-free system could also account for the reported partial lack of reversal of cation-induced stimulation of PEPCK, provided that added EGTA would complex this bivalent cation rather than binding the known PEPCK activators Mn²⁺, Fe²⁺, Co²⁺ or Zn²⁺.

In consideration of the precautions taken while preparing all the solutions used for the experiments (see the Experimental section), it is likely that, if a cation was present as an impurity in the experimental system, it would be added together with the cytosol and not with the incubation mixture. To exclude this possibility, similar experiments to those described in Table 2 were performed with a cytosol prepared with an homogenization buffer containing 0.2 mM EGTA in addition to the 0.1 mM EDTA routinely used. Since the results were the same as in Table 2, the presence of bivalent cations in the cytosol is excluded.

Table 2 Effect of EGTA on the Mn^{2+} -induced activation of PEPCK

PEPCK activity was assayed in the standard incubation mixture described in the Experimental section (i.e. in the presence of 3 mM MgSO_4). The final concentration of oxaloacetate was $12.7 \pm 1.7 \mu\text{M}$ (mean $\pm$ S.D., $n = 3-5$). The resulting concentrations of free Mn^{2+} calculated on the basis of the stability constants for the Me^{2+} -ITP and Me^{2+} -EGTA complexes (see the Experimental section) are indicated in parentheses. An exact calculation of the free Fe^{2+} concentration was not possible, since literature data on the stability constants were not available. Results are expressed as means $\pm$ S.E.M. from 3-5 independent experiments.

Addition	EGTA added (μM) ...	PEPCK activity ($\mu\text{mol/min per g of liver}$)					
		0	0.5	1.0	2.0	3.0	5.0
None		0.12 ± 0.01					
$0.5 \mu\text{M Fe}^{2+}$		0.16 ± 0.01	0.16 ± 0.01	0.19 ± 0.02	0.18 ± 0.02		
$1.0 \mu\text{M Fe}^{2+}$		0.22 ± 0.02	0.26 ± 0.05	0.20 ± 0.02	0.23 ± 0.02	0.23 ± 0.02	
$2.0 \mu\text{M Fe}^{2+}$		0.31 ± 0.02	0.29 ± 0.03	0.32 ± 0.02	0.31 ± 0.03	0.32 ± 0.04	
$0.5 \mu\text{M Mn}^{2+}$		0.59 ± 0.05	0.58 ± 0.06	0.57 ± 0.06	0.51 ± 0.07		0.27 ± 0.07
		($0.034 \mu\text{M}$)	($0.017 \mu\text{M}$)	($0.010 \mu\text{M}$)	($0.005 \mu\text{M}$)		($0.002 \mu\text{M}$)
$1.0 \mu\text{M Mn}^{2+}$		0.89 ± 0.08	0.84 ± 0.10	0.80 ± 0.08	0.81 ± 0.08	0.79 ± 0.10	0.54 ± 0.15
		($0.068 \mu\text{M}$)	($0.044 \mu\text{M}$)	($0.027 \mu\text{M}$)	($0.012 \mu\text{M}$)	($0.008 \mu\text{M}$)	($0.004 \mu\text{M}$)
$2.0 \mu\text{M Mn}^{2+}$		1.21 ± 0.07	1.17 ± 0.08	1.16 ± 0.08	1.11 ± 0.08	1.07 ± 0.08	0.78 ± 0.07
		($0.157 \mu\text{M}$)	($0.107 \mu\text{M}$)	($0.081 \mu\text{M}$)	($0.041 \mu\text{M}$)	($0.019 \mu\text{M}$)	($0.010 \mu\text{M}$)

Figure 2 Effect of EGTA on the Mn^{2+} -induced activation of arginase (a) and PEPCK (b)

●, activation curves (i.e. Mn^{2+} -dependence in the absence of EGTA); ○, inactivation curves (i.e. simultaneous addition of Mn^{2+} and increasing concentrations of EGTA). (a) Arginase activity was assayed as described previously (Maggini et al., 1992). (b) PEPCK activity was assayed in the standard incubation mixture described in the Experimental section (i.e. in the presence of 3 mM MgSO_4). The final concentration of oxaloacetate was $13.0 \pm 0.4 \mu\text{M}$ (mean $\pm$ S.D., $n = 5$). The resulting concentrations of free Mn^{2+} were calculated on the basis of the stability constants for the EGTA- Me^{2+} and the ITP- or ATP- Me^{2+} complexes reported in the Experimental section. Results are expressed as means $\pm$ S.E.M. from 5 independent experiments, except where indicated.

