Physiological concentrations of 2-oxoglutarate regulate the activity of phosphoenolpyruvate carboxykinase in liver

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2-Oxoglutarate was found to inhibit purified rat liver phosphoenolpyruvate carboxykinase when the assay was performed in the direction of either phosphoenolpyruvate or oxaloacetate synthesis. The inhibition was competitive with respect to oxaloacetate or phosphoenolpyruvate, the K_i values being 0.32 ± 0.04 mM and 0.63 ± 0.19 mM respectively. 2-Oxoglutarate inhibited non-competitively when tested against GTP or Mn^{2+} . The reported cytosolic concentrations of 2-oxoglutarate in rat hepatocytes are such that the enzyme is likely to be significantly inhibited under basal conditions. The cytosolic concentration of 2-oxoglutarate is known to fall precipitously under the influence of glucagon and other hormones that stimulate gluconeogenesis, and it is suggested that the hormone-induced decrease in 2-oxoglutarate content would alleviate the inhibition of phosphoenolpyruvate carboxykinase and stimulate flux from oxaloacetate to phosphoenolpyruvate. The implications of this finding to the rationalization of the role of pyruvate kinase in the stimulation of gluconeogenesis in the fasted state are discussed.

INTRODUCTION

It has been established for many years that gluconeogenic hormones acutely increase glucose synthesis from C_3 precursors by an acceleration of the conversion of pyruvate into phosphoenolpyruvate (PEP), the intracellular concentration of pyruvate decreasing while that of PEP increases (Exton & Park, 1969; Williamson *et al.*, 1969; Ui *et al.*, 1973a). More recent studies using control strength analysis and curve-subtraction techniques have suggested that, although the maximal flux through gluconeogenesis is limited by pyruvate carboxylase activity, the effect of hormones to stimulate the pathway can be accounted for by an increased conversion of oxaloacetate (OAA) into PEP (Groen *et al.*, 1983; Sistare & Haynes, 1985). This could be the result of either an increased formation of PEP from OAA by a stimulation of PEP carboxykinase (PEPCK) or a decreased utilization of PEP by pyruvate kinase. In the absence of any known acute effects of glucagon on PEPCK activity, the latter was attributed almost exclusively to an inhibition of pyruvate kinase activity (Groen *et al.*, 1983; Sistare & Haynes, 1985). This model would fit with the known phosphorylation and inactivation of pyruvate kinase activity by both glucagon and Ca^{2+} -dependent hormones (Feliu *et al.*, 1976; Garrison & Borland, 1978; Chan & Exton, 1978; Riou *et al.*, 1978; Garrison *et al.*, 1979; Garrison & Wagner, 1982), although the ability of the latter to cause a significant inhibition of pyruvate kinase activity is questionable (Hue *et al.*, 1978; Chan & Exton, 1978; Blair *et al.*, 1979). However, this model contrasts with the conclusions drawn from direct measurements of pyruvate kinase flux using isotopic-tracer techniques, which suggest that it is the forward reactions which are important in control, i.e. the formation of OAA or the conversion of OAA into PEP by PEPCK (Rognstad & Katz, 1977; Haynes & Picking, 1990). These studies suggest that pyruvate kinase flux is low in cells prepared from the starved animal and that either there is no significant change with glucagon or Ca^{2+} -dependent hormones (Rognstad & Katz, 1977) or there is a small inhibition in the presence of glucagon which is insufficient to account for most of the stimulation of gluconeogenesis (Haynes & Picking, 1990).

The apparent inconsistencies between these two models can be rationalized if the effects of the hormones are to increase PEPCK flux, either in addition to or instead of decreasing pyruvate kinase flux, as these cannot be distinguished by the curve-subtraction techniques. A stimulation of PEPCK could occur either by increasing the supply of OAA, or by a direct effect of the hormone at the level of PEPCK, or both (Haynes & Picking, 1990). There is no consistent evidence for a rise in the cytosolic concentration of OAA after treatment of the cells with glucagon (Haynes & Picking, 1990, and references therein), suggesting the possibility of kinetic control of PEPCK, although evidence for this is lacking. The enzyme is a monomer of M_r 72000 (Brinkworth *et al.*, 1981), which can be activated by the presence of Mn^{2+} and Fe^{2+} ions (Holten & Nordlie, 1965; Foster *et al.*, 1967; Snoke *et al.*, 1971). No rapid effects of glucagon have been observed on PEPCK activity (Eisenstein & Strack, 1968), nor have any effects *in vitro* been observed after incubation of cell-free extracts with cyclic AMP under a wide variety of conditions (Wicks *et al.*, 1972). A rise in cytosolic Ca^{2+} produced by hormones has been proposed to activate PEPCK by a redistribution of either Fe^{2+} (Merryfield & Lardy, 1982) or Mn^{2+} (Karczmarewicz *et al.*, 1985); however, these studies have not been confirmed or elaborated further.

