3-Mercaptopicolinate
A REVERSIBLE ACTIVE SITE INHIBITOR OF AVIAN LIVER PHOSPHOENOLPYRUVATE CARBOXYKINASE*

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The inhibition of chicken liver phosphoenolpyruvate carboxykinase by 3-mercaptopicolinic acid (3-MP) has been investigated. Kinetic studies show 3-MP to be a noncompetitive inhibitor relative to all substrates and to the activator, Mn**.

EPR studies demonstrate that Mn** binding to the enzyme is unaffected by 3-MP. Proton relaxation rate studies demonstrate that 3-MP binds to the binary E'Mn complex with a $K_D$ of $0.5 \times 10^{-4} M$ and gives a ternary enhancement of 8.0. Additional proton relaxation rate studies detected formation of the quaternary complexes E'Mn-IDP-3-MP, E'Mn-ITP-3-MP, and E'Mn'CO2-3-MP.

High resolution 3'H nuclear relaxation rate studies suggest that 3-MP binds in close proximity to the activator cation, Mn**, but not in the first coordination sphere. Active site models suggest that the 3-MP-binding site may partially overlap the phosphoenolpyruvate-binding site. The NMR studies, which detected formation of the quaternary E'Mn-3-MP-phosphoenolpyruvate complex, also demonstrated that the binding of one of these ligands affects the interactions of the other ligand with E'Mn.

Calorimetric studies of the E'Mn complex demonstrated that 3-MP causes an increase in the transition temperature midpoint without an increase in enthalpy. These results indicate that 3-MP causes a conformational change in the enzyme but does not increase the thermostability of the ternary complex.

The experiments reported herein suggest that inhibition by 3-MP is due to specific and reversible binding within the active site of phosphoenolpyruvate carboxykinase.

In vertebrates, phosphoenolpyruvate carboxykinase (GTP:oxalacetate carboxylase (transphosphorylating), EC 4.1.1.32) catalyzes an essential reaction in the formation of glucose from three and four carbon precursors: the conversion of oxalacetate to P-enolpyruvate. Mitochondrial and cytosolic forms of the enzyme are known, and the cellular distribution is species-dependent (Utter and Kolenbrander, 1972). Studies have shown the mitochondrial and cytosolic forms to be different proteins in rat liver (Ballard and Hanson, 1969), in monkey liver (Utter and Chuang, 1978), and in guinea pig liver. The two forms are thought to be subject to different forms of control.

The reaction catalyzed by P-enolpyruvate carboxykinase is thought to be a key regulatory site for gluconeogenesis, yet little is known regarding the control of either form of the enzyme. The cytosolic enzyme from rat liver has been extensively studied, and several factors have been reported to play a role in the control of its activity. These include: diabetes, fasting and hormones (Shrago et al., 1976), and metal ion levels (Colombo et al., 1981; Schramm et al., 1981). Divalent metal ions have also been suggested as playing a regulatory role in mitochondrial P-enolpyruvate carboxykinase from avian liver (Lee et al., 1981).

Tryptophan metabolites and structurally related derivatives of picolinic acid have been reported to alter the rate of gluconeogenesis both in rats and in vitro studies (Snoke et al., 1971).

Particular interest has centered on 3-mercaptopicolinic acid since it was first shown to be a potent hypoglycemic agent in laboratory animals and that it inhibits glucose synthesis from three common precursors in vitro (DiTullio et al., 1974). Later studies demonstrated that 3-MP specifically inhibits gluconeogenesis at the level of P-enolpyruvate carboxykinase (Kostos et al., 1975).

Studies of rat liver cytosolic P-enolpyruvate carboxykinase show 3-MP to be a noncompetitive inhibitor of the P-enolpyruvate formation reaction with a $K_i = 0.5 \mu M$, and it was suggested that 3-MP removes a tightly bound inhibitory metal other than Mn** (Domain-Baum et al., 1976). MacDonald (1978) has reported that an Fe**-3-MP complex inhibits crude fractions of cytosolic or mitochondrial P-enolpyruvate carboxykinase from five species equally well. His studies suggested that 3-MP will not bind well to a Mn**-containing P-enolpyruvate carboxykinase. Reynolds (1980) has reported 3-MP to be a competitive inhibitor with respect to P-enolpyruvate using P-enolpyruvate carboxykinase from rat liver and from tapeworm.

Other investigators have reported that the cytosolic enzyme is more sensitive than the mitochondrial enzyme to inhibition by 3-MP from studies of enzymes from guinea pig liver (Robinson and Oei, 1975) and from kidney tubules of five

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1 P. Markowitz, personal communication.