In order to test the ability of EGTA to bind Me^{2+} under the described experimental conditions, experiments were performed with arginase. Arginase is the last enzyme of the urea cycle, and it catalyses the hydrolysis of arginine to urea and ornithine. Rat liver arginase is, like PEPCK, a Mn^{2+} -dependent enzyme, and recently its K_a (Mn^{2+}) was reported to be $8 \mu\text{M}$, when enzyme

Figure 3 Eadie-Hofstee plot of the data of Figure 2(b)

Results are expressed as means from 5 independent experiments.

activity was assayed under near-physiological conditions (Maggini et al., 1992).

Figure 2 shows a comparison of the effects resulting from concomitant addition of EGTA and of Mn^{2+} on the activities of arginase and PEPCK. For arginase, a decrease in the free Mn^{2+} concentration caused by EGTA was shown to inactivate the enzyme as expected. Furthermore, the activation curve (normal Mn^{2+} -dependence in the absence of EGTA) and the inactivation curve (simultaneous addition of Mn^{2+} and increasing EGTA concentrations) were identical. In other words, Mn^{2+} -induced stimulation of arginase activity could be completely reversed by EGTA. For PEPCK, on the other hand, the activation and inactivation curves were not identical. Addition of EGTA concentrations in the range 10-40 μM together with 30 μM Mn^{2+} left PEPCK activity almost unaffected, in spite of a 30-fold decrease in the free metal concentration. Higher EGTA concentrations ($> 50 \mu\text{M}$), resulting in a free Mn^{2+} concentration of about $0.05 \mu\text{M}$, were necessary to inhibit enzyme activity. One should note that for these experiments higher concentrations of both Mn^{2+} and EGTA were used compared with the experiments of Table 2. This was necessary to allow a direct comparison with arginase, since the K_a (Mn^{2+}) of the latter enzyme is about 10-fold higher than that of PEPCK.

In conclusion, these results show that the partial lack of reversal of Me²⁺-induced activation of PEPCK is not due to the inability of EGTA to bind Me²⁺ in the cell-free incubation system.

Figure 3 shows an Eadie–Hostee plot of the data reported in Figure 2(b) and indicates the presence of two Mn²⁺-binding sites with K_a (Mn²⁺ free) values of about 10 and 40 nM.

DISCUSSION

The kinetics of purified hepatic PEPCK have been extensively investigated by means of initial-rate measurements and fixed time assays with saturating or sub-saturating substrate concentrations (Bentle and Lardy, 1976; Bentle et al., 1976; Colombo and Lardy, 1981; Brinkworth et al., 1981; Lee et al., 1981; Schramm et al., 1981).

An alternative to the experiments with purified enzyme preparations is represented by steady-state assays carried out in cell-free systems. Such systems leave the enzymes in a more physiological environment. This aspect is particularly important for PEPCK, since it was observed that purification of this enzyme results in changes in the oxidation state of the thiol groups necessary for proper cation-induced activation (Ballard and Hanson, 1969; Bentle and Lardy, 1976). The importance of carrying out experiments on metal-induced activation under near-physiological conditions has been further emphasized by Schramm (1986).

The kinetic parameters for rat liver PEPCK determined in the present work under near-steady-state conditions were in good agreement with literature data. On addition of Mn²⁺, a 3.5-fold increase in V_{max} was measured, whereas no effect on the K_m for oxaloacetate could be detected. Schramm et al. (1981) reported a 3–4-fold increased V_{max} upon Mn²⁺ addition and no significant effect of the cation on the K_m (oxaloacetate) (10 μ M) for the purified rat liver enzyme. Thus the results show the suitability of our experimental system for studying the kinetics of PEPCK under near-physiological conditions.

The activation constant for Mn²⁺ (total) for highly purified PEPCK preparations under incubation conditions similar to those of our cell-free system (e.g. in the presence of millimolar concentrations of Mg²⁺) has been reported to be 1.7–5.4 μ M for the cytosolic rat liver enzyme (Schramm et al., 1981; Titheradge et al., 1992) and 2 μ M (Lee et al., 1981) for the chicken liver mitochondrial enzyme. In our system, the K_a (total) for Mn²⁺ was 0.62 ± 0.08 μ M, corresponding to a K_a (free) of 45 ± 6 nM. The free concentration of Mn²⁺ determined in hepatocytes isolated from fed rats is in the range 0.6–0.8 μ M, and decreased to 0.2–0.3 μ M upon fasting (Ash and Schramm, 1982).

For Fe²⁺, activation constants (total) between 5 and 10 μ M are found in the literature (Bentle and Lardy, 1977), whereas in our system a K_a (total) of 3.37 ± 0.67 μ M was determined. The concentration of free Fe²⁺ and Fe³⁺ in living cells is thought to be very small, because many biological materials are known to have a high affinity for these ions (Lardy and Hughes, 1984; Richardson and Baker, 1992). However, it has been shown that mobilization of mitochondrial Fe²⁺ through the action of hormones and/or Ca²⁺ could provide a total Fe²⁺ concentration of 3 μ M in the liver cytosol (Lardy and Hughes, 1984).

The K_a values (total) for Zn²⁺ and Co²⁺ measured for the avian enzyme were 10 μ M and 4.8 μ M respectively (Lee et al., 1981), being thus 2–7-fold higher than our values (Table 1). The total concentration of Co²⁺ has been reported to be 1–2 μ M for the intact liver (Snoke et al., 1971). For Zn²⁺ reliable values for hepatocytes are still lacking, but the free concentration of this cation is probably below 1 μ M (Brand and Heinickel, 1991).

Furthermore, little is known about the subcellular localization of the free pools of these cations. It is thus not clear yet whether these two cations are suitable candidates for controlling PEPCK activity *in vivo*.

From our data, the most likely candidates for regulation of PEPCK activity *in vivo* are Mn²⁺ and Fe²⁺. Especially, variations in the free concentrations of these cations should account for PEPCK activation *in vivo* as proposed by several authors (Schramm, 1986; Nowak, 1986; Lardy and Hughes, 1984).

In order to decrease the free Me²⁺ concentration, EGTA was added to the cell-free system. Arginase was used as a control to test the EGTA-sensitivity of the experimental system. Recently, the kinetics of rat liver arginase have been investigated under near-physiological conditions by means of a cell-free system sharing several similarities to the one used for the PEPCK assay (for details see Maggini et al., 1992). Striking differences were observed in the activation (normal Mn²⁺-dependence without added EGTA) and inactivation curves (addition of both Mn²⁺ and EGTA) for the two enzymes (Figure 2). Activation of arginase by Mn²⁺ could be completely reversed by EGTA, whereas a decrease in the free Mn²⁺ concentration from 1.5 μ M to 0.05 μ M did not influence PEPCK activity. The Eadie–Hofstee plot shown in Figure 3 indicates the existence of two binding sites for Mn²⁺.

Since only one Mn²⁺-binding site is present on the monomeric PEPCK enzyme (Nowak, 1986), and since there is no evidence in the literature concerning the existence of different cytosolic isoenzymes, our results (Figure 3) could rather indicate the presence of two molecular forms of PEPCK, e.g. differing in their oxidation states. Höppner et al. (1991) have also suggested that alterations in the oxidation state of the thiol groups of PEPCK in response to the redox state of the cell could generate two forms of PEPCK with different sensitivity for bivalent cations.

The results presented here show (a) the existence of a PEPCK form with a high-affinity binding site for Mn²⁺ [K_a (free) = 10 nM, corresponding to a K_a (total) $\approx$ 0.15 μ M] (see Figure 3) and (b) the relative insensitivity of PEPCK to changes in the free Mn²⁺ concentration over the physiological range (0.05–1.5 μ M; Figure 2b). It is therefore concluded that variations in the free concentration of Mn²⁺ like those occurring under non-pathological conditions *in vivo* do not result in significant regulation of the flux through the PEPCK reaction.

We are indebted to Dr. A. Jakob for the programming for the calculations of the free metal concentrations. We thank Mrs. Hanni Zürcher and Mrs. Ruth Bolz for skilful technical assistance.

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Received 19 October 1992/31 December 1992; accepted 15 January 1993