The most consistent observation reported after acute treatment of either perfused livers (Williamson *et al.*, 1969; Ui *et al.*, 1973a,b; Haussinger & Sies, 1984) or hepatocytes (Siess *et al.*, 1977, 1982; Ochs, 1984; Staddon & McGivan, 1984) with gluconeogenic hormones is a dramatic fall in the tissue 2-oxoglutarate concentration. As this is a homologue of OAA, we speculated that this might act as a competitive inhibitor of PEPCK, although Utter & Kolenbrander (1972) stated that tricarboxylic-acid-cycle intermediates were ineffective as inhibitors or activators when tested against limiting concentrations of OAA. The decline in 2-oxoglutarate produced in liver by hormones that stimulate glucose synthesis from lactate/pyruvate could provide the kinetic control of PEPCK by releasing the enzyme from a basal, inhibited, state, provided that the activity of PEPCK is sensitive to changes in the concentrations of 2-

Abbreviations used: PEP, phosphoenolpyruvate; PEPCK, PEP carboxykinase (EC 4.1.1.32); OAA, oxaloacetate.

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oxoglutarate present in control and hormone-stimulated hepatocytes. The data presented in this paper indicate that the available data are consistent with this hypothesis.

EXPERIMENTAL

Materials

GTP-agarose, 2-oxoglutarate, 3-oxoglutarate, malate, glutamate, GTP, PEP, nucleoside-5'-diphosphate kinase (50% glycerol suspension) and malate dehydrogenase (50% glycerol suspension) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Other reagents were of AnalaR grade or similar from BDH Chemicals, Poole, Dorset, U.K., or Sigma.

Methods

PEPCK was purified from fasted male Sprague-Dawley or Wistar rats weighing 300–400 g. The purification procedure was essentially as described by Philippidis *et al.* (1972) using $(\text{NH}_4)_2\text{SO}_4$ fractionation, followed by Sephadex G-100 and DEAE-cellulose column chromatography. The enzyme was precipitated with 70% satd. $(\text{NH}_4)_2\text{SO}_4$ and the activity extracted from the precipitate with decreasing concentrations of $(\text{NH}_4)_2\text{SO}_4$ (Ballard & Hanson, 1969). The final purification step was replaced by adsorption on and elution from GTP-agarose in the absence of added Mn^{2+} as described by Brinkworth *et al.* (1981). The enzyme had an activity of 8 units or greater per mg of protein as measured in the direction of PEP synthesis when freshly prepared. Analysis of the purified enzyme by SDS/PAGE indicated that the most predominant band was PEPCK, with a M_r of approx. 70000, together with two minor components of M_r approx. 50000 and 37000. Similar trace impurities were also evident in the preparation of Brinkworth *et al.* (1981). However, in contrast with the former study, no evidence was found for contamination of the preparation with the M_r -29000 protein, which has subsequently been identified as glutathione peroxidase (Punekar & Lardy, 1987).

Enzymic activity in the direction of PEP synthesis was assayed spectrophotometrically at 37°C in a basic assay medium containing 50 mM-Hepes (pH 7.0 or 8.0), 50 μM -GTP, 75 μM - MnCl_2 , 1 mM-ATP, 1.5 mM- MgCl_2 , 7.5 units of malate dehydrogenase/ml and 0.2 unit of nucleoside-diphosphate kinase/ml. Malate and NAD^+ were added in equal concentrations to provide the desired concentration of OAA. A value of 6.4×10^{-13} M for the malate dehydrogenase equilibrium constant was used to determine the amounts of malate and NAD^+ added (Yoshida, 1965). The concentrations of OAA generated were confirmed by measuring the increase in A_{339} on addition of malate dehydrogenase (Bergmeyer, 1974). The reaction was initiated by adding PEPCK, and the increase in A_{339} was monitored. The reaction was linear for at least 2 min in the presence of the GTP-regenerating system, and doubling the amount of malate dehydrogenase did not increase the rate of the PEPCK reaction, indicating that the malate dehydrogenase was not rate-limiting in the system. Spontaneous decarboxylation of the OAA in the assay appeared to be minimal, as a steady-state absorbance was achieved in the presence of malate dehydrogenase over the time of the PEPCK assay. In some experiments PEPCK activity was determined directly by the measurement of the accumulation of PEP in neutralized HClO_4 extracts during 2 min incubations (Lowry & Passonneau, 1972). PEPCK activity was measured spectrophotometrically in the direction of OAA formation as described by Bente & Lardy (1976), except that the concentrations of MnCl_2 and MgCl_2 were altered to give final concentrations of 75 μM and 1.5 mM respectively.

Data were analysed by using an iterative non-linear-regression

data-analysis computer program that tested for closeness of fit to the rate equations for competitive, non-competitive and uncompetitive inhibition of single-substrate reactions (Enzfitter; Biosoft, Cambridge, U.K.). This was justified in that, in all cases, the second substrate and activator were held constant at near-saturating levels while one substrate or activator was varied. In the double-reciprocal plots the lines were drawn by using the K_i , V_{max} and K_m values derived from the fitted data with the smaller variance. The results shown are representative of two to four replicates using three separate enzyme preparations.

RESULTS

Fig. 1(a) shows the effect of increasing concentrations of 2-oxoglutarate on PEPCK activity measured in the direction of PEP synthesis at pH 8.0, the standard pH used in the definitive studies of the kinetics of this enzyme and its inhibition by 3-mercaptopycolinate (Jomain-Baum *et al.*, 1976; Jomain-Baum & Schramm, 1978; Schramm *et al.*, 1981). The K_m for OAA was comparable with that described previously for measurements where spontaneous OAA decarboxylation was minimized by maintaining it in equilibrium with malate using the malate dehydrogenase reaction (Jomain-Baum *et al.*, 1976; Jomain-Baum & Schramm, 1978; Schramm *et al.*, 1981), and is the same order of magnitude as the cytosolic concentrations of OAA (Williamson *et al.*, 1969; Siess *et al.*, 1977; Groen *et al.*, 1983; Rognstad, 1987; Haynes & Picking, 1990). At all concentrations of OAA tested in the physiological range, the addition of 2-oxoglutarate significantly inhibited PEPCK activity. The inhibition was competitive with respect to OAA, with a K_i of 0.26 ± 0.04 mM.

As it has been suggested that tricarboxylic-acid-cycle intermediates were ineffective as inhibitors of PEPCK (Utter & Kolenbrander, 1972), it was necessary to prove that the effect of 2-oxoglutarate was at the level of PEPCK and not at the level of the malate dehydrogenase monitoring system. Therefore PEP formation was measured directly by fluorimetric analysis, and the results are shown in the inset (Fig. 1b). It is evident that there

Fig. 1. Effect of 2-oxoglutarate on PEPCK activity with variable concentrations of OAA

PEPCK activity was measured at pH 8 as described in the Experimental section by either spectrophotometric (a) or chemical means (b). The K_m for OAA was determined as 3.6 ± 0.6 μM by the spectrophotometric method and 5.4 ± 0.7 μM by the chemical method. By using the model for competitive inhibition, the K_i values for 2-oxoglutarate were found to be 0.26 ± 0.04 mM and 0.36 ± 0.04 mM respectively by the two methods.

Fig. 2. Effect of 2-oxoglutarate on PEPCK activity with variable concentrations of GTP

PEPCK activity was measured as described in the Experimental section with the concentrations of OAA and Mn^{2+} fixed at $20 \mu\text{M}$ and $75 \mu\text{M}$ respectively. The K_m for GTP was determined to be $7.5 \pm 1.5 \mu\text{M}$ and the K_i for 2-oxoglutarate as $1.8 \pm 0.2 \text{ mM}$, by using the model for non-competitive inhibition.

Fig. 3. Effect of 2-oxoglutarate on PEPCK activity with variable concentrations of Mn^{2+}

PEPCK activity was measured as described in the Experimental section with the concentrations of OAA and GTP fixed at $20 \mu\text{M}$ and $50 \mu\text{M}$ respectively. The K_m for Mn^{2+} was determined to be $3.0 \pm 0.6 \mu\text{M}$ and the K_i for 2-oxoglutarate as $1.6 \pm 0.2 \text{ mM}$ by using the model for non-competitive inhibition.

is no significant difference between the two assay methods, the K_m for OAA and the K_i for 2-oxoglutarate being very similar, indicating that the effect of 2-oxoglutarate is not at the level of the monitoring system. Further evidence that 2-oxoglutarate was not active in this system by inhibiting malate dehydrogenase was shown as follows: (1) 2-oxoglutarate did not inhibit malate dehydrogenase when measured in the direction of malate synthesis; (2) the position and time taken to reach the pre-reaction equilibrium of the dehydrogenase reaction were not affected by 2-oxoglutarate; (3) 2-oxoglutarate did not compromise the linearity of the PEPCK reaction; (4) when the malate dehydrogenase reaction was used as a source of OAA for the citrate

Fig. 4. Effect of 2-oxoglutarate on PEPCK activity at pH 7 with variable concentrations of OAA

PEPCK activity was measured at pH 7 as described in the Experimental section. The K_m for OAA (a) was determined as $2.4 \pm 0.7 \mu\text{M}$ and the K_i value for 2-oxoglutarate was $0.25 \pm 0.06 \mu\text{M}$, by using the model for competitive inhibition. The inset (b) shows the Dixon plot for 2-oxoglutarate inhibition. The K_i was determined as $0.32 \pm 0.04 \text{ mM}$.

synthase reaction, 2-oxoglutarate did not inhibit the rate of citrate formation.

To provide additional assurance that the effect of 2-oxoglutarate was the result of specific competition with OAA, kinetic studies were also carried out with Mn^{2+} and GTP as variable activator and variable substrate. Figs. 2 and 3 show that 2-oxoglutarate did not compete with either Mn^{2+} or GTP for active sites on the enzyme, but acted non-competitively against Mn^{2+} and GTP. The K_m values obtained for Mn^{2+} and GTP were in good agreement with previously published values (Jomain-Baum *et al.*, 1976; Jomain-Baum & Schramm, 1978; Schramm *et al.*, 1981).

The experiments above were all carried out at pH 8.0; however, if inhibition of this enzyme is to be physiologically significant, it must also occur at a pH near 7.0. Fig. 4 shows that the effect of 2-oxoglutarate persisted at all concentrations of OAA at physiological pH, the estimated K_i being essentially the same as at pH 8.0. To determine the K_i for the competition with OAA more accurately, the effect of 2-oxoglutarate was measured over a greater range of inhibitor concentrations and the data were transformed to a Dixon (1953) plot. The results are shown in the inset (Fig. 4b). The results agreed with previous data, which showed competitive inhibition with respect to OAA with a K_i for 2-oxoglutarate of $0.33 \pm 0.03 \text{ mM}$. 2-oxoglutarate also competitively inhibited the reverse reaction of PEPCK, i.e. the carboxylation of PEP to OAA, with a similar K_i to that of the forward reaction. The K_m for PEP was determined as $43 \pm 8 \mu\text{M}$ and the K_i for 2-oxoglutarate was $0.63 \pm 0.19 \text{ mM}$.

To see if the inhibition by 2-oxoglutarate is selective for this compound and not just a general property of dicarboxylic acids of similar size, succinate, glutamate, 3-oxoglutarate and 2-oxoglutarate were compared at a final concentration of 1 mM . PEP production from $20 \mu\text{M}$ -OAA was found to be inhibited as follows: succinate, 8%; glutamate, 8%; 3-oxoglutarate, 38%; and 2-oxoglutarate, 43%. Because malate is a component of the usual assay system, it had to be tested as a potential inhibitor in a modified assay. For this purpose, OAA was initially added at $50 \mu\text{M}$; GTP was $50 \mu\text{M}$, with no OAA or GTP-regenerating systems present. Malate concentrations as high as 9.9 mM did not

inhibit PEP formation. This is in contrast with the report by Meyuhas *et al.* (1971).

DISCUSSION

It is clearly established that hormones which increase gluconeogenesis from substrates which feed into the pathway via OAA cause a rapid decline in the cellular content of 2-oxoglutarate and glutamate (Williamson *et al.*, 1969; Ui *et al.*, 1973a,b; Haussinger & Sies, 1984; Staddon & McGivan, 1984). In these studies total cellular 2-oxoglutarate has been reported to fall by 40–60% in the presence of glucagon, phenylephrine or vasopressin. This is now believed to result from an activation of the 2-oxoglutarate dehydrogenase by Ca^{2+} (McCormack, 1985). Using digitonin fractionation, Siess *et al.* (1977) determined that in cells utilizing lactate/pyruvate, the cytosolic 2-oxoglutarate concentration was 1.2 mM and that this decreased by 70% within 10 min of glucagon administration. By using a value of 1.57 ml of water/g dry wt. of cells and the fact that 76% of the total cellular 2-oxoglutarate is cytosolic (Siess *et al.*, 1982), a comparable value of 1.45 mM can be calculated for the cytosolic 2-oxoglutarate concentrations in cells metabolizing proline, which also decreased by approx. 70% in the presence of vasopressin (Staddon & McGivan, 1984). Similar values can be obtained from the perfused-liver studies, by assuming an average cytosolic water content of 2.0 ml/g dry wt. (Siess *et al.*, 1982), with cytosolic concentrations of 1.08 mM (Williamson *et al.*, 1984) and 0.84 mM (Haussinger & Sies, 1984) for cells metabolizing lactate or glutamine respectively. Therefore, from the results presented above, it is evident that 2-oxoglutarate acts as a competitive inhibitor of PEPCK and that the cytosolic concentration of 2-oxoglutarate is in the correct range for it to exert a significant inhibition of the enzyme in unstimulated cells. Stimulation of 2-oxoglutarate dehydrogenase activity with a concomitant fall in the cytoplasmic 2-oxoglutarate concentration by either glucagon or the Ca^{2+} -dependent hormones might therefore be expected to relieve the inhibition and increase flux through PEPCK and enhance gluconeogenesis from lactate/pyruvate and other substrates feeding in at the level of OAA. Assuming that the cytoplasmic concentration of OAA is 15 μM and that this is not changed by glucagon (Ochs & Lardy, 1983; Haynes & Picking, 1990), it can be predicted from the above kinetic constants that a change in the cytosolic 2-oxoglutarate concentration from 1.2 mM to 0.4 mM after glucagon stimulation (Siess *et al.*, 1977) would increase PEPCK flux by approx. 35%. Direct measurements of PEPCK flux *in situ* indicate that the flux is increased by 22–31% (Rognstad & Katz, 1977; Haynes & Picking, 1990), suggesting that the observed fall in 2-oxoglutarate is sufficient to account for the effects *in vivo*.

The correlation between increased 2-oxoglutarate oxidation and enhanced flux through PEPCK and gluconeogenesis is not new, and was originally proposed by Ui *et al.* (1973a). In the absence of known effectors of PEPCK, it was suggested that the linkage might be the result of an elevation of GTP through increased succinyl thiokinase activity, although it was recognized that this was unlikely because of the problems with compartmentation. 2-Oxoglutarate has also been shown to act as a competitive inhibitor of aspartate efflux from the mitochondria, and therefore it has been proposed that a fall in the mitochondrial 2-oxoglutarate concentration may enhance gluconeogenesis from reduced substrates by accelerating the efflux of aspartate from the mitochondria (Strzelecki *et al.*, 1988). Thus there may be a concerted mechanism to enhance the flux of carbon from pyruvate to PEP brought about by an activation of 2-oxoglutarate dehydrogenase. A fall in both mitochondrial and cytosolic 2-oxoglutarate would act to enhance the translocation of OAA from the mitochondria as aspartate and increase the

conversion of OAA into PEP by PEPCK, while the associated decrease in mitochondrial glutamate concentration would ensure adequate OAA provision by increasing the flux through pyruvate carboxylase (Scrutton & White, 1974). This model would not be inconsistent with the data obtained by the control strength analysis or the curve-subtraction techniques (Groen *et al.*, 1983; Sistare & Haynes, 1985), and would not necessitate the requirement for the majority of control to reside at the level of pyruvate kinase in the fasted rat.

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