Pyruvate Carboxylase

VII. A POSSIBLE ROLE FOR TIGHTLY BOUND MANGANESE*

(Received for publication, February 10, 1966)

ALBERT S. MILDVAN,† MICHAEL C. SCRUTON, AND MERTON F. UTTER§

From The Johnson Research Foundation, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104, and the Department of Biochemistry, Western Reserve University, School of Medicine, Cleveland, Ohio 44106

SUMMARY

Pyruvate carboxylase contains firmly bound manganese which affects the longitudinal relaxation rate of the protons of water as measured by pulsed nuclear magnetic resonance. The manganese shows an enhanced effect ($E_b = 4.2$) on the relaxation rate when bound to the enzyme. Studies on the effect of added substrates and inhibitors on the proton relaxation rate have shown that components of the second partial reaction, e.g. the carboxylation of pyruvate by the enzyme-biotin $\sim$ CO$_2$ complex, interact with the bound manganese of the enzyme and cause a reduction in the enhancement. In contrast, components of the first partial reaction, the formation of enzyme-biotin $\sim$ CO$_2$ from adenosine triphosphate and HCO$_3^-$, do not significantly affect the enhancement. Examination of the enhancement of the bound manganese as a function of substrate or inhibitor concentration has permitted the determination of dissociation constants and enhancement values for the enzyme-substrate and enzyme-inhibitor complexes. The rate of inactivation of pyruvate carboxylase by avidin is increased by pyruvate and oxalacetate, and decreased by certain dissociable inhibitors, e.g. oxalate. While dissociable inhibitors such as oxalate probably act by direct chelation of the bound manganese since the enhancement of the enzyme-inhibitor complex has a very low value ($E_r < 0.3$), the corresponding values for the enzyme-substrate complexes are considerably higher ($E_r = 1.5$ to 2.5), suggesting either that fewer metal-H$_2$O ligands are displaced by substrates or that conformational changes in the protein contribute to the situation in a different way with the substrates and the inhibitors. Several possible mechanisms for the involvement of the bound manganese in this reaction are discussed, and a mechanism is suggested which is compatible with the kinetic and magnetic resonance data.

---

Reactions 1, 2, and 3 show a requirement for activation by a divalent cation which is required for this reaction and which participates in the formation of the enzyme-biotin $\sim$ CO$_2$ intermediate from HCO$_3^-$ and ATP.

Studies reported in the preceding paper (1) showed that pyruvate carboxylase (pyruvate-Co$_2$ ligase (ADP), EC 6.4.1.1) purified from chicken liver mitochondria contains tightly bound manganese in a stoichiometry approximating 1 mole of manganese per mole of biotin or 4 moles of manganese per mole of enzyme. Earlier studies on the exchange reactions catalyzed by highly purified pyruvate carboxylase and isolation of the enzyme-biotin $\sim$ CO$_2$ intermediate have permitted the formulation of a minimal mechanism for this enzyme, involving two partial reactions (Reactions 1 and 2) (2, 3).

Enzyme-biotin + ATP + HCO$_3^-$ $$\rightarrow$$

$\text{acetyl-CoA, Mg}^{2+}$

$$\text{envelope-biotin} \sim \text{CO}_2 + \text{ADP} + \text{Pi} \quad (1)$$

$$\begin{align*}
\text{Enzyme-biotin} \sim \text{CO}_2 + \text{pyruvate} & \rightarrow \\
\text{enzyme-biotin} + \text{oxalacetate} & \quad (2)
\end{align*}$$

Sum: Pyruvate + ATP + HCO$_3^-$ $$\rightarrow$$

$\text{acetyl-CoA, Mg}^{2+}$

$$\text{oxalacetate} + \text{ADP} + \text{Pi} \quad (3)$$

Reactions 1 and 3 show a requirement for activation by a divalent cation which may be satisfied by the addition of either magnesium...
or manganese (4). However, the divalent cation required for activation may be removed by dialysis or chelating agents, and is therefore not related to the bound metal ion component of pyruvate carboxylase.

The bound manganese of pyruvate carboxylase has an enhanced effect ($e_b = 4.2$) on the proton relaxation rate of water (1). By qualitative and quantitative studies of the effects of added substrates and inhibitors on the enhancement, it has been possible to show a functional role for manganese in Reaction 2 and to obtain dissociation constants for enzyme-substrate and enzyme-inhibitor complexes which can be compared with Michaelis, inhibitor, and dissociation constants obtained by other methods.

These studies and possible mechanistic interpretations are presented below.

A preliminary account of some of these findings has already appeared (5).

**Methods**

Pyruvate carboxylase was prepared as described previously (6) and assayed spectrophotometrically in the direction of CO$_2$ fixation (2). Oxalacetate decarboxylation was measured spectrophotometrically by estimating ATP production with hexokinase and glucose 6-phosphate dehydrogenase. Specific activities are expressed as micromoles per min per mg of protein at 25°C. Exchanges of pyruvate-14C with oxalacetate and of 32P from AMP with ATP were assayed as described previously (6). Protein was estimated spectrophotometrically (7).

