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EVIDENCE THAT Fe^{2+} COMPLEXES OF 3-AMINOPICOLINATE AND 3-MERCAPTOPICOLINATE ACTIVATE AND INHIBIT PHOSPHOENOLPYRUVATE CARBOXYKINASE

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SUMMARY: 3-Mercaptopicolinic acid is known to be an inhibitor of phosphoenolpyruvate carboxykinase and 3-aminopicolinic acid permits Fe^{2+} to activate the enzyme. The potency of mercaptopicolinate is increased by incubating the enzyme with Fe^{2+} prior to assaying for activity. In the present work, the average combining ratio of either pyridine carboxylate with Fe^{2+} at pH 7.5 was determined to be 2:1 when measured by the method of continuous variation of Job or by elemental analysis of the isolated pyridine carboxylate-Fe^{2+} complexes. The ratio of 3-mercaptopicolinate or 3-aminopicolinate to Fe^{2+} that caused the greatest inhibition or activation of purified phosphoenolpyruvate carboxykinase was 2:1. In the absence of Fe^{2+}, neither pyridine carboxylate altered the activity of the enzyme. These results indicate that the two pyridine carboxylates can interact with phosphoenolpyruvate carboxykinase as Fe^{2+} coordination complexes.

INTRODUCTION: Quinolinic acid, 3-mercaptopicolinic acid and 3-aminopicolinic acid are structurally similar effectors of the gluconeogeneic enzyme phosphoenolpyruvate carboxykinase (E.C. 4.1.32). The first two compounds inhibit the enzyme *in vitro* (1-9), inhibit glucose synthesis in the perfused liver (1,5, 10) or in liver slices (11) and cause hypoglycemia in intact animals (11,12). 3-Aminopicolinate permits Fe^{2+} to activate purified phosphoenolpyruvate carboxykinase (7,8) and in this respect it is a model for the naturally-occurring hepatic protein named ferroactivator (6). 3-Aminopicolinate increases glucose synthesis and causes hyperglycemia in intact animals (8,13,14).

Lardy and coworkers (1,2,7-9) and McDaniel *et al.* (3) maintain that because Fe^{2+} enhances the inhibition of the carboxykinase by quinolinate and 3-mercaptopicolinate it is likely that these agents inhibit the enzyme by forming coordination complexes with Fe^{2+}. Others have postulated that 3-mercapto-

ABBREVIATIONS: Ferrozine, 3-(2-pyridyl)-5,6-diphenyl-1, 2, 4-triazine-p, p'-disulfonic acid. Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid.

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picolinate inhibits the carboxykinase by withdrawing a metal from the enzyme (5). Because 3-aminopicolinate is without effect on phosphoenolpyruvate carboxykinase in the absence of transition metal ions (7,8), it too may interact with the carboxykinase by forming a complex with a transition metal ion. Whether 3-mercaptopicolinate and 3-aminopicolinate can alter the activity of phosphoenolpyruvate carboxykinase by interacting with the enzyme as Fe^{2+} coordination complexes has been investigated.

EXPERIMENTAL PROCEDURES.

Materials. 3-Aminopicolinic acid hydrochloride and 3-mercaptopicolinic acid were gifts of Theodore Resnick and Dr. Harry Saunders, respectively, both of Smith Kline Corp., Phil., Pa. The purity of these agents was established by thin layer chromatography, nuclear magnetic resonance spectroscopy and their carbon and nitrogen contents. The chloride content of the 3-aminopicolinate hydrochloride was determined in order to estimate its formula weight. One mole of 3-aminopicolinate was contained in 180 grams of its hydrochloride. The molecular weight of 3-mercaptopicolinic acid was taken as 155. Ferrozine was from Aldrich Chemical Co. Phosphoenolpyruvate carboxykinase was purified from rat liver cytosol according to the methods of Ballard and Hanson (15,16) with modifications (17). The specific enzyme activity of the preparation used was 9 μmoles product x min^{-1} mg $protein^{-1}$ when assayed at 25°C in the absence of effectors and in the direction of phosphoenolpyruvate formation (represented as 100% enzyme activity in Figures 2, 3 and 4).