2 The abbreviations used are: 3-MP, 3-mercaptopicolinic acid; PRK, solute proton longitudinal relaxation rate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
species (Watford et al., 1980).

Initial observations in our laboratory suggested that 3-MP was the only derivative of picolinic acid which specifically affected chicken liver mitochondrial P-enolpyruvate carboxykinase activity, causing inhibition at micromolar concentrations.

The inhibitor 3-MP could be a useful tool in understanding the mechanism of regulation of this enzyme. A detailed kinetic and spectroscopic investigation of the inhibition of chicken liver mitochondrial P-enolpyruvate carboxykinase by 3-MP was undertaken in an effort to elucidate the mechanism by which enzymic activity, and thereby the rate of glucose synthesis, is controlled in vertebrates.

The results presented here suggest that 3-MP inhibits avian liver P-enolpyruvate carboxykinase activity by reversibly and specifically binding within the active site. This binding causes a conformational change which is unfavorable for catalysis.

**EXPERIMENTAL PROCEDURES**

**Materials**

P-enolpyruvate carboxykinase was purified from chicken liver as previously described by Habeeb and Nowak (1982a) and modified by Duffy (1982) and Lee (1983). Lactate dehydrogenase, malate dehydrogenase, and pyruvate kinase were purchased from Boehringer Mannheim. P-enolpyruvate, oxaloacetate, ATP, GDP, and NADH were purchased from Sigma. 3-Mercaptopicolinic acid and the other derivatives tested were generous gifts from Drs. Harry Saunders and Nicholas DiTullio, Smith Kline and French Laboratories, Philadelphia, PA, and were prepared fresh daily by dissolving in H2O and neutralizing to pH 7 with KOH prior to final dilution. All other reagents were of the highest grade commercially available. Distilled, deionized H2O was used routinely to prepare all solutions.

**Methods**

**Kinetic Assays**—Initial velocity studies of the carboxylation of P-enolpyruvate to form oxaloacetate were done using the continuous assay described by Habed and Nowak (1982a), which couples P-enolpyruvate carboxykinase to lactate dehydrogenase. The disappearance of NADH absorbance at 340 nm was monitored with either a Gilford 240 or 250 spectrophotometer. Initial velocity studies of the P-enolpyruvate formation reaction were done by monitoring the decrease in absorption of NADH at 340 nm using an assay which couples P-enolpyruvate carboxykinase to pyruvate kinase and lactate dehydrogenase (Lee et al., 1981). Enzyme activity is expressed as micromoles of NADH formed per ml/min/mg of protein (units/mg).

All kinetic studies were performed at room temperature, 22 ± 1 °C. Enzyme concentrations were determined spectrophotometrically at 290 nm using the reported extinction coefficients εMnH = 18.3 and εMn = 72,000 (Habeeb and Nowak, 1982a).

**Enzyme Preparation for Physical Studies**—Prior to all physical studies, enzyme was prepared by desalting on a Bio-Rad P-6-DG column (1.1 × 25 cm), having 2 cm of Chelex 100 on the top, which had been equilibrated in 65 mM Tris-HCl buffer, pH 7.4. The enzyme was concentrated using either an Amicon Model 80C ultifiltration system with microvolute accessary or a Minicon B-15 concentrator. All solutions used in the physical studies, with the exception of MnCl2, were passed through a Chelex 100 column.

**3-MP Concentration**—When an exact determination of 3-MP concentration was required, it was determined spectrophotometrically by reaction with 5,5′-dithiobis(2-nitrobenzoic acid). Aliquots of 3-MP were added to an excess of 5,5′-dithiobis(2-nitrobenzoic acid) (0.4 ml final volume), and the change in absorption at 412 nm, due to release of the nitromercaptopentobenzate anion, was monitored. Using the reported extinction coefficient of 13,600 M−1 cm−1 (Habeeb, 1972), the moles of anion released could be calculated. The amount of anion released is stoichiometric with the amount of 3-MP.

**Mn2+ Binding Studies**—Mn2+ binding to P-enolpyruvate carboxykinase in the presence of 3-MP was investigated using EPR techniques (Cohn and Traversend, 1964; Mildvan and Cohn, 1963). A Varian model 4500 spectrometer was used, and the probe temperature was maintained at 22 ± 1 °C by N2 flow. Individual samples (0.05 ml final volume) contained varying Mn2+ concentrations and fixed concentrations of enzyme and 3-MP in 65 mM Tris-HCl, 100 mM KCl, pH 7.4. The control samples lacked enzyme. The samples were drawn into 1-mm (inner diameter) quartz capillary tubes and the spectra were taken. The peak height of the Mn2+ spectrum was used as a measure of free Mn2+, and it was assumed that bound Mn2+ makes no contribution to the observed signal height. Bound Mn2+ was calculated as the difference in concentration of total Mn2+ and free Mn2+. Samples were treated and measured as described by Ploplis et al. (1981). The same procedures were used to measure the binding of Mn2+ to 3-MP.

**Proton Relaxation Rate Studies**—Formation of the ternary E-Mn-3-MP complex and quaternary E-Mn-3-MP-S complexes was investigated using PRR techniques (Mildvan and Cohn, 1970; Nowak, 1981). Proton relaxation rates were measured at 22 ± 1 °C on a Seimco pulsed NMR spectrometer operating at 24.3 MHz, using the Carr-Purcell (1954) 180°-90° sequence.

Sample tubes contained, in a final volume of 0.05 ml, a fixed concentration of enzyme and Mn2+ in 65 mM tris-HCl, 100 mM KCl, pH 7.4. A sample prepared identically but which also contained 3-MP was titrated into the first tube, and the PRR was measured with each addition. The reverse titrations were also performed. In this case, sample tubes which contained fixed concentrations of enzyme, Mn2+, and 3-MP were titrated with an identical sample of Mn2+ which also contained the substrate to be titrated. The PRR was measured at each addition.

The dissociation constant (Kd) of ligand from the enzyme-Mn or the enzyme-Mn-ligand complex was obtained by generating a "best fit" to the data. Titration data were fit using the program of Reed et al. (1970) which has been adapted for the Wang calculator and which varies K0 until a minimum per cent standard deviation is obtained.

**High Resolution 1H NMR**—The 1H NMR spectra were obtained at 300 MHz using a Nicolet NTC-300 spectrometer equipped with a 298A pulse system and a Nicolet 1100E computer. The spectra taken at 300 MHz were obtained with a Varian XL-100-15 spectrometer interfaced to a Nicolet 1080 computer. The spectrometer was locked on 2H, present as the solvent, H2O. The Ti values of the 3-MP protons were measured by the inversion recovery method (Vold et al., 1968; Allerhand et al., 1971). Microliter quantities of Mn2+ were titrated into buffered solutions of 3-MP or enzyme and 3-MP. The longitudinal relaxation rates (1/TI) were measured at each addition. 1/TI values were plotted against [Mn2+], and the paramagnetic contribution to the relaxation rate (1/TI) was calculated as the difference in concentrations of total Mn2+ and free Mn2+. Data were treated by the Scatchard (1949) method. The same dissociation constant (Kd) of ligand from the enzyme-Mn or the enzyme-Mn-ligand complex was obtained by generating a "best fit" to the data. Titration data were fit using the program of Reed et al. (1970) which has been adapted for the Wang calculator and which varies K0 until a minimum per cent standard deviation is obtained.

**3-Mercaptopicolinate Inhibition**

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3-Mercaptopicolinate Inhibition

**RESULTS**

**Kinetic Studies**—Several derivatives of picolinic acid were screened for their effect on chicken liver P-enolpyruvate carboxykinase activity. The compounds tested were 3-MP, 3-aminopicolinic acid, quinolinic acid, 3-S-methylpicolinic acid, 3-S-phenylpicolinic acid, 3-hydroxypicolinic acid, 3-bromopicolinic acid, and 2-hydroxymethyl-3-mercaptopicolinic acid. All compounds tested inhibited P-enolpyruvate carboxykinase activity, but only 3-MP caused inhibition at concentrations of 0.1 mM or less. None of the other compounds showed effects at concentrations less than 0.25 mM. At higher concentrations, these compounds caused abrupt losses in enzyme activity (data not shown). This phenomenon is believed to be due to some nonspecific effect, such as removal of the activator, Mn²⁺, rather than specific inhibition of enzymatic activity.

Detailed kinetic studies of 3-MP inhibition against P-enolpyruvate carboxykinase, oxalacetate, and the nucleotide substrates exhibit mixed noncompetitive inhibition. The results of one such study are shown in Fig. 1A. 3-MP affects both the Kₘ and the Vₘₐₓ. If the experiments are performed against HCO₃⁻ or Mn²⁺, only a Vₘₐₓ effect is observed, a pattern typical of "classical" noncompetitive inhibition. The results of one such experiment are shown in Fig. 1B. The slope and the intercept replots are linear in all the experiments performed (i.e., insets of Fig. 1, A and B). Table I summarizes the results of the inhibition studies.

One plausible reason for the inhibition of P-enolpyruvate carboxykinase by 3-MP could be the formation of a mixed disulfide, E-S-S-3-MP complex. To test this possibility, kinetic inhibition studies were done in the presence of 0.143 M β-mercaptoethanol and in the absence of additional thiol. The kinetic constants obtained were the same in the presence of β-mercaptoethanol as those obtained using an assay system without reducing agent. These results suggest inhibition by 3-MP is not due to mixed disulfide bond formation. The inhibition of enzyme activity by 3-MP is reversible. Enzyme was incubated with excess 3-MP for 5 min and enzymatic activity was lost. The sample was then passed over a Bio-Rad P-6-DG desalting column. The enzyme was separated from free 3-MP under these conditions and full enzymatic activity was recovered.

Experiments which involved high concentrations of 3-MP and P-enolpyruvate carboxykinase could occasionally become hazy after several hours at room temperature. Enzyme activity could not be recovered in these cases. Whenever this phenomenon was observed, the data were discarded.

**Mn²⁺ Binding**—Mn²⁺ binding to chicken liver P-enolpyruvate carboxykinase in the presence of 3-MP was investi-
contrast to the positive temperature dependence of PRR and lack of a substantial temperature effect suggests that the measured with the enzyme from pig liver which suggests that be fit equally well when the data was obtained with the ternary Mn complex with a value of 1.3–1.7 for n. These values are in good agreement with those reported by Jomain-Baum et al. (1976) and MacDonald and Lardy (1978).

**Formation of Quaternary E·Mn·3-MP·S Complexes—PRR**

**Fig. 2. Scatchard plot of Mn**

The observed enhancement ($e^*$) is plotted as a function of 3-MP concentration. The closed circles represent the titration of the binary $E$·Mn complex with 3-MP. The sample contained 0.065 M Tris-HCl, pH 7.4, 0.1 M KCl, 0.02 M dithiothreitol, 1.1 × 10⁻⁴ M enzyme, and 5.02 × 10⁻⁶ M MnCl₂. The best fit to the data is obtained using $K_3 = 0.5 × 10^{-6}$ M. The open circles represent the titration of the ternary $E$·Mn·IDP complex with 3-MP. The experimental conditions were as described above except that the sample also contained 2.1 × 10⁻⁴ M IDP. This titration data was fit with $K_3 = 5.0 × 10^{-6}$ M.

in that case the water is in slow exchange (Miller et al., 1968). For the $E$·Mn·3-MP ternary complex, $\tau_r$ was estimated to be $3.4 \times 10^{-6}$ s at 24.3 MHz. The value of $f(\tau_r)$ can be calculated, and by assuming a value of 2.87 Å for the distance from Mn²⁺ to solvent protons, one can then use the following equation

$$\frac{1}{pT_2} = \frac{1}{T_{2M}} = q \left(\frac{\delta I_2}{\pi^2}\right) f(\tau_r)$$

to estimate $q$, the number of water molecules in the first coordination sphere of Mn²⁺ in the ternary complex. A value of 0.7 was calculated for $q$. This value is approximately one-half the value reported for the binary $E$·Mn²⁺ complex, and it is identical with that reported for the $E$·Mn·P-enolpyruvate ternary complex (Duffy, 1982).

These results suggest that 3-MP binding causes the same type of change in the environment of enzyme-bound Mn²⁺ as P-enolpyruvate binding does: displacement or immobilization of one-half of the water molecules bound in the first coordination sphere of Mn²⁺.

For P-enolpyruvate, there was no evidence for quaternary $E$·Mn·P-enolpyruvate·3-MP complex formation by either type of titration. No change in observed enhancement ($e^*$) was detected upon the addition of inhibitor to the $E$·Mn·P-enolpyruvate complex ($e^* = 5.5$) or upon the addition of P-enolpyruvate to the $E$·Mn·3-MP complex ($e^* = 4.9$). High resolution ¹H NMR experiments (vide infra) demonstrate that the quaternary $E$·Mn·P-enolpyruvate·3-MP complex does form, however. Since P-enolpyruvate binding and 3-MP binding to enzyme·Mn cause the same type of change in the

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environment of bound Mn$^{2+}$, addition of the second ligand does not cause any further perturbations in the environment of bound Mn$^{2+}$; therefore, the quaternary complex cannot be detected by PRR techniques. When 3-MP was titrated into E·Mn·IDP, a titration curve was obtained (Fig. 3). Computer fits to the data give an $e_r$ of 3.5 and a $K_o$ of approximately $5 \times 10^{-6}$ M. Titration of IDP into E·Mn·3-MP causes no change in $e_r$ ($e_r = 4.3$).

Addition of 3-MP to E·Mn·ITP resulted in a titration curve which was fit with a $K_o$ of $0.1-1.0 \times 10^{-6}$ M and an $e_r$ of 1.4. Titration of ITP into E·Mn·3-MP showed no change in the observed enhancement ($e_r = 4.2$).

Formation of the quaternary E·Mn·CO$_2$·3-MP complex was detected by PRR techniques. The 3-MP binds to the ternary complex and causes a decrease in the observed enhancement. The titration curve was fit with a $K_o$ of $0.1 \times 10^{-6}$ M and an $e_r$ of 9.6 was obtained. HCO$_3^-$ was titrated into the E·Mn·3-MP complex and a small increase in enhancement was observed ($e_r = 4.5$). A graphical estimation of the dissociation constant for HCO$_3^-$ from E·Mn·3-MP gives a value of $2.0 \times 10^{-3}$ M. This value is in good agreement with the $K_o$ of HCO$_3^-$ and the $K_o$ and $K$ values for HCO$_3^-$ binding to E·Mn previously reported (Hebda and Nowak, 1982a and 1982b).

This result is consistent with the kinetic experiments which suggest that HCO$_3^-$ binding to P-enolpyruvate carboxykinase is unaffected by 3-MP. As previously stated, with tight binding ligands, good fits to the data can be obtained even when $K_o$ values vary by a factor of 5-10. For example, the per cent standard deviation varies by only 2% when $K_o$ varies from $0.1 \times 10^{-6}$ M to $1 \times 10^{-6}$ M for 3-MP binding to E·Mn·ITP.

High Resolution $^1$H NMR—A proton NMR spectrum of 3-MP in D$_2$O (pD 7.4) is shown in Fig. 4. Resonance peak assignments were made on the basis of chemical shifts, coupling constants, and homonuclear decoupling experiments. The effect of added Mn$^{2+}$ on the $1/T_1$ values of the 3-MP protons in the presence and absence of enzyme was measured as described under "Methods," and the $1/pT_1$ values were determined. The relaxation rates of the three ring protons of 3-MP were differentially affected by the paramagnetic Mn$^{2+}$ ion (Fig. 5, A and B).

The effect of Mn$^{2+}$ on the relaxation rates of the protons of 3-MP in the binary Mn·3-MP and ternary E·Mn·3-MP complexes is summarized in Table III. The relaxation rates for the ternary complex were measured at both 100 and 300 MHz. For the binary complex a value of $3.48 \times 10^{-11}$ s for $r_e$ was used to calculate $r$. In these calculations it was assumed that for small complexes $r_e$ equals $r$, the rotational correlation time. The $r$ for Mn(H$_2$O)$_5^{2+}$ (3 $\times 10^{-11}$ s) was estimated to increase by approximately 15% upon formation of the Mn$^{2+}$.

![3-Mercaptopicolinate Inhibition](image)

**Fig. 4.** Proton NMR spectrum of 0.16 M 3-MP in D$_2$O (pD 7.4), obtained at 300 MHz. Sixteen scans were taken. The y axis is labeled in parts/million. The resonance peaks shown are downfield from the HDO peak, which has been assigned a value of 0 ppm.
ligand complex, proportional to a change in molecular weight of the complex. A $\tau_c$ of $3.9 \times 10^{-9}$ s was used to calculate $r$ in the ternary complex. This value was obtained from the frequency dependence of the relaxation rates of the 3-MP protons in the ternary complex (Table III) and assuming no frequency dependence of $\tau_c$. Assuming a maximum frequency dependence of $r$, it yields a value for $r_c$ which is smaller by a factor of 8 ($0.5 \times 10^{-9}$ s). This value is reflected in a 15% smaller value of $f(\tau_c)$ at 100 MHz and a factor of 4 smaller value at 300 MHz. Resultant variations in the distances calculated are reflected in the error levels given in Table III.

Studies of several ternary P-enolpyruvate carboxykinase complexes in our laboratory have shown that $\tau_c$ varies little in the ternary complexes investigated, regardless of the method used to determine $\tau_c$ (Duffy, 1982; Lee, 1983).

In calculating the Mn nuclei distances, it was assumed that the ligand is in fast exchange ($\tau_m \ll T_{1M}$, where $\tau_M$ is the lifetime of the ligand in the complex); therefore, $1/pT_{1p} = 1/T_{1M}$. This assumption is based on the observation that the three protons of 3-MP experience a different paramagnetic effect (Fig. 5, A and B) and a frequency dispersion of $1/T_{1p}$ is measured (Table III). If the ligand was in slow exchange, $1/T_{1M}$ would dominate $1/pT_{1p}$ and identical $1/pT_{1p}$ values would be expected for all three protons. Furthermore, if the larger value of $1/pT_{1p}$ measured (H6 proton at 100 MHz) is partially limited by chemical exchange, $1/pT_{1p} < 1/T_{1M}$, the calculation of $\tau_c$ would then lead to an apparent shorter value for $\tau_c$. This is not the case as a value of $\tau_c = 3.9$ ns is calculated from this data.

The distances were also calculated based on the assumption that $q$, the number of 3-MP molecules associated with $E-Mn$, is 1. Two lines of evidence support this assumption. First, the PRR titrations were fit using a model where $q = 1$, and good fits ($\pm 10\%$ S.D.) were obtained for all the titrations. Furthermore, in the PRR experiments where well defined titration curves were obtained (i.e., Fig. 3), the maximum effect is observed when the concentration of 3-MP is approximately the same as the concentration of $E-Mn$, suggesting that 3-MP forms a 1:1 complex with the $E-Mn$ complexes. The other assumption used is that all of the Mn$^{2+}$ added to the $^1H$ NMR experiment forms an $E-Mn$-3-MP complex. This assumption is consistent with the frequency dispersion of $1/pT_{1p}$ observed (Table III). The binary Mn-3-MP complex shows no frequency dispersion in this frequency range. Also, a calculation of the Mn$^{2+}$ distribution indicates that $>97\%$ of the Mn$^{2+}$ is in the enzyme complex.

The Mn $^1H$ distances calculated for the ternary complex (Table III) are larger than those measured for the binary complex. This indicates that 3-MP binds close to the activator cation Mn$^{2+}$ in chicken liver P-enolpyruvate carboxykinase, although it does not bind with the same coordination scheme as it does with free Mn$^{2+}$.

Models of $E-Mn$-3-MP (Fig. 6) were constructed based on the interatomic distances reported herein. Similar models of the $E-Mn$-P-enolpyruvate complex were constructed based on data reported elsewhere (Duffy, 1982). These models suggested that the 3-MP-binding site could possibly overlap the P-enolpyruvate-binding site. Since the quaternary $E-Mn$-P-enolpyruvate-3-MP complex was not detected by PRR techniques, studies were done to determine if the quaternary complex does form, or whether P-enolpyruvate and 3-MP binding to $E-Mn$ is mutually exclusive.

Attempts to do direct P-enolpyruvate binding studies, using radiolabeled P-enolpyruvate, were unsuccessful. The enzyme is unstable over the time course of an equilibrium dialysis experiment and would frequently precipitate. This occurred in the presence or absence of 3-MP. P-enolpyruvate carboxykinase elution from gel filtration columns is retarded (Hebda and Nowak, 1982a), and this phenomenon is thought to interfere with Hummel-Dreyer-type binding studies. Skewed peaks and unequal peak and trough sizes were consistently obtained when binding studies were attempted using this method.

![Fig. 6. Structure of the ternary E+Mn+3-MP complex.](image)

![Fig. 7. Effect of 3-MP on the $^1H$ relaxation rates of P-enolpyruvate in the ternary complex measured at 300 MHz.](image)
3-Mercaptopicolinate Inhibition

A high resolution $^1$H nuclear relaxation rate study of the quaternary complex was performed. If 3-MP was competitive with P-enolpyruvate for a binding site on the enzyme, increasing concentrations of 3-MP would displace P-enolpyruvate from the enzyme, and the relaxation rates of the P-enolpyruvate protons would approach the values for free P-enolpyruvate.

3-MP was titrated into a buffered solution of $E\cdot Mn\cdot P\text{-enolpyruvate}$, and the effect on the relaxation rates of P-enolpyruvate protons was monitored at each addition. The results are shown in Fig. 7. In the ternary $E\cdot Mn\cdot P\text{-enolpyruvate}$ complex, the $1/T_1$ value for the cis proton is approximately 1.2 s$^{-1}$, and this value remains constant upon addition of 3-MP. The $1/T_1$ value for the trans proton of P-enolpyruvate decreases upon the addition of 3-MP. These data indicate that although 3-MP perturbs the environment of enzyme-bound P-enolpyruvate, it does not displace P-enolpyruvate from its binding site. Both the substrate and the inhibitor bind, forming a quaternary $E\cdot Mn\cdot P\text{-enolpyruvate}\cdot 3\text{-MP}$ complex. The protons of the substrate are estimated to be greater than 9 Å from the Mn$^{2+}$ in the quaternary complex.

In the ternary $E\cdot Mn\cdot P\text{-enolpyruvate}$ complex, the distances from Mn$^{2+}$ to the cis and the trans protons are ~5.7 and 6.6 Å, respectively (Duffy, 1982).

The relaxation rates of the 3-MP protons were determined from the same experiment. In the quaternary complex these protons all experience a smaller paramagnetic effect than in the ternary complex (data not shown). If one uses these values to calculate Mn$^{2+}$ $^1$H distances using the assumptions made previously and the same value of $\tau_s$, the values obtained are all approximately 2 Å larger than those calculated for the ternary complex. This again suggests that while both 3-MP and P-enolpyruvate bind to form the quaternary complex, their interactions with $E\cdot Mn$ are affected by the presence of the other ligand.

During the time course of the $^1$H NMR experiments, 3-MP appears to exist in solution as a monomer, as evidenced by a single set of resonance peaks. Over extended periods of time, however, a second set of resonance peaks appears, shifted downfield by ~0.3 ppm. This phenomenon occurs both in the absence and presence of enzyme and is possibly due to formation of 3-MP dimers. The relative concentrations of these are minor, however.

**Differential Scanning Calorimetry**—Transition endotherms for enzyme $\cdot$ Mn, enzyme $\cdot$ Mn $\cdot$ 3-MP, and enzyme $\cdot$ Mn $\cdot$ P-enolpyruvate are shown in Fig. 8. In each case only one thermal denaturing transition is observed. The presence of saturating 3-MP causes an increase in the $T_M$ from 54.4 to 58.3 °C without an increase in the measured calorimetric enthalpy. Saturating P-enolpyruvate, however, causes a large increase in the $T_M$ to 63.8 °C which is accompanied by an increase in the calorimetric enthalpy, indicative of an increase in thermostability of the ternary complex over the binary $E\cdot Mn$ complex. The binding of 3-MP does not elicit the same effects on the protein as does the substrate P-enolpyruvate.

**DISCUSSION**

Mixed type kinetic inhibition may be thought of as partial competitive inhibition ($K_M$ effects) and pure noncompetitive inhibition ($V_{max}$ effects), although no simple unified model for such inhibition exists. The competitive portion of this effect suggests at least partial overlap of the inhibitor and the substrate for the active site. With P-enolpyruvate, oxaloacetate, and the nucleotide substrates, $K_{I,S} \neq K_{I,E}$, which indicates that 3-MP has a different affinity for $E'\cdot S$ than for $E'\cdot S$, where $S$ is the variable substrate and $E'$ represents enzyme in the presence of fixed substrates and cofactors. Since $K_{I,S} < K_{I,E}$, this suggests that these substrates affect formation of the $E'\cdot 3$-MP complex. The $E'\cdot S$ complex has a lower affinity for 3-MP than does free $E$. The inhibitor constants were corrected for the presence of the fixed substrate to obtain a $K_I$ value (Table I). The $K_I$ values calculated for the reaction measured in the direction of P-enolpyruvate formation ($K_I = 3-4 \mu M$) are lower than the values measured in the direction of oxaloacetate formation ($K_I = 40-50 \mu M$). Kinetic constants, where comparable, are lower when measured in the direction of P-enolpyruvate formation apparently due to differences in experimental conditions for the two assays. Analogous behavior has been reported previously with rat liver P-enolpyruvate carboxykinase (Jomain-Baum et al., 1976) and with the en-
zyme from rat, guinea pig, dog, rabbit, and man (Watford et al., 1980). The enzyme from chicken liver has been reported to have different requirements for metal ion and sulfhydryl reagent in the forward and reverse reactions (Lee et al., 1981; Hebda and Nowak, 1982a). The differences in magnitude of the $K_t$ value for 3-MP in the forward and reverse reaction indicate that the interactions of 3-MP with the enzyme are influenced by assay conditions.

The value of $K_t$ measured for 3-MP in the P-enolpyruvate formation direction, is approximately the value of $K_t$ for 3-MP (Table II). The presence of IDP weakens the binding of 3-MP, and HCO$_3^-$ causes no effect as suggested by inhibition kinetics. The fit to the binding data in the presence of ITP is not sufficiently precise to detect a factor of 4 increase in $K_0$ for 3-MP.

The presence of 3-MP affects the formation of the E-S complexes, with 3-MP increasing the $K_M$ for these substrates. These kinetic results are consistent with a model of 3-MP partially overlapping the P-enolpyruvate (oxa1acetate)- and the nucleotide-binding site. The $K_M$ value of HCO$_3^-$ is unaffected by 3-MP; $K_{t,s} = K_{t,s}$. This indicates that the interactions of 3-MP and CO$_2$ (the true substrate for this enzyme) with the enzyme are mutually independent. These results were confirmed by the PRR titration data and suggest that the CO$_2$ is located on the enzyme at a site more remote from the 3-MP site. The kinetic and the binding data are consistent with a single mode of 3-MP binding, indicating that the same phenomenon is being investigated.

The reversibility of inhibition and lack of evidence for mixed disulfide bond formation support the hypothesis that inhibition of chicken liver P-enolpyruvate carboxykinase is due to a specific and reversible binding of the inhibitor to the enzyme. The relatively weak binding of 3-MP to Mn$^{2+}$ further supports this idea, as inhibition by 3-MP at the concentrations reported herein could not be due to simple removal of the activator cation by 3-MP. The kinetic studies show that 3-MP and Mn$^{2+}$ interact independently with the enzyme, while the EPR and PRR experiments demonstrate that both Mn$^{2+}$ and 3-MP specifically bind to the enzyme. The lack of a positive synergistic effect of 3-MP on Mn$^{2+}$ binding to P-enolpyruvate carboxykinase is contrary to the effect observed by P-enolpyruvate (Hebda and Nowak, 1982b), suggesting different conformational effects by these two ligands although similar $c$ values are induced.

The PRR studies also display evidence for the formation of the quaternary complexes E-Mn-CO$_2$-3-MP, E-Mn-JTP-3-MP, and E-Mn-IDP-3-MP. Although not demonstrated by PRR techniques, $^1$H nuclear relaxation techniques demonstrated that the quaternary E-Mn-P-enolpyruvate-3-MP complex also forms. While the environment of the cis protons of P-enolpyruvate is unchanged in the quaternary complex from the ternary complex, the trans proton undergoes a substantial change in its environment upon 3-MP binding such that it experiences a negligible paramagnetic effect from the activator, Mn$^{2+}$. Thus, although 3-MP and P-enolpyruvate bind to E-Mn, it is suggested that their binding sites are in very close proximity, possibly overlapping each other. Partial overlap of binding sites would be consistent with the $K_M$ effects observed and would also be consistent with the $^1$H relaxation rate studies which demonstrated that the interactions of both P-enolpyruvate and 3-MP with E-Mn are affected by the presence of the other ligand. Direct binding studies of P-enolpyruvate to E-Mn, in the presence and absence of 3-MP, would be helpful in clarifying whether the interactions of P-enolpyruvate with the enzyme are weaker in the presence of 3-MP. As discussed above, however, this type of experiment has not been successful to date.

While an overlap of binding sites may explain the $K_M$ effects, it is not sufficient to explain the $V_{max}$ effects. A classical noncompetitive inhibitor is generally thought to bind at a remote allosteric site where it causes a conformational change which is detrimental to catalysis. Both the E-S and the E-S-I (where I is inhibitor) complexes form, but the E-S-I complex is nonproductive. The inhibition of chicken liver P-enolpyruvate carboxykinase by 3-MP is believed to be due to 3-MP binding within the active site, not at a remote site. Not only is there an overlap of substrate- and inhibitor-binding sites, but 3-MP binding also causes a conformational change which is unfavorable for catalysis.

That 3-MP binding clearly causes a conformational change is shown by the increase in $T_m$ in the differential scanning calorimetry studies. Although tight binding of 3-MP to E-Mn is demonstrated by the PRR studies, it is unaccompanied by an increase in enthalpy. This suggests that no additional stabilizing interactions, such as hydrogen bonding, hydrophobic interactions, or electrostatic interactions, occur when 3-MP binds to E-Mn. When the substrate P-enolpyruvate binds to E-Mn, there is an increase in the enthalpy, indicating the formation of such additional interactions. These interactions increase the thermosability of the ternary complex.

The mixed type kinetic behavior of 3-MP inhibition of chicken liver P-enolpyruvate carboxykinase can thus be described in terms of specific enzyme-ligand interactions occurring within the active site of the enzyme: overlapping of the inhibitor and substrate sites ($K_M$ effects) and an unfavorable conformational change ($V_{max}$ effects). The specific and reversible nature of 3-MP inhibition distinguishes it from other reported types of inhibitors, such as EDTA and sulfhydryl reagents, which elicit kinetic effects which appear noncompetitive.

The experiments reported here do not rigorously rule out the possibility that 3-MP binds at two independent sites, one affecting substrate binding and the second causing the unfavorable conformational change. A two-site model can only be ruled out by direct binding studies of 3-MP to the enzyme. The one-site model is the simplest model consistent with the data presented here.

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