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SHEEP KIDNEY PHOSPHOENOLPYRUVATE CARBOXYLASE: CONTROL OF ENZYMIC ACTIVITY BY OXALOACETATE

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SUMMARY

A kinetic analysis of sheep kidney mitochondrial phosphoenolpyruvate carboxylase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) was carried out. When the concentration of particular pairs of substrates were varied, *e.g.*, phosphoenolpyruvate and IDP or IDP and HCO_3^- or phosphoenolpyruvate and HCO_3^-, initial velocity profiles revealed non-hyperbolic saturation kinetics indicative of a system exhibiting negative cooperativity with respect to substrate binding. However, the data did not fully describe negative cooperativity; (a) although R_s values greater than 81 were obtained, in some instances, the value varied with the concentration of the fixed non-saturating substrate, (b) the biphasic double reciprocal plots were eliminated if a coupled assay system was used, and (c) only one molecule of nucleotide diphosphate bound to the enzyme.

The similarity between the *in vivo* conditions and the uncoupled assay with respect to the concentration of phosphoenolpyruvate and oxaloacetate suggested that oxaloacetate could be an important effector controlling the balance between the carboxylation of phosphoenolpyruvate and the decarboxylation of oxaloacetate by mitochondrial phosphoenolpyruvate carboxylase.

INTRODUCTION

Considering the key position phosphoenolpyruvate (PEP) carboxylase (GTP: oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) in the gluconeogenic pathway[1,2], it would be expected that the activity of this enzyme would be subject to one or more control mechanisms. There are numerous reports of changes in the level of activity of the enzyme in response to perturbations in the metabolic and/or hormonal state of an organism. In general the changes which occur are in the same direction as the change in the gluconeogenic flux rate[1–10]. On the other hand, reports concerning metabolic effectors are rare and previous attempts to detect these using purified en-

Abbreviation: PEP, phosphoenolpyruvate.

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zymes isolated from mammalian tissues have failed. One example of an acute control in microorganisms has been reported, *viz.*, an allosteric inhibition by NADH in *Escherichia coli*[11].

In this report, evidence is presented that in the direction of carboxylation, oxaloacetate is a powerful inhibitor suppressing its own biogenesis.

MATERIALS AND METHODS

Enzymic assays

The carboxylation of PEP was followed in the uncoupled system by the incorporation of $^{14}CO_2$ into oxaloacetate. The reaction mixture (0.5 ml) contained (in μmoles): imidazole chloride (pH 6.5) (adjusted at 30 °C) or *N*-ethylmorpholine chloride (pH 7.5), 50; GSH, 0.8; phosphoenolpyruvate carboxylase, 0.01–0.015 unit; $MnCl_2$, PEP, IDP and $NaH^{14}CO_3$ ($6 \cdot 10^5$ cpm/μmole) as indicated in the figure legends. The reaction was started by the addition of the enzyme and incubated for 5 min at 30 °C. Under these conditions the activity was a linear function with respect to time with up to 0.015 unit of enzyme. The reaction was stopped by the addition of 0.05 ml of 6 M HCl saturated with 2,4-dinitrophenylhydrazine. Each assay solution was processed in triplicate as described previously for the $^{14}CO_2$: oxaloacetate exchange reaction[12].

In coupled assay systems, the oxaloacetate was converted to, either aspartate, using 10 μmoles of glutamate, 0.02 μmole of pyridoxal phosphate and 4.5 units of aspartate transaminase (EC 2.6.1.1), or malate, using 1 μmole of NADH and 4 units of malate dehydrogenase (EC 1.1.1.37).

Reaction velocity data were processed on a CDC 6400 digital computer using the HYPER programs of Cleland[13]. In those cases where biphasic double reciprocal plots of velocity against substrate concentration were obtained, the data corresponding to the two linear regions were analysed separately.

GDP binding

The apparatus and method used was that described by Colowick and Womack[14]. PEP carboxylase, dissolved in 0.025 M imidazole chloride (pH 6.5) and [β-^{32}P]GDP were added to the upper chamber, while 0.025 M imidazole chloride (pH 6.5) was pumped through the lower chamber at the rate of 8 ml per min. The concentration of GDP in the upper chamber was increased with 5–10-μl aliquots of unlabelled GDP every 2 min. The effluent was collected in 2-ml fractions and the radioactivity of each fraction determined.

The sources of all reagents and enzymes have been described previously[12,15].

RESULTS

Initial velocity studies

The data shown in Fig. 1 were obtained when PEP and IDP were the variable and fixed variable substrates, respectively. The deviation from a rectangular hyperbola was consistently observed and became more pronounced as the concentration of IDP decreased. The same data presented as a double reciprocal plot yield biphasic plots with inflection points at 0.2 mM PEP (Fig. 2). R_s values (the ratio of PEP con-

Fig. 1. Initial velocity plotted as a function of PEP concentration with Mn^{2+} at pH 6.5. PEP was varied at three fixed IDP concentrations: △—△, 0.8 mM; ▲—▲, 0.2 mM; ○—○, 0.1 mM. The points represent the experimental values, the lines are drawn from the computer-determined constants of best fit using the HYPER program of Cleland[13]. Assay mixtures contained 0.1 M imidazole chloride pH 6.5 (adjusted at 30 °C), 2 mM $MnCl_2$; 10 mM $NaH^{14}CO_3$; 1.6 mM GSH; PEP and IDP as indicated; PEP carboxylase 0.005 unit. The reaction mixture was incubated for 5 min at 30 °C and stopped with 0.05 ml of 6 M HCl saturated with 2,4-dinitrophenylhydrazine.

Fig. 2. Double reciprocal plot of initial velocity against PEP concentration. The data are the same as in Fig. 1. □—□, 0.8 mM IDP; ▽—▽, 0.2 mM IDP; ○—○, 0.1 mM IDP.

centrations at 90% and 10% of the maximum velocity) of 93.5, 155 and 174 were obtained at IDP concentrations of 0.8 mM, 0.2 mM and 0.1 mM, respectively, compared with a value of 81 for a reaction exhibiting hyperbolic kinetics. Hill plots of the initial velocity data are presented in Fig. 3. With 0.8 mM IDP, the plot was linear with unit slope whereas with 0.2 mM IDP, a triphasic curve was obtained with the slope decreasing to 0.37 in the region of 0.2 mM PEP.

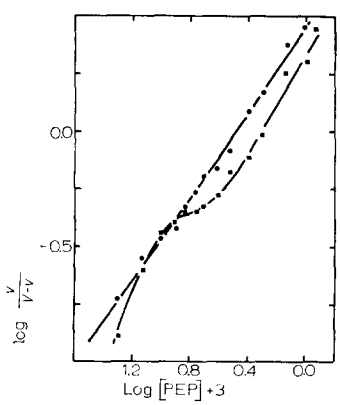
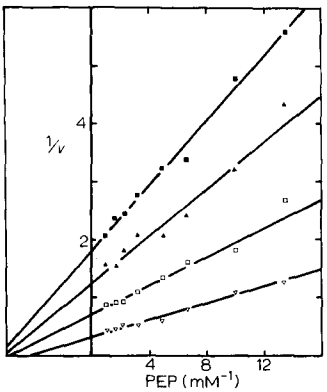


Fig. 3. Hill plot of the data presented in Fig. 1. ●—●, 0.8 mM; ■—■, 0.2 mM.

Biphasic double reciprocal plots were not obtained when PEP and HCO_3^- were the variable and fixed variable substrates (Fig. 4) but were when IDP and HCO_3^- were covariables (Fig. 5). Once again, the inflection point in the double reciprocal plot occurred at 0.2 mM substrate although a constant R_s value (125) indicated that the magnitude of the deviation from hyperbolic kinetics was apparently independent of



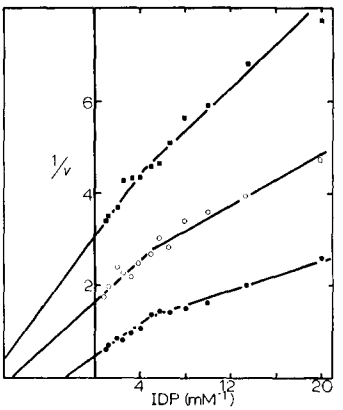


Fig. 4. Double reciprocal plots of initial velocity against varying PEP concentrations. The PEP concentration was varied at four fixed levels of HCO_3^-; ▽—▽, 8.0 mM; □—□, 3.0 mM; ▲—▲, 1.5 mM; ■—■, 1.0 mM. The assay conditions were the same as described in Fig. 1 but with 1 mM PEP and IDP and HCO_3^- as indicated.

Fig. 5. Double reciprocal plots of initial velocity against IDP varying concentrations. The IDP concentration was varied at three fixed levels of HCO_3^-: ●—●, 8.0 mM; ○—○, 2.0 mM; ■—■, 1.0 mM. The assay conditions were the same as in Fig. 1 but with 1 mM PEP and IDP and HCO_3^- as indicated.

the HCO_3^- concentration. It would appear that biphasic double reciprocal plots are only obtained when IDP is non-saturating while the R_8 value is constant if PEP is held constant but varies if PEP varies. When the metal ion concentration was varied along with each of the three substrates, non-hyperbolic kinetics were only shown when PEP was the covariable. No explanation can be put forward for the lack of any apparent interaction when IDP and Mn^{2+} were the covariables.

If the reason for the biphasic double reciprocal plots was negative cooperativity with respect to IDP or PEP binding, the kinetic patterns should not be altered using a coupled assay system. With PEP carboxylase, conversion of oxaloacetate to aspartate by the addition of aspartate transaminase, glutamate and pyridoxal phosphate

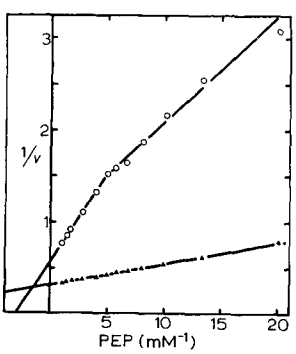


Fig. 6. Comparison of the coupled and uncoupled assay procedures. ▲—▲, coupled assay procedure; ○—○, uncoupled assay procedure. Assay mixtures contained (in μmoles): imidazole chloride, pH 6.5 (adjusted at 30 °C), 50; IDP, 0.5; PEP, as indicated; $MnCl_2$, 2; $NaH^{14}CO_3$ (0.5 Ci per μmole), 10; GSH, 0.8; enzyme, 0.01 unit. To the coupled assay, sodium glutamate, 10; pyridoxal phosphate, 0.02 and aspartate transaminase, 4.5 units were added.

resulted in linear double reciprocal plots as well as a marked increase in the observed enzymic activity (Fig. 6). Addition of the coupling components either separately or in various combinations had no effect on the kinetics of the uncoupled assay unless the complete coupling system was present. This effect was verified using a second coupled system where oxaloacetate was converted to malate and results similar to those obtained with the aspartate transaminase couple were obtained. A comparison of the activities obtained with the coupled and uncoupled assay systems under equivalent conditions (Fig. 6) indicates that oxaloacetate can act as a powerful inhibitor of its own synthesis. It is significant that, in reports concerning mammalian PEP carboxylase, coupled carboxylation assays have always been used.

Effect of pH

We have previously reported that the optimum pH of the PEP carboxylase reaction was dependent on the activating metal ion used[12]; pH 6.5 using Mn^{2+} and pH 7.5 using Mg^{2+}. To examine whether the biphasic kinetics were influenced by either the pH or the divalent cation used, the initial study presented in Fig. 1 was repeated at pH 7.5 using Mn^{2+} and Mg^{2+}. Essentially the same behaviour was obtained in each case with the inflection point at 0.2 mM PEP and with the R_S value increasing with decreasing IDP concentrations (*cf.* Fig. 2).

Binding studies

Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase[16] and *E. coli* alkaline phosphatase[17] both exhibit biphasic kinetic features. Furthermore, both enzymes have been shown to exhibit negative cooperativity with respect to substrate binding. In the former case, four moles of NAD^+ per mole of enzyme are bound with progressively decreasing affinity. In the latter case, two moles of PP_i per mole of enzyme are bound in a discontinuous manner with the second molecule bound less readily than the first. Since varying IDP with either PEP or HCO_3^- produced biphasic kinetic patterns, the binding of the nucleotide to PEP carboxylase was investigated. The results indicated that only one mole of nucleotide is bound per mole of enzyme ($n = 0.92$, Fig. 7). (Since GDP and IDP exhibit similar biphasic kinetic patterns, GDP was used in the

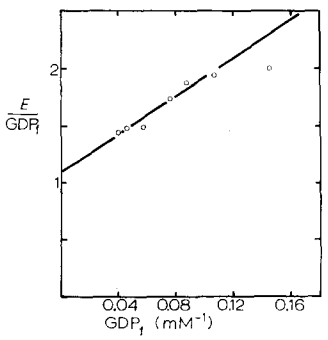


Fig. 7. Binding of GDP to PEP carboxylase. A Hughes–Klotz[21] plot of the data obtained for the binding of [β-^{32}P]GDP to PEP carboxylase using the apparatus and method described by Colowick and Womack[14]. The buffer used was 0.025 M imidazole (pH 6.5) at 20 °C. The intercept on the ordinate gives $1/n$, where n is the number of GDP binding sites, and the intercept on the abscissa gives $-1/K_D$ where K_D is the dissociation constant for the enzyme–GDP complex.

binding studies). This result is contrary to that expected for negative cooperativity since this concept requires a minimum of two molecules of substrate per enzyme molecule to achieve an interaction between binding sites.

DISCUSSION

From the data presented, it would appear that sheep kidney mitochondrial PEP carboxylase satisfies the criteria defined by Levitzki and Koshland[18] for negative cooperativity with respect to substrate binding; (a) the profile of initial velocity plotted as a function of substrate concentration exhibits an intermediary plateau region, (b) the double reciprocal plot of velocity against substrate concentration is biphasic with increasing slope at high substrate concentrations, (c) the R_s value is greater than 81, and, (d) the Hill plot is non-linear with an intermediate region of slope much less than unity.

However, PEP carboxylase exhibits a somewhat different response from that shown by other enzymes reported to illustrate negative cooperativity. Firstly, the presence of plateau regions in initial velocity plots and biphasic double reciprocal plots does not seem to be associated with any particular substrate or pair of covariable substrates. Second, the observation that the R_s value, in some instances, increases as the level of the fixed variable decreases is atypical. For example, glutamate dehydrogenase exhibits negative cooperativity with respect to NAD^+ (ref. 19) although normalized plots of velocity against NAD^+ concentration at various glutamate concentrations become identical. Similarly, with CTP synthetase, the R_s value for glutamine is independent of the concentration of GTP[18] and in the case of pyruvate carboxylase, the R_s value for pyruvate is unchanged over a ten-fold concentration range of either acetyl-CoA or $MgATP^{2-}$ (ref. 20). Thirdly, from binding studies and Hill plots it is concluded that only one mole of GDP was bound per mole of enzyme. Finally, the biphasic double reciprocal plots were eliminated if oxaloacetate was removed using a coupled assay system.

These data could be explained by a system where two forms of the enzyme exist, one of which is stabilized by oxaloacetate, or alternatively, by the induction of a second enzyme form following oxaloacetate binding. The form stabilized by oxaloacetate would gradually accumulate in the uncoupled system but not in the coupled system resulting in the situation where the two enzyme species are present. Depending on the magnitude and direction of the differences in kinetic properties between the two enzyme forms, deviations from hyperbolic kinetics could be expected. A metabolic effector of PEP carboxylase could quite feasibly act by increasing or decreasing the response to oxaloacetate and since such responses could not be detected with a coupled assay, these effectors may have missed detection although they have the capability of altering the balance between carboxylation and decarboxylation.

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