Analyses. Determinations of absorption spectra of solutions of Fe^{2+} and pyridine carboxylates were made with a DW-2 UV/VIS Spectrophotometer (American Instrument Co.) using the split beam mode. Iron was determined by atomic absorption spectroscopy and chloride with kit No. 830-T from Sigma. Total organic carbon was determined with a Dohrmann DC-50 carbon analyzer (18) and total Kjeldahl nitrogen by an ultra micro method (19) by the Water Chemistry Division, Wisconsin State Laboratory of Hygiene.

Enzyme assay. Phosphoenolpyruvate carboxykinase activity was assayed in the direction of phosphoenolpyruvate formation as previously described (6-9,17). Prior to assaying for activity, 2.0 to 3.0 μg of purified enzyme were incubated for 10 minutes with or without pyridine carboxylates or $FeCl_2$ in a final volume of 0.2 ml of 5 mM Hepes buffer pH 7.5 and 1 mM dithiothreitol. One tenth milliliter of the incubation mixture was added to 0.9 ml of the enzyme reaction mixture to start the enzyme reaction.

RESULTS

Combining ratios. Fe^{2+} coordination complexes with 3-mercaptopicolinate and 3-aminopicolinate are colored and have absorption maxima at wavelengths in the visible region. The colors and absorption maxima at pH 7.5 in water are: with 3-mercaptopicolinate, dark blue-green, a broad peak at 625; and with 3-aminopicolinate, orange, 466 nm. The iron in the complexes with 3-mercapto-

picolinate and with 3-aminopicolinate is Fe(II) and not Fe(III). When $FeCl_3$ is mixed with 3-aminopicolinate at pH 7.5, a magenta-colored complex which has an absorption maximum at 525 nm, is formed. If dithiothreitol, a reducing agent, is added to the Fe^{3+}-3-aminopicolinate solution, the color of the solution changes to orange and the absorption maximum at 525 nm disappears over two to three minutes as an absorption maximum at 466 nm appears, indicating that the dithiothreitol reduced the Fe(III) coordinated with 3-aminopicolinate to Fe(II). Solutions of $FeCl_3$ when mixed with an excess of 3-mercaptopicolinate, form a yellow-red color that changes within seconds to the dark-blue green color identical to the color seen when $FeCl_2$ is mixed with 3-mercaptopicolinate indicating that the Fe(III) is reduced by the 3-mercaptopicolinate. This reaction is too fast to follow by conventional spectrophotometric methods.

The average combining ratios of the pyridine carboxylates with Fe^{2+} were determined by the method of continuous variation of Job (20,21). Various concentrations of $FeCl_2$ and pyridine carboxylate (keeping the sum of Fe^{2+} and pyridine carboxylate constant) were mixed and the absorbance immediately measured at the absorption maxima of the complexes. The highest absorption readings were centered around 0.67 mole fraction of pyridine carboxylate indicating that with each of the pyridine carboxylates, the average combining ratio is two molecules of pyridine carboxylate to one of Fe^{2+} (Fig. 1).

Complexes formed by mixing Fe^{2+} with 3-aminopicolinate or with 3-mercaptopicolinate and submitting them to elemental analysis indicated that the average combining ratio was the same as that determined with spectrophotometric techniques. Fe(II) complexes of 3-aminopicolinate and 3-mercaptopicolinate were isolated by mixing concentrations of Fe^{2+} and pyridine carboxylate that exceeded the solubility products of the complexes. Precipitates that were formed in water after mixing 10 to 20 mM of $FeCl_2$ and pyridine carboxylate at pH 7.5 (pH adjusted with NaOH) were washed five to ten times with water and were analyzed for carbon, nitrogen, iron and chlorine. The colors of the

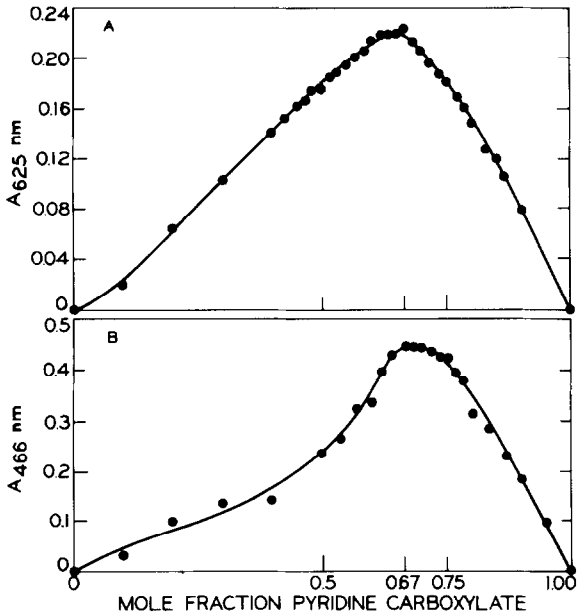

Fig. 1:
Combining ratios for Fe^{2+}, 3-mercaptopicolinate (A) and 3-aminopicolinate (B) determined by the method of continuous variation. $FeCl_2$ and one of the pyridine carboxylates were mixed in various ratios in Hepes-NaOH buffer, pH 7.5, keeping the sum of the Fe^{2+} and the pyridine carboxylate constant. Immediately after mixing, the optical density was measured. The sum of the concentrations of $FeCl_2$ plus 3-mercaptopicolinate or 3-aminopicolinate were 0.3 and 1.5 mM, respectively. The concentration of the Hepes buffer was 50 mM in the experiment with 3-aminopicolinate and 5 mM with 3-mercaptopicolinate.

isolated complexes were the same as those in dilute solutions. No chlorine was detected in the complexes. The number of gram atoms of the other three elements found in either the 3-aminopicolinate or the 3-mercaptopicolinate complex indicated that the average combining ratios were most likely two molecules of pyridine carboxylate to one of Fe (Table I). Complexes formed by mixing marked excesses of either $FeCl_2$ or of pyridine carboxylate had identical compositions.

Enzyme activity. The method of continuous variation of Job was also used to determine whether 3-mercaptopicolinate and 3-aminopicolinate inhibit and activate phosphoenolpyruvate carboxykinase by forming complexes with Fe^{2+}. Various ratios of Fe^{2+} and 3-mercaptopicolinate were incubated with the purified carboxykinase before assaying for enzyme activity (Fig. 2). The combinations of Fe^{2+} and 3-mercaptopicolinate (their sum constant at 50 μM or at 100 μM)

TABLE I

Elemental analysis of Fe^{2+} complexes of 3-aminopicolinate and of 3-mercaptopicolinate

Pyridine Carboxylate	Ratios in gram atoms[a]		Probable combining ratio[b]
	C:Fe	N:Fe	Pyridine carboxylate:Fe
3-Aminopicolinate	11.9 ± 0.3	4.0 ± 0.3	2:1
3-Mercaptopicolinate	11.7 ± 3.6	1.60	2:1

[a]Results are means (± S.D.) of analyses of 3 different preparations except for the N:Fe of 3-mercaptopicolinate in which n = 1.

[b]With either pyridine carboxylate C:Fe ratios of 6:1, 12:1 and 18:1 indicate pyridine carboxylate:Fe ratios of 1:1, 2:1 and 3:1 respectively. N:Fe ratios of 2:1, 4:1 and 6:1 with 3-aminopicolinate and 1:1, 2:1 and 3:1 with 3-mercaptopicolinate indicate pyridine carboxylate:Fe ratios of 1:1, 2:1 and 3:1, respectively.

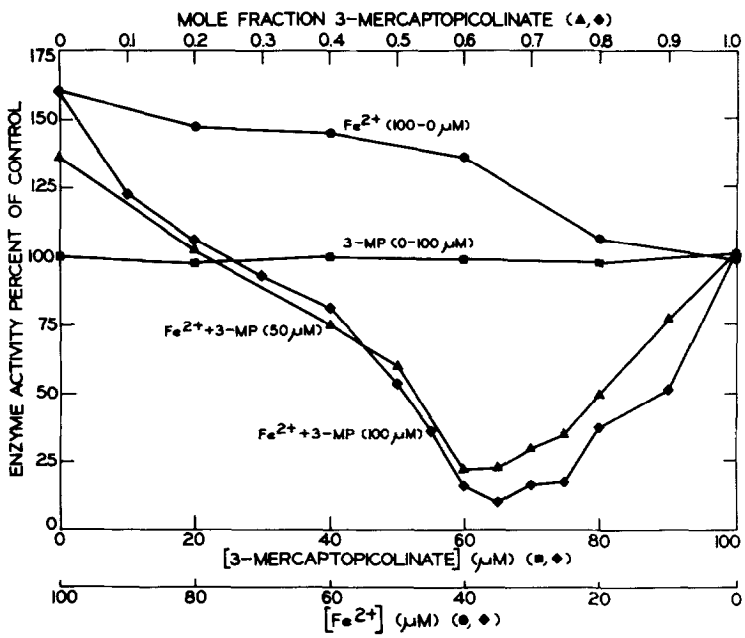

Fig. 2:
Effect of continuously varying the ratio of 3-mercaptopicolinate to Fe^{2+} on the inhibition of phosphoenolpyruvate carboxykinase activity. Various ratios of 3-mercaptopicolinate and $FeCl_2$ [sum of both effectors equaled 50 μM (▲) or 100 μM (◆)] were incubated with the purified carboxykinase prior to assaying for enzyme activity. The effects of several concentrations of $FeCl_2$ alone (100 to 0 μM, ●) and of 3-mercaptopicolinate alone (0 to 100 μM, ■) are also shown. Each point is the mean of six or more determinations.

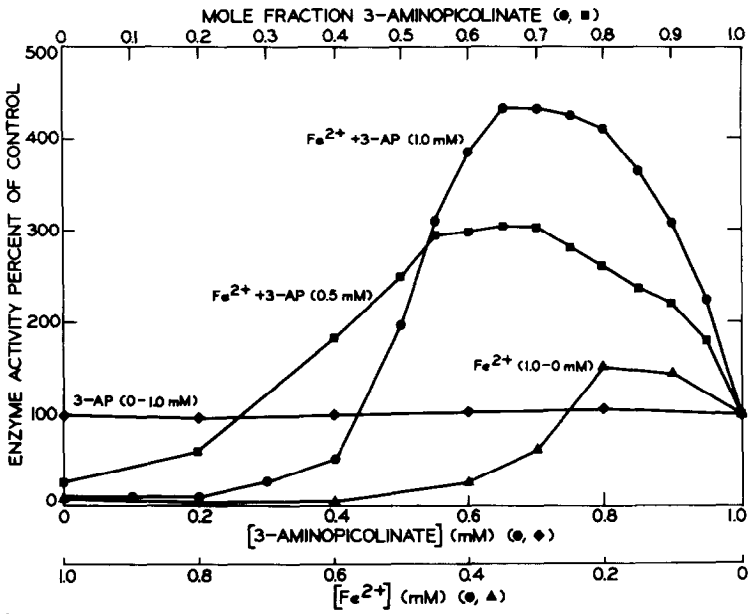

Fig. 3:
Effect of continuously varying the ratio of 3-aminopicolinate to Fe^{2+} on the activation of phosphoenolpyruvate carboxykinase. Various ratios of 3-aminopicolinate and $FeCl_2$ [sum of both effectors equaled 0.5 mM (■) or 1.0 mM (●)] were incubated with the purified carboxykinase prior to assaying for enzyme activity. The effects of several concentrations of $FeCl_2$ alone (1.0 to 0 mM, ▲) and 3-aminopicolinate alone (0 to 1.0 mM, ◆) are also shown. Each point is the mean of six or more determinations.

that caused the greatest inhibition of the purified carboxykinase were centered around a mole fraction of 0.67 3-mercaptopicolinate indicating that the average combining ratio of the complex(es) that inhibit the carboxykinase is two molecules of 3-mercaptopicolinate to one of Fe^{2+}. In the absence of added Fe^{2+}, concentrations of 3-mercaptopicolinate up to 300 μM in the incubation mixture were without effect on the activity of the carboxykinase. Fe^{2+} slightly enhanced the activity of the enzyme in the absence of 3-mercaptopicolinate. Fig. 3 shows the effect of incubating various ratios of 3-aminopicolinate and Fe^{2+} with the purified carboxykinase before assaying for enzyme activity while keeping the sum of the 3-aminopicolinate and the Fe^{2+} concentrations constant at 0.5 or 1.0 mM. The peak of activation of the enzyme was centered around a 2:1 ratio of 3-aminopicolinate to Fe^{2+} and ratios of 1:1 or 3:1 gave lower activations. Fe^{2+} alone in very high concentrations inhibited or inactivated the enzyme as reported previously (7) and low concentrations caused a slight activation of the enzyme.

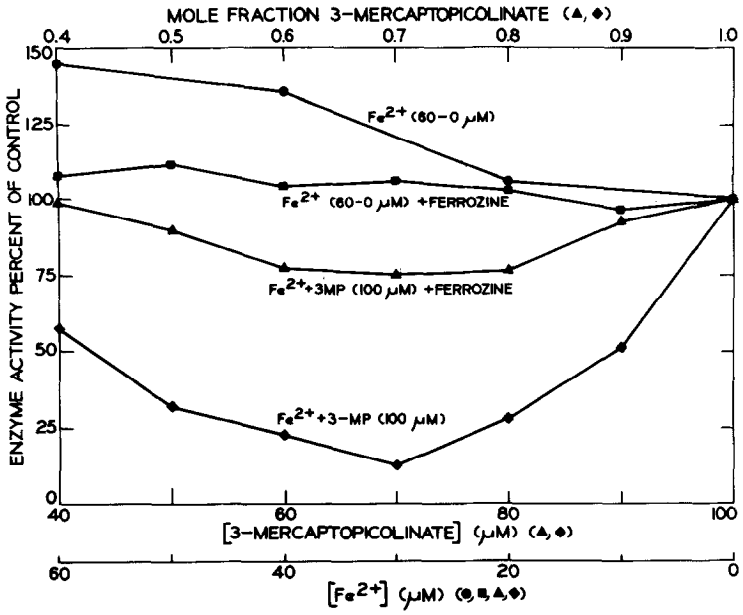

Fig. 4:
Effect of a strong chelating agent on inhibition of phosphoenolpyruvate carboxykinase by various ratios of 3-mercaptopicolinate to Fe^{2+}. 3-Mercaptopicolinate and Fe^{2+} (the sum of both effectors was kept constant at 100 μM) were incubated with the purified carboxykinase in the presence (▲) or absence (◆) of 1 mM Ferrozine - a strong chelator of Fe^{2+}. The effects of Fe^{2+} (60 to 0 μM) on enzyme activity in the presence (■) or absence (●) of 1 mM Ferrozine are also shown. Conditions were the same as in Figure 2. Each point is the mean of four or more determinations.

Ferrozine, a strong metal chelating agent, was added to the incubation mixture containing 3-mercaptopicolinate, Fe^{2+} and phosphoenolpyruvate carboxykinase (Fig. 4). By complexing the Fe^{2+} ferrozine (1 mM) prevented the 3-mercaptopicolinate and/or the carboxykinase access to the Fe^{2+} and interfered with the inhibition of the enzyme by Fe^{2+}-3 mercaptopicolinate. The fact that the strong chelating agent interfered with inhibition by 3-mercaptopicolinate, instead of increasing the inhibition, is another indication that 3-mercaptopicolinate does not inhibit the carboxykinase by removing a metal ion from the enzyme.

DISCUSSION: The fact that a specific ratio of 3-mercaptopicolinate or 3-aminopicolinate to Fe^{2+} causes maximal inhibition or activation of purified phosphoenolpyruvate carboxykinase indicates that the two pyridine carboxylates act on the carboxykinase as metal-ion complexes for if they did not act as

complexes their effects would increase as their concentrations are increased and would be independent of the pyridine carboxylate:Fