Communication

A Simple and Accurate Spectrophotometric Assay for Phosphoenolpyruvate Carboxylase Activity

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ABSTRACT

The rate of phosphoenolpyruvate carboxylase activity measured through the conventional coupled assay with malate dehydrogenase is underestimated due to the instability of oxaloacetate, which undergoes partial decarboxylation into pyruvate in the presence of metal ions. The addition of lactate dehydrogenase to the conventional assay allows the reduction of pyruvate formed from oxaloacetate to lactate with the simultaneous oxidation of NADH. Then, the enzymic determination of substrate and products shows that the combined activities of malate dehydrogenase and lactate dehydrogenase account for all the phosphoenolpyruvate consumed. The net result of the improved assay is a higher $V_{max}$ with no apparent effect on $K_m$. The free divalent cation concentration appears to be the major factor in the control of the rate of oxaloacetate decarboxylation.

PEPC$^3$ (EC 4.1.1.31) catalyzes the Me$^{2+}$-dependent conversion of PEP to OAA. The most widely used method for the measurement of PEPC activity is the spectrophotometric coupled assay with MDH (EC 1.1.1.37) which reduces the OAA formed to malate (7). The simultaneous oxidation of NADH is followed at 340 nm.

However, methods based on OAA measurement have been proved to be inaccurate in the presence of metal cations due to the formation of various OAA-Me$^{2+}$ complexes (3, 4, 8) and the subsequent decarboxylation of OAA into pyruvate (4, 5, 8, 10). The problems associated with OAA stability in enzymic assays are well known (1) and have been recently readdressed with regard to PEPC (12). In this study, the potential inhibitory effects of the intermediate OAA-enol forms on the MDH reaction were also emphasized (12). However, as these intermediate forms are in equilibrium, the presence of an excess of added MDH should be able to displace this equilibrium and allow for the estimation of essentially all the OAA present in the assay medium. Such an assumption is fully supported by the fact that the sum of malate plus pyruvate formed accounts for all the PEP consumption (12). No evidence for the presence in significant amount of a form of OAA unavailable to MDH during PEPC assay can be found in the study (12). More simply, depending on the experimental conditions (6), a certain percentage of the OAA produced complexes with Mg$^{2+}$ and then undergoes decarboxylation to pyruvate thus becoming unavailable to MDH. Radiochemical assays (based on $^{14}$CO$_2$-fixation) would similarly underestimate the rate due to OAA decarboxylation.

Unfortunately, the measurement of inorganic orthophosphate that has been proposed in order to overcome the problem of OAA instability (11, 12) has major drawbacks, including the necessity of phosphate free media, potential interfering reactions, and time consuming assay prohibiting routine kinetic studies.

We present here an assay which overcomes this problem and gives a rapid measurement of PEPC activity which accounts completely for the rate of PEP consumption.

MATERIALS AND METHODS

Enzyme Preparation. PEPC was purified from leaves of Croton argentea Thunb. as previously described (14) with the following changes. Day leaves were homogenized in 2 ml per g fresh weight of grinding medium consisting of 50 mM HEPES (pH 8.0), 1 mM EDTA, 1 mM DTT, 1% (w/v) PVP, 0.1 mg/ml 2-mercaptopethanol Na, 200 mM KCl, and 1% (w/v) PEG 6000. The resuspended 5 to 12% PEG precipitate was dialyzed overnight against 50 mM HEPES (pH 7.0), 1 mM EDTA, 1 mM DTT. This fraction was applied to a Fractogel TSK DEAE 650 m column in the same buffer and eluted with a 50 to 150 mM NaCl gradient. Active fractions were pooled, precipitated in 60% (NH$_4$)$_2$SO$_4$, resuspended and dialyzed overnight with 50 mM K-phosphate (pH 7.4), and 1 mM DTT. This fraction was applied to a hydroxylapatite column (HTP, Biorad) and PEPC eluted with 100 to 400 mM K-phosphate gradient (pH 7.4). Active fractions were again pooled, precipitated with 60% (NH$_4$)$_2$SO$_4$, and dialyzed with 50 mM HEPES (pH 7.2) and 1 mM DTT. Aliquots of the concentrated enzyme were stored frozen at -70°C and diluted in appropriate buffer for subsequent assay. PEPC eluted from the HTP column was found to have a specific activity of 19 IU/mg showing one band on a SDS-PAGE gel. Protein concentration was determined by the method of Bradford (2).

Enzymes and Substrate. Pig heart MDH, hog muscle LDH (EC 1.1.1.27), and rabbit muscle PK (EC 2.7.1.40) of the highest available purity were purchased from Boehringer Mannheim. The MDH dilution (12 IU/ml) was found to contain no significant LDH activity. The trisodium salt of PEP was purchased from Sigma and was found to contain no pyruvate contamination. Maize PEPC with a specific activity of 3 IU/mg protein was purchased from Calbiochem. Wheat PEPC with an approximate specific activity of 5 IU/mg was purchased from Boehringer Mannheim.

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3 Abbreviations: PEPC, phosphoenolpyruvate carboxylase; ACES, N-2-acetamido-2-aminoethanesulfonic acid; LACT, lactate; LDH, lactate dehydrogenase; MAL, malate; MDH, malate dehydrogenase; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate; PK, pyruvate kinase; ME$^{2+}$, metal (divalent cation); G-6-P, glucose 6-phosphate.
PEPC Assay. The activity of PEPC was assayed in a Beckman DU-50 spectrophotometer by monitoring the decrease in absorbance at 340 nm in an assay system coupled with MDH (7) and in an identical assay system including both MDH and LDH. The temperature was maintained at 25°C by a circulating water bath. Assay mixtures included 50 mM ACES (pH 7.0), 5 mM free Mg²⁺ (from MgSO₄), 5 mM NaHCO₃, 0.16 mM NADH, and 12 IU of MDH or 12 IU of MDH plus 5.5 IU of LDH. This was a greater than 50-fold excess of MDH in order to maximize OAA utilization by the conventional coupled assay. When present, a similar excess of LDH was used in order to maximize pyruvate utilization. However, a 10-fold excess of both enzymes is adequate to account for total PEPC activity. For routine assay 1 mM PEP (free anion concentration) was used. Assays were based on constant levels of free anions and Mg²⁺. The problem of maintaining known concentrations of uncomplexed assay constituents was solved by a computer program, written in BASIC, which takes account of pH and ionic strength effects on the complexing of acids with the metal ions and calculates the volume of the stock solution of each constituent necessary to achieve the required free concentrations in each assay, writing protocols for the desired conditions (13). Reactions were initiated by the addition of PEPC. Rates were typically calculated 0 to 10 min and were linear with correlation coefficients greater than 0.995. Kinetic parameters were determined by fitting the rates obtained at varying concentrations of PEP to the Michaelis-Menten equation modified to provide estimates of the Hill number as well as Vₘₐₓ and Kₘ (15).

Enzymic Determination of Products and Substrate. The concentrations of OAA, pyruvate, and PEP during the time course of a standard PEPC reaction were enzymatically determined by the oxidation of NADH with the consecutive additions of MDH, LDH, and PK, respectively (1, 9). The oxidation of NADH was followed in a Cary 219 spectrophotometer. At given time points, aliquots of the standard reaction mixture were rapidly transferred to an assay for product measurement consisting of 50 mM ACES (pH 7.0), 5 mM MgSO₄, 2 mM ADP, 0.2 mM NADH, and 12 IU of MDH. Thus, level of OAA in the sample was immediately measured by NADH oxidation, via the MDH present in the assay. Next, LDH (15 IU) was added to the assay and pyruvate levels determined by the oxidation of NADH. Finally, PK (6 IU) was added and, coupled with the LDH present, allowed the measurement of PEP by NADH oxidation. In all cases, the decrease in absorbance was rapid and a steady baseline was established prior to the addition of the next enzyme.

Effect of Metal Cations. Assays (MDH and MDH + LDH) were run at 1 mM total concentrations for MgSO₄, MnSO₄, CoCl₂, and ZnCl₂. Total concentrations of other assay constituents were 50 mM ACES, 5 mM HCO₃⁻, 1 mM PEP, and 0.2 mM NADH. In all cases, the rates of PEPC activity for the substituted cations were lower than those of Mg²⁺, the presumed acting in vivo metal (data not shown). This may be due to several factors (inhibition, lack of activation, low cation binding to PEPC, etc.) but has no bearing on the percent difference between the MDH and the MDH + LDH assay (total activity).

RESULTS AND DISCUSSION

Figure 1A presents an enzymic determination of the changes in the concentration of substrate (PEP) and products (OAA, pyruvate) of the reaction catalyzed by PEPC from *C. argentea*. This determination was carried out under the conditions of a conventional coupled assay, i.e. in the presence of NADH (0.16 mM) and MDH (12 IU). After about 10 min (the time during which a regular spectrophotometric assay would be performed) all the NADH initially added to the reaction mixture has been oxidized via MDH using the OAA produced by PEPC activity.

Based on PEP consumption, the activity of PEPC was 19.5 nmol/min. It is clear that a significant amount of pyruvate (about 25% of the NADH oxidized) was produced during these 10 min. It is noteworthy that the amount of NADH consumed (i.e. OAA produced) and pyruvate produced accounts for all the PEP consumed. Thus, the MDH coupled assay, as previously shown (12), underestimates the rate equal to the rate of pyruvate production. After all NADH has been exhausted, both OAA and pyruvate (from OAA decarboxylation) build up. These conditions (no NADH present) correspond to those used for determination of PEPC activity from direct spectrophotometric measurement of OAA at 270 to 285 nm (3). It is shown that under these conditions (10–30 min), pyruvate production is higher due to the accu-

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**Fig. 1.** Measurement of PEPC activity from *C. argentea*. A. Enzymic determination of substrate and products during time course of a conventional PEPC assay in the presence of MDH alone; B, similar determination carried out during the time course of a PEPC assay in the presence of MDH and LDH. Although the traces represent changes in concentrations (nm), numbers along the traces in A and B are initial rates of PEP consumption in nmol/min to enable direct comparison to rates in C. C. Spectrophotometric assay of PEPC based on the oxidation of NADH in the presence of MDH alone (a) or in the presence of both MDH and LDH (b). Numbers along the traces are nmol NADH oxidized/min. The arrow indicates the addition of PEPC. Experimental conditions are similar for all the experiments reported in this Figure and are described in greater detail under "Materials and Methods."
mulation of OAA in the medium. Direct measurement of OAA at 270 to 285 nm would be further complicated by the different extinction coefficients of the various species and complexes with Mg²⁺ (3, 4, 8). At late time points (+30 min), as OAA reaches a higher constant value (0.2 mM), the rate of PEP consumption actually approaches the rate of pyruvate produced by the decarboxylation of OAA. However, it should be again noticed that under all the conditions, presence or absence of NADH, the rate of OAA and pyruvate production accounts for all the PEP consumption.

A similar experiment was carried out but in the presence of LDH in addition to MDH. Under these conditions, in the presence of NADH, the products of PEP consumption (OAA and pyruvate) were respectively reduced to malate and lactate. Figure 1B shows the changes in the concentrations of substrate and products during the first 20 min. The rate of PEP consumption is stoichiometric with the rate of NADH oxidation (i.e. OAA and pyruvate production). As previously shown in Figure 1A, buildup of OAA + pyruvate occurs after all the NADH has been consumed. The rest of the time course was essentially identical to the one of Figure 1A and therefore not presented here. The rates of PEP consumption in the presence of MDH alone (19.5 nmol/min, Fig. 1A) and of MDH and LDH (20.0 nmol/min, Fig. 1B) are in good agreement; similar amounts of PEP were consumed under these two conditions. It follows that the presence of LDH or of lactate arising from pyruvate reduction did not affect the rate of PEPC activity. Moreover, control experiments have shown that no effect of lactate on PEPC activity could be observed for concentrations under 0.1 mM even at moderate PEP concentrations (0.1 mM). This lactate concentration is higher than the level that could be potentially reached during the approximate 10 min of a standard PEPC assay (160 μM NADH).

As (a) OAA and pyruvate were simultaneously produced during assay of PEPC and as (b) the presence of LDH or of lactate does not affect the activity of PEPC, the possibility of a simultaneous measurement of OAA and pyruvate during PEPC carboxylation was investigated (Fig. 1C). In trace a, as in Figure 1A, PEPC activity was followed by the conventional spectrophotometric coupled assay through the reduction of OAA taking place in the presence of NADH and MDH alone. In trace, b, as in Figure 1B, the activity was followed through the simultaneous measurement of OAA and pyruvate reduction taking place in the presence of NADH and MDH + LDH. It is shown that the presence of the two enzymes (MDH + LDH) in the assay medium reveals a higher (about 25%) apparent PEPC activity than the activity measured by the conventional assay with MDH alone. This is in good agreement with the amount of pyruvate produced in the experiment of Figure 1A (about 25% of the NADH oxidized). The higher rate observed in the presence of MDH + LDH is due to the oxidation of NADH as the pyruvate produced is then reduced to lactate by LDH. It should be mentioned that the rate of the activity measured in the presence of both enzymes (19.8 nmol/min, Fig. 1C) corresponds to the rate of PEP consumption measured by the enzymatic determination (20.0 nmol/min, Fig. 1B and also to the rate with MDH alone (19.5 nmol/min, Fig 1A). It therefore appears that using such an assay method overcomes the problem of nonenzymatic OAA decarboxylation giving a continuous measure of the true rate of PEPC activity.

Given that the MDH coupled assay underestimates the true rate of PEPC, it was necessary to determine whether the measured kinetic parameters of the enzyme were modified using a MDH/LDH coupled assay. Figure 2 shows two PEPC isotherms. The first, (line a), was carried out in the presence of MDH alone whereas the second, (line b), was performed in the presence of both MDH and LDH. As anticipated, there is a significant in-
for G-6-P binding to Mg\(^{2+}\). Despite this high total Mg\(^{2+}\) concentration, no increase in the rate of OAA decarboxylation could be detected.

The rates of PEPC activity obtained in the presence of other metal cations, such as Mn\(^{2+}\), Co\(^{2+}\), and Zn\(^{2+}\), from the MDH coupled assay (measuring OAA reduction to malate) and from the MDH + LDH assay (total activity) were compared and the amount of OAA decarboxylated, calculated as in Table II (see “Materials and Methods” for details). The percent of OAA decarboxylated was 10% for 1 mM Mg\(^{2+}\) (total). This rose to 27, 35, and 88% for Mn\(^{2+}\), Co\(^{2+}\), and Zn\(^{2+}\), respectively. This was anticipated as these metal cations are known to promote higher rates of OAA decarboxylation (10). A greater percentage of the OAA being produced by the PEPC is being decarboxylated so there is a greater difference between the MDH coupled assay and the MDH + LDH assay. Further, the order of metal decarboxylating activity obtained in our system is in complete agreement with the Irving-Williams natural stability order for metal complex formation (4); Zn\(^{2+}\) being the most active followed by Co\(^{2+}\), Mn\(^{2+}\), then Mg\(^{2+}\). This was also expected as metal-OAA complex formation is a necessary intermediate in the metal catalyzed decarboxylation.

Under our standard conditions (5 mM free Mg\(^{2+}\)), the level of OAA decarboxylation reached about 20% of the OAA reduced to malate by MDH. We have obtained similar results for commercial maize and wheat PEPC indicating that OAA decarboxylation was not an artifact linked to our enzyme preparation. In addition, we found that in ACES buffer (pH 6–8), the pH of the assay did not seem to have a large effect on the rate of decarboxylation (15–25%). It is difficult to assign an exact value for the percentage of OAA decarboxylation probably because of its extreme sensitivity to free metal concentration.

As emphasized by Krebs (6), the rate of nonenzymatic OAA decarboxylation shows considerable variation, probably due to trace impurities (essentially metal cations) that affect the stability of OAA.

### Table II. Effect of Various Experimental Conditions and Effectors on PEPC Activity and on the Rate of Nonenzymic OAA Decarboxylation during PEPC Assay

Experimental conditions are described under “Materials and Methods.”

<table>
<thead>
<tr>
<th>Conditions</th>
<th>MDH + LDH PEPC Activity</th>
<th>MDH PEPC Activity</th>
<th>(MDH + LDH) – MDH OAA Decarboxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, (5 mM Mg(^{2+}), 25°C)</td>
<td>9.28</td>
<td>7.89</td>
<td>1.39 (18%)</td>
</tr>
<tr>
<td>5 mM Mg(^{2+}), 34°C</td>
<td>20.20</td>
<td>16.9</td>
<td>3.3 (19.5)</td>
</tr>
<tr>
<td>5 mM Mg(^{2+}), 25°C.</td>
<td>14.33</td>
<td>12.47</td>
<td>1.86 (15)</td>
</tr>
<tr>
<td>5 mM G-6-P</td>
<td>6.00</td>
<td>4.97</td>
<td>1.03 (21)</td>
</tr>
<tr>
<td>5 mM malate</td>
<td>6.30</td>
<td>4.89</td>
<td>1.40 (22)</td>
</tr>
<tr>
<td>10 mM Mg(^{2+}), 25°C</td>
<td>9.46</td>
<td>6.29</td>
<td>3.17 (50)</td>
</tr>
</tbody>
</table>

\(^{2}\)Percent of OAA converted to malate by MDH.

### LITERATURE CITED