Tritiated pyruvate was prepared and purified as described by Rose (8). The solutions were stored at -20°C and used for no more than 1 week after preparation. Tritium release was assayed in a system containing, in 0.5 ml, 100 μmoles of Tris-sulfate (pH 7.8), 3.5 μmoles of tritiated pyruvate (8.2 X 10$^4$ cpm per μmole), 1.2 μmoles of ATP, 2.5 μmoles of MgSO$_4$, 8 μmoles of KHCO$_3$, and 0.1 μmole of acetyl-CoA. Incubation was performed for 15 min at 23°C, after which the reaction was stopped by addition of trichloracetic acid (final concentration, 3%), and denatured protein was removed by centrifugation. The supernatant fraction was neutralized to pH 7 with KOH and then placed on a Dowex 1-Cl- column (8 X 1 cm) which had previously been washed with 10 ml of 1 N HCl followed by H$_2$O until the effluent pH was approximately 5 to 6. A 10-ml fraction, collected after adding the supernatant fraction and washings to the column followed by distilled water, was found to contain all the radioactivity released from tritiated pyruvate. One-milliliter aliquots from this fraction were added to 15 ml of dioxane-naphthalene scintillation mixture (9) and radioactivity was estimated in a Packard Tri-Carb scintillation spectrometer.

**Analysis of Proton Relaxation Rate Data**—The PRR was measured and the observed enhancement ($e^*$) was defined as described previously (1). Since in this system all the manganese is bound to protein, $e^*$ is equal to $e_b$, the enhancement of the bound manganese. When substrates or inhibitors are added to pyruvate carboxylase, the observed enhancement ($e^*$) decreases and approaches a limiting value ($e_o$), the enhancement of the enzyme-substrate or enzyme-inhibitor complex.

The interaction of substrates (A) or inhibitors (A) with enzyme-bound manganese ($E$-Mn) may be described by

\[ E-Mn + A \rightarrow E-Mn-A \]

and, hence,

\[ K_d = \frac{[E-Mn][A]}{[E-Mn-A]} \]

Also,

\[ [E-Mn]_t = [E-Mn] + [E-Mn-A] \]

The observed enhancement ($e^*$) has been shown (10) to be a weighted average of the enhancements due to all forms of manganese. In the present case, we may write

\[ e^* = \frac{[E-Mn]}{[E-Mn]_t} e_b + \frac{[E-Mn-A]}{[E-Mn]_t} e_o \]

Solving Equation 7 for $[E-Mn]$ gives

\[ [E-Mn] = [E-Mn]_t \left( \frac{e_o - e^*}{e_b - e^*} \right) \]

From the definition of the dissociation constant ($K_d$) of $[E-Mn]$ (Equation 5), we may write

\[ [E-Mn] = [E-Mn]_t \left( \frac{[A]}{[A] + K_d} \right) \]

where [A] is the concentration of free substrate or inhibitor. Setting Equation 8 equal to Equation 9, taking reciprocals, and dividing by $(e_b - e_o)$ gives

\[ \frac{1}{(e_o - e^*)} = \frac{1}{(e_b - e^*)} + \left( \frac{1}{(e_b - e_o)} \right) \frac{K_d}{[A]} \]

If $([A]$ is greater than $[E-Mn]_t$, $(A)$ approximates $([A]_t$ and a plot of $1/(e_b - e^*)$ against $1/(A)$ should be linear. Examination of the plot permits evaluation of $e_o$ from the ordinate intercept and $K_d$ from the slope. Equation 10 is similar in form to an equation derived previously for the interaction of bovine serum albumin with manganese (10).

**Analysis of Effect of Substrates and Inhibitors on Rate of Inactivation by Avidin**—Dissociation constants ($K_d$) for substrates and inhibitors have also been obtained by measurement of the effects of these substances on the rate of inactivation of pyruvate carboxylase by avidin. In this method, which has been described in detail previously (11), pyruvate carboxylase and avidin are incubated in the presence of various concentrations of the substrate or inhibitor and the rate of irreversible inactivation of the enzyme by avidin is determined by removal of small aliquots for assay by the previously described spectrophotometric assay system for CO$_2$ fixation (2). The following relationship has previously been shown to apply to the rates of inactivation by avidin in the presence of a substrate (A) or inhibitor (A) (11).

\[ \frac{v_o}{v_i} = \frac{k_2 + K_d}{k_1} \left( 1 - \frac{v_i}{v_o} \right) \]

Here $v_o$ and $v_i$ are the slopes of the pseudo-first order plots obtained for the inactivation of enzyme sites by excess avidin in the presence and absence of $A$, respectively; $K_d$ is the dissociation constant of the $EA$ complex; and $k_2/k_1$ is the ratio of the fractional order rate constants for the inactivation of $EA$ and $E$ by avidin. The units of $v_o$ and $v_i$ are min$^{-1}$, and $A$ is expressed as molar. When $v_i/v_o$ is plotted against $(1 - v_i/v_o)/A$, the slope...
gives $K_d$ and the ordinate intercept gives the $k_o/k_i$ ratio. Data of this type are shown for pyruvate (Fig. 2) and oxalate (Fig. 5) (see below). Other studies have shown that the reaction orders in enzyme sites and avidin were unaffected by the presence of either pyruvate or oxalate. Unchanged reaction orders in the presence of $A$ are a necessary prerequisite for the derivation and application of Equation 11.

Methylmalonyl-CoA-oxalacetate transcarboxylase (EC 2.1.3.1) (specific activity, approximately 40) was the gift of Dr. H. G. Wood. Redistilled $\alpha$-ketobutyric acid was the gift of Dr. D. S. Kerr. Sodium fluoropyruvate was obtained from K and K Laboratories; mesoxalic acid (A grade) from Calbiochem; oxamic acid and glyoxal from Matheson, Coleman, and Bell; and malonic acid from Eastman Organic Chemicals. All other reagents were reagent grade materials or were obtained as described previously (3, 6).

RESULTS

Effect of Reaction Components on Enhancement of Bound Manganese—Examination of the effect of reaction components on the PRR of a solution of pyruvate carboxylase (Table I) showed that only the components of Reaction 2 (pyruvate and oxalacetate) caused a marked decrease in the enhancement of the bound manganese from the initial value, $e_0 = 4.2$. Addition of the components of Reaction 1 with the exception of KHCO$_3$ had no significant effect on this parameter. The decrease caused by KHCO$_3$ was significant, but occurred only in the presence of very high concentrations of this substrate. The concentration range (about 0.1 M) in which KHCO$_3$ caused 50% reduction in the enhancement of the bound manganese greatly exceeded the observed $K_m$ ($1 \times 10^{-4}$ M) for this substrate in $CO_2$ fixation. Therefore, the binding of KHCO$_3$ observed by the reduction in the enhancement of the bound manganese probably represents the formation of an inactive complex between enzyme and HCO$_3^-$ which is not involved in the main reaction pathway. Additionally, no reduction in enhancement of the bound manganese occurred on formation of the enzyme-biotin- $CO_2$ complex from ATP + HCO$_3^-$ in the presence of Mg$^{2+}$ and acetyl-CoA.

Dissociation Constants for Enzyme-Substrate Complexes Obtained by PRR Titration and by Analysis of Rates of Inactivation by Avidin—By the use of the property of reduction in enhancement, the dissociation constant ($K_d$) of the enzyme-substrate complexes may be measured by titration of the bound manganese with pyruvate and oxalacetate as described by Cohn (12) and Mildvan and Cohn (13). The titration with pyruvate plotted according to Equation 10 is shown in Fig. 1. By analyses of the slope and ordinate intercept of this plot, $K_d$ was obtained as $3.3 \times 10^{-3}$ M and $e_0$ as 1.65.

Pyruvate and oxalacetate accelerated the rate of inactivation of pyruvate carboxylase by avidin, and analysis of the data according to Equation 11 permitted determination of $K_d$ by this method. Experimental data showing the potentiation of the bound manganese with pyruvate and oxalacetate as described by Cohn (12) and Mildvan and Cohn (13). The titration with pyruvate plotted according to Equation 11 is shown in Fig. 2. Analyses of the slope and ordinate intercept gave $K_d$ as $4.8 \times 10^{-3}$ M and the $k_o/k_i$ ratio as 1.32.

The $K_d$ values obtained by these two methods for pyruvate and oxalacetate are compared with Michaelis ($K_m$) and inhibitor ($K_i$) constants for these substrates in Table II. Pyruvate carboxylase has also been shown to carboxylate $\alpha$-ketobutyrate at approximately 3% of the rate of $CO_2$ fixation on pyruvate (4), and

\[ K_d = 4.8 \times 10^{-3} \text{ M} \]

\[ e_0 = 1.65 \]

\[ K_m = 1 \times 10^{-4} \text{ M} \]

\[ k_o/k_i = 1.32 \]

\[ K_m = 1 \times 10^{-3} \text{ M} \]

\[ K_i = 1 \times 10^{-3} \text{ M} \]

\[ e_0 = 4.2 \]

\[ K_d = 3.3 \times 10^{-3} \text{ M} \]

\[ e_0 = 1.65 \]

\[ K_d = 4.8 \times 10^{-3} \text{ M} \]

\[ k_o/k_i = 1.32 \]

\[ K_m = 1 \times 10^{-3} \text{ M} \]

\[ K_i = 1 \times 10^{-3} \text{ M} \]

\[ e_0 = 4.2 \]

\[ K_d = 3.3 \times 10^{-3} \text{ M} \]

\[ e_0 = 1.65 \]

\[ K_d = 4.8 \times 10^{-3} \text{ M} \]

\[ k_o/k_i = 1.32 \]

\[ K_m = 1 \times 10^{-3} \text{ M} \]

\[ K_i = 1 \times 10^{-3} \text{ M} \]

\[ e_0 = 4.2 \]

\[ K_d = 3.3 \times 10^{-3} \text{ M} \]

\[ e_0 = 1.65 \]
Michaelis, inhibitor, and dissociation constants for this substrate are also included in Table II. For pyruvate and oxalacetate, the $K_a$ values obtained by the two methods agree closely, but differ from $K_a$ and $K_i$ determinations obtained in initial rate studies of the over-all reaction by one to two orders of magnitude. In contrast, all the constants obtained for $\alpha$-ketobutyrate were in the same range. The latter substrate differed also in that the enhancement of the enzyme-substrate complex ($\epsilon$) is less than 1 as compared with the $\epsilon$ for pyruvate and oxalacetate, which are greater than 1. Possible differences between the interaction of pyruvate carboxylase with $\alpha$-ketobutyrate as compared with the interaction of this enzyme with pyruvate and oxalacetate are discussed in a later section.

The $K_d$ values obtained for pyruvate and $\alpha$-ketobutyrate represent true dissociation constants for the enzyme-substrate complexes, but the value found for oxalacetate is more complex since pyruvate carboxylase reacts with oxalacetate in the absence of other reaction components.

Enzyme-biotin + oxalacetate $\rightleftharpoons$ enzyme-biotin-oxalacetate $\rightleftharpoons$

\[
\text{enzyme-biotin} \sim \text{CO}_2 \text{-pyruvate} \rightleftharpoons (12) \\
\text{enzyme-biotin} \sim \text{CO}_3 + \text{pyruvate}
\]

Furthermore, Reaction 12 does not come to a true equilibrium because of the nonenzymatic decomposition of enzyme-biotin $\sim$ CO$_3$ to enzyme-biotin $+$ CO$_2$. Therefore, the apparent $K_d$ for oxalacetate determined by either the PRR or avidin inactivation methods is not a true dissociation constant for the enzyme-oxalacetate complex, but also contains a factor providing for the remaining reactions shown in Reaction 12 and terms due to the nonenzymatic breakdown of enzyme-biotin $\sim$ CO$_3$. Similarly, the observed $\epsilon$ is not a true enhancement of the enzyme-oxalacetate complex. The relative contributions of these factors and terms to the observed $K_d$ and $\epsilon$ cannot be assessed at present.

Properties of Certain Inhibitors of Pyruvate Carboxylase Which Interact with Bound Manganese—Further evidence relating to the role of the bound manganese in pyruvate carboxylase was obtained by examination of the properties of inhibitors of the over-all reaction, the action of which is localized in the second partial reaction, the carboxylation of pyruvate by enzyme-biotin $\sim$ CO$_3$ (Reaction 2). These compounds have been found to reduce the enhancement of the bound manganese and also to protect pyruvate carboxylase against inactivation by avidin. The inhibitor and dissociation constants obtained by examination of the effects of these inhibitors on the initial rate of the over-all reaction, on the rate of inactivation by avidin, and on the enhancement of the PRR are summarized in Table III. Comparison of the inhibitor constants and dissociation constants obtained by the three inde-
TABLE III
Comparison of inhibitor constants and dissociation constants for some inhibitors of pyruvate carboxylase

The $K_i$ values were obtained by measurement of inhibition of initial rate of CO$_2$ fixation as a function of pyruvate concentration, except for mesoxalate, when the inhibition of the initial rate of oxalacetate decarboxylation was measured as a function of oxalacetate concentration. For Type II and III inhibitors, $K_i$ was estimated from a plot of $1/V_i$, against inhibitor concentration as described by Webb (14). For Type I inhibitors, $K_i$ was obtained from secondary plots of the ordinate intercepts or slopes as shown for oxalate in Fig. 7. Other values were obtained as described in Table II.

<table>
<thead>
<tr>
<th>Inhibitor$^*$</th>
<th>Catalytic activity ($k_2$)</th>
<th>Avridin inactivation</th>
<th>PRR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_1/k_2$</td>
<td>$K_d$</td>
<td>$k_1/k_2$</td>
</tr>
<tr>
<td>Type I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxalate</td>
<td>$11.0 \times 10^{-6}$</td>
<td>0.2</td>
<td>$8.9 \times 10^{-6}$</td>
</tr>
<tr>
<td>Oxamate</td>
<td>$1.6 \times 10^{-3}$</td>
<td>0.12</td>
<td>$1.4 \times 10^{-3}$</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>$5.6 \times 10^{-4}$</td>
<td>0.12</td>
<td>$6.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>Fluoropyruvate</td>
<td>$1.7 \times 10^{-4}$</td>
<td>0.05</td>
<td>$1.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>Type II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesoxalate</td>
<td>$2.1 \times 10^{-3}$</td>
<td>0.14</td>
<td>$1.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>Malonate</td>
<td>$2.2 \times 10^{-2}$</td>
<td>0.2</td>
<td>$8.2 \times 10^{-3}$</td>
</tr>
<tr>
<td>Type III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Malate</td>
<td>$6.5 \times 10^{-4}$</td>
<td>0</td>
<td>$2.3 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

$^*$ Other inhibitors, the properties of which have been examined, are: (a) Type I, bromopyruvate, glyoxalate, and oxalate; (b) Type II, tartronate; and (c) Type III, $\alpha$-ketoglutarate, isocitrate, maleate, and fumarate.

$^b$ Measured as a function of pyruvate concentration (see Fig. 3A).

$^a$ Measured as a function of oxalacetate concentration (see Fig. 3B).

Although all the inhibitors described here affect the second partial reaction of pyruvate carboxylase, a closer examination reveals that the details of the mechanism of inhibition may differ. For convenience, these inhibitors have been subdivided into three types in Table III.

**Type I Inhibitors, e.g. Oxalate—**The inhibition of the initial rate of the over-all reaction by Type I inhibitors was uncompetitive with respect to pyruvate (Fig. 3A), but was competitive when measured as a function of the oxalacetate concentration (Fig. 3B). Furthermore, the relationship of $1/(k_2b_2)$ with $1/(inhibitor)$ was nonlinear at high concentrations of inhibitor (Fig. 4) and the enzyme-inhibitor complex was only partially protected against inactivation by avidin (0 < $k_2b_1$ < 1) (Fig. 5; Table III).

**Type II Inhibitors, e.g. Mesoxalate—**These compounds resembled the Type I inhibitors in showing nonlinearity of the relationship of $1/(k_2b_2)$ with $1/(inhibitor)$ at high inhibitor concentrations and an enzyme-inhibitor complex which was partially protected against inactivation by avidin. However, the initial rate of the over-all reactions measured as a function of either pyruvate or oxalacetate concentration showed a mixed type of inhibition by these compounds (14), in contrast to the competitive or uncompetitive behavior observed with Type I compounds (Fig. 3). The mixed inhibitor behavior of mesoxalate on oxalacetate decarboxylation measured as a function of oxalacetate concentration is shown in Fig. 6.

**Type III Inhibitors, e.g. L-Malate—**These compounds showed a mixed type of inhibition of the initial rate of the over-all reaction when this parameter was measured as a function of pyruvate concentration. However, they resembled pyruvate (Fig. 1) in their effect on the enhancement of the bound manganese since the relationship of $1/(k_2b_2)$ with $1/(inhibitor)$ was linear at all inhibitor concentrations. Additionally, the enzyme-inhibitor complex was completely protected against avidin inactivation.

In Table IV, effect of some carboxylic acids on rates of CO$_2$ fixation, exchange of pyruvate-$^4$C with oxalacetate, and exchange of $^{32}$P with ATP by pyruvate carboxylase CO$_2$ fixation was measured spectrophotometrically and ATP-P$_i$ and pyruvate-oxalacetate exchange were measured as described under "Methods."

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>CO$_2$ fixation</th>
<th>ATP-P$_i$ exchange</th>
<th>Pyruvate-oxalacetate exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 $\times 10^{-4}$ M malonate</td>
<td>98.5</td>
<td>99.5</td>
<td>91.0</td>
</tr>
<tr>
<td>1.5 $\times 10^{-4}$ M malonate</td>
<td>70</td>
<td>94.8</td>
<td>71.5</td>
</tr>
<tr>
<td>2.7 $\times 10^{-4}$ M oxalate</td>
<td>8.7</td>
<td>98.0</td>
<td>14</td>
</tr>
</tbody>
</table>

In Table III, a closer examination revealed that the details of the mechanism of inhibition may differ. For convenience, these inhibitors have been subdivided into three types.
Fig. 3. Inhibition of pyruvate carboxylase by oxalate. The initial rates of CO$_2$ fixation and oxalacetate decarboxylation were measured spectrophotometrically as described in "Methods." Oxalate was present at the concentrations ($\times 10^{-5}$ M) indicated by the figures.

Fig. 4. Dissociation constant for oxalate by PRR analysis. The titrating agent contained, in 0.1 ml, 5 moles of Tris-Cl (pH 7.8), 8.5 moles of Tris-Cl (pH 7.2), 34 moles of KCl, 0.77 mg of pyruvate carboxylase (specific activity, 10.6), and 0.025 mmole of potassium oxalate. Titration was performed as described for Fig. 1. The final pH was 7.8.

Fig. 5. Dissociation constant for oxalate by measurement of protection against avidin inactivation. The system was as described for Fig. 2 but with various oxalate concentrations. Analysis was performed as described for Fig. 2.
Secondary plots of the ordinate intercepts of Fig. 3A and the slopes of Fig. 3B against the oxalate concentration (Fig. 7) showed that a linear relationship existed in each case. This relationship is characteristic of simple uncompetitive (Fig. 7A) and simple competitive (Fig. 7B) inhibition. These results suggest that, in contrast to Type II and III inhibitors, the only significant interaction of Type I inhibitors is with the free enzyme to give $E_I$, and that the formation of $E_I \sim CO_2$ is not a kinetically significant pathway. The relationship of pyruvate with Type I inhibitors may be compared with the results obtained in initial rate studies of the relationship between pyruvate and the substrates of Reaction 1 (ATP and HCO$_3^-$). For these latter studies, an uncompetitive relationship was also observed, and is explained if pyruvate interacts significantly only with $E \sim CO_2$. The binding of pyruvate to the free enzyme as shown by PRR (Fig. 1) and avidin inactivation (Fig. 2) studies, therefore, represents the formation of a complex which is not a part of the main reaction pathway. The observed difference between $K_m$ and $K_s$ for pyruvate (Table II) is therefore not unexpected.

A possible deviation from the linear relationship shown in Fig. 7A may be found for the value of the ordinate intercept at the highest concentration of oxalate which is less than that pre-

---

*Fig. 6. Inhibition of pyruvate carboxylase by mesoxalate. The initial rate of oxalacetate decarboxylation was measured spectrophotometrically as described in "Methods." Mesoxalate was present at the concentrations ($\times 10^{-5}$ M) indicated by the figures.

*Fig. 7. Secondary plots of the ordinate intercepts from Fig. 3A and slopes of Fig. 3B against oxalate concentration

---

4 M. C. Scrutton and M. F. Utter, unpublished observations.
dined. Similar deviations at the highest inhibitor concentration used have been observed for all Type I inhibitors except bromopyruvate.

Enolization of Pyruvate—The results presented in the previous sections are entirely consistent with the proposal that the bound manganese of pyruvate carboxylase is involved in the carboxylation of pyruvate by the enzyme-biotin \( \sim \text{CO}_2 \) complex and in the formation of this complex from enzyme-biotin and oxalacetate (Reaction 2). Since these are transcarboxylation reactions, the role of the bound manganese may be analogous to that suggested by Steinberger and Westheimer (15) for the catalysis of the decarboxylation of dimethyl oxalacetate by metal ions. The intermediates proposed by Steinberger and Westheimer (15) (as written for pyruvate and oxalacetate) were those shown in Reaction 13. Spectral evidence for complexes of this type with divalent manganese had previously been presented by Kornberg, Ochoa, and Mehler (16). In the decarboxylation reaction (15), the most effective catalysts were \( \text{Fe}^{3+}, \text{Al}^{3+}, \) and \( \text{Cu}^{2+} \), and, although the rate of decarboxylation in the presence of \( \text{Mn}^{2+} \) was only 4\% of that found for \( \text{Al}^{3+} \), the results suggest that \( \text{Mn}^{2+} \) might be a more effective catalyst. Reaction 13 predicts that pyruvate carboxylase should cause enolization of pyruvate in the absence of other reaction components, which might be detected by a release of tritium from the methyl group of tritiated pyruvate on incubation with the enzyme.

The assay for tritium release described under “Methods” has been studied as a function of time and enzyme concentration in a system which contained the components necessary for the overall carboxylation reaction (Fig. 8). The release of tritium was linear with time to 20 min in the presence of 0.88 unit of enzyme (Fig. 8A) and with enzyme concentration to 1.1 units when a 15-min incubation period was used (Fig. 8B). Table V shows that release of tritium from tritiated pyruvate occurred only in the presence of the complete reaction mixture (Experiment A) or of oxalacetate (Table VB). Enolization of pyruvate in this system appeared, therefore, to require the presence of the enzyme-biotin \( \sim \text{CO}_2 \) complex. These results provide no support for the mechanism shown in Reaction 13, but are not necessarily inconsistent with such a mechanism. The equivocal nature of the results arises from the possibility that the identical proton,

![Fig. 8. Characteristics of tritium release from tritiated pyruvate by pyruvate carboxylase. The incubation system was as described in “Methods.” For examination of the linearity with time (A), the reaction was initiated with 32 \( \mu \text{g} \) of pyruvate carboxylase (specific activity, 18.2) and incubation was conducted for the time indicated. The linearity of response to enzyme concentration was examined with an incubation time of 15 min and the reaction was initiated with various concentrations of pyruvate carboxylase (specific activity, 18.2) (B) as indicated. The total counts per min released have been corrected for nonenzymic release of \( \text{H}^+ \) from tritiated pyruvate.](http://www.jbc.org/content/241/9/3495/F1.large.jpg)
which is removed from pyruvate on formation of the enzyme-
pyruvate complex, may be replaced on this substrate when the
complex dissociates. This phenomenon of proton retention
has been observed for several enzymes (17, 18).

Preliminary results also suggested that pyruvate was not
bound to the enzyme as a Schiff base intermediate, since the
presence of pyruvate did not increase the extent of inactivation
of pyruvate carboxylase by NaBH₄ at pH 7.8 as tested by the
procedure of Rutter (19).

**DISCUSSION**

The data presented here strongly support the postulate that
the bound manganese of pyruvate carboxylase is involved in the
transcarboxylation portion of the reaction. Any detailed hypo-
thesis describing the role of the metal ion in carboxyl group
transfer from 1'-N-carboxybiotin-enzyme to pyruvate is neces-
sarily speculative, but the experimental observations suggest
certain guidelines for such hypotheses and for future experi-
mentation. Any proposed mechanism must, as a minimum, be
consistent with (a) the difference in the enhancements (εₑ)
of the enzyme-substrate (Table II) and enzyme-inhibitor (Table
III) complexes, (b) the failure to observe a reduction in the en-
hancement of the bound manganese when the enzyme-biotin ∼
CO₂ complex is formed from ATP + HCO₃⁻ (Table I, Experi-
ment B), and (c) the simple competitive relationship between
oxalate and oxalacetate observed in initial rate studies of the
overall reaction (Figs. 3B and 7B).

Luz and Meiboom (20) have shown that the PRR of water
in the presence of a complex of a paramagnetic ion, and hence
the enhancement value of the complex, is directly proportional
to the number of water molecules in the coordination sphere
of the ion. Additionally, the PRR is inversely proportional to
both the lifetime of the water molecules in the coordinated state
and the rotation rate of the hydration sphere (13, 21). An in-
crease in the rotation rate of the hydration sphere such as might
result from a conformational change of the protein would reduce
the enhancement to a value no less than 1, the enhancement of
free manganese. For inhibitors such as oxalate, actual de-en-
hancement of the bound manganese is observed in the enzyme-
inhibitor complex (εₑ < 0.3), and this finding strongly suggests
that the inhibitors coordinate directly to the bound manganese
reducing the number of water ligands. The enhancement value
of the complex is similar to that found for the complex of Mn(II)
with EDTA (22), in which only 1 water molecule remains in the
coordination sphere of the metal (23). Therefore, in the en-
zyme-inhibitor complexes, only 1 water molecule may remain
in the manganese coordination sphere, and, since inhibitors such
as oxalate can provide two ligands to the metal, the structure
of the complex may be drawn as shown in Fig. 9, with the pro-
tein providing the remaining three ligands.

In contrast to the inhibitors, actual de-enhancement is not
observed in the E-pyruvate and E-oxalacetate complexes (εₑ >
1.0), and the possibility must be considered that these substrates
do not interact with the bound manganese but bind at another
site of the protein. In the latter case, the reduction in enhance-
ment would be explained if binding of the substrate caused a
conformational change which resulted in facilitation of the rota-
tion of the manganese hydration shell or a decrease in the rate
of exchange of the coordinated water (13, 20, 21). Unequivocal
evidence for or against direct coordination of these substrates to
the metal ion requires further experimentation, e.g. examination
of the effect of the presence of the enzyme on the nuclear mag-
netic resonance spectrum of the methyl protons of pyruvate.

Despite these reservations, the present data suggest that a direct
interaction between the bound manganese and these substrates
does occur since oxalate, which is believed to coordinate directly
with the metal ion, shows a simple competitive relationship with
oxalacetate (Figs. 3B and 7B). The enhancement of the enzyme-
pyruvate complex (εₑ = 1.7) then suggests that pyruvate pro-
vides only one ligand to the bound manganese (Fig. 9), leaving 2
water molecules in the manganese coordination sphere.

The proposed role of the metal ion in the formation of oxal-
acetate from E ∼ CO₂ and pyruvate is shown in Fig. 10. The
carboxylation of free enzyme (A) by ATP + HCO₃⁻ to give
1'-N-carboxybiotin-enzyme (B) does not result in interaction
of the 1'-N-carboxyl group with the bound manganese, in accord
with the finding that the enhancement was unaffected when the
intermediate was formed in this way (Table I, Experiment B).
The addition of pyruvate to B to give E ∼ CO₂-pyruvate (C)
causes a conformational change which brings the biotin ring
into close proximity to the bound manganese. The conversion
of E ∼ CO₂-pyruvate (C) to E-oxalacetate (D) is then shown as
a concerted process in which the proton leaves pyruvate syn-
chronously with carboxyl group transfer to pyruvate from the
1'-N-carboxybiotin moiety. In this process, the electron-with-
drawing effect of the metal would both facilitate the departure
of a proton from the methyl group of pyruvate and would also
assist the formation of a carbonium ion from 1'-N-carboxybiotin.

Thus, in the mechanism shown in Fig. 10, the metal ion has a
dual role (see below). Additionally, the nucleophilic sulfur atom
might also aid the required polarization of the carboxyl
ation of the effect of the presence of the enzyme on the nuclear mag-
netic resonance spectrum of the methyl protons of pyruvate.

Despite these reservations, the present data suggest that a direct
interaction between the bound manganese and these substrates
does occur since oxalate, which is believed to coordinate directly
with the metal ion, shows a simple competitive relationship with
oxalacetate (Figs. 3B and 7B). The enhancement of the enzyme-
pyruvate complex (εₑ = 1.7) then suggests that pyruvate pro-
vides only one ligand to the bound manganese (Fig. 9), leaving 2
water molecules in the manganese coordination sphere.

The proposed role of the metal ion in the formation of oxal-
acetate from E ∼ CO₂ and pyruvate is shown in Fig. 10. The
carboxylation of free enzyme (A) by ATP + HCO₃⁻ to give
1'-N-carboxybiotin-enzyme (B) does not result in interaction
of the 1'-N-carboxyl group with the bound manganese, in accord
with the finding that the enhancement was unaffected when the
intermediate was formed in this way (Table I, Experiment B).
The addition of pyruvate to B to give E ∼ CO₂-pyruvate (C)
causes a conformational change which brings the biotin ring
into close proximity to the bound manganese. The conversion
of E ∼ CO₂-pyruvate (C) to E-oxalacetate (D) is then shown as
a concerted process in which the proton leaves pyruvate syn-
chronously with carboxyl group transfer to pyruvate from the
1'-N-carboxybiotin moiety. In this process, the electron-with-
drawing effect of the metal would both facilitate the departure
of a proton from the methyl group of pyruvate and would also
assist the formation of a carbonium ion from 1'-N-carboxybiotin.

Thus, in the mechanism shown in Fig. 10, the metal ion has a
dual role (see below). Additionally, the nucleophilic sulfur atom
might also aid the required polarization of the carboxyl

A in a Dreiding molecular model of biotin, the sulfur atom was
found to be 3.9 Å from the carboxyl carbon atom. The 3d orbi-
tals of sulfur, which contain the unbonded electrons, extend well
beyond this distance.
A difference in the environment of the biotin ring in pyruvate carboxylase is further suggested by the contrasting effects on the rate of inactivation of the enzyme by avidin shown by pyruvate or oxalacetate (increased rate of inactivation) as compared with ATP (decreased rate of inactivation) (11).

Figs. 9 and 10 are entirely consistent with the observed relationships of the inhibitors with pyruvate and oxalacetate but the structure of the E-oxalacetate complex (Fig. 10D), in which this substrate provides two ligands to the bound manganese, suggests that the enhancement value of this complex should be similar to that observed for the E-oxalate complex ($e_e < 0.3$). The observed value for the E-oxalacetate ($e_e = 2.45$) is, however, much higher, and an apparent discrepancy exists, which may be rationalized in several ways. (a) As noted previously, it is not possible to obtain a true value for the enhancement of the E-oxalacetate complex since it is in equilibrium (Reaction 12) with several other structures (Fig. 10, B and C) and the observed value for $e_e$ is a weighted average of these various structures. (b) A change in the environment of the manganese may occur in the E-oxalacetate complex which hinders the rotational rate of the one remaining coordinated water molecule. (c) The rapid chemical exchange shown for the methylene protons of oxalacetate (Table V, Experiment B) may affect the longitudinal relaxation rate of the water protons in a manner analogous to an additional water molecule. (d) Disruption of one of the metal-protein ligands may occur on formation of the E-oxalacetate complex, permitting the coordination of an additional water molecule by the manganese and resulting, therefore, in the net
displacement of only 1 water molecule. (e) Manganese may coordinate an additional water molecule as a seventh ligand (23) on formation of the $E\cdot$oxalacetate complex. While none of these alternatives can be excluded at present, either $a$, $b$, or $c$ is considered to provide the more probable explanation.

The data obtained for $\alpha$-ketobutyrate (Table II), as compared with pyruvate and oxalacetate, suggest that $E\cdot$ketobutyrate complex resembles the $E\cdot$oxalacetate complex more closely than the $E\cdot$pyruvate complex. Thus, for $\alpha$-ketobutyrate, $e = 0.4$, suggesting that this substrate is a bidentate ligand for manganese, as has been proposed for oxalate, and therefore forms a complex analogous to that depicted in Equation 13. A structural difference between the $\alpha$-ketobutyrate complex and the pyruvate complex is compatible with the very different rates of CO$_2$ fixation by pyruvate carboxylase on these two substrates (4).

The results reported here, together with Caplow's studies (24), suggest that bound metal ions may be present in pyruvate carboxylases purified from other sources and in other biotin enzymes. Indirect evidence from inhibitor studies affords some support for this postulate since Losada, Canovas, and Ruiz-Amil (26) have shown that pyruvate carboxylase in bakers' yeast is inhibited by oxalate; Seubert and Huth (27) have found inhibition of pyruvate carboxylase in rat liver by oxalate, oxamide, and L-malate with inhibitor constants similar to those reported here for the chicken liver enzyme; and methylmalonyl-CoA-oxalacetate transcarboxylase from Propionibacterium shermanii is inhibited by oxalate. Methylmalonyl-CoA-oxalacetate transcarboxylase has been examined for bound metal by the PRR method, and, while a small effect is found, the PRR after denaturation of an enzyme sample with perchloric acid gave an effect equivalent to only 0.1 µmole of manganese per µmole of biotin. Manganese cannot, therefore, be involved in this biotin enzyme, but the presence of some other metal with a much less significant effect on the PRR of water cannot be excluded.

No information is available which permits direct deductions concerning the environment of bound manganese in pyruvate carboxylase. The absence of de-enhancement by large chelating agents, e.g. EDTA (1), in contrast to the very marked effect of small bidentate ligands, e.g. oxalate, suggests that steric restrictions on access to the metal ion may exist. While the PRR data strongly suggest that oxalate combines directly with the bound manganese, experiments with $^{45}$Mn-pyruvate carboxylase show unequivocally that the $^{45}$Mn content is unaffected by incubation with oxalate. The unusually tight binding of manganese to this protein makes the investigation of the ligands involved a subject of considerable interest and studies on this aspect are now under way.

Acknowledgment—The authors are exceptionally indebted to Dr. M. Cohn for her advice and encouragement.

REFERENCES

7. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1941).
Pyruvate Carboxylase: VII. A POSSIBLE ROLE FOR TIGHTLY BOUND MANGANESE
Albert S. Mildvan, Michael C. Scrutton and Merton F. Utter


Access the most updated version of this article at http://www.jbc.org/content/241/15/3488

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/241/15/3488.full.html#ref-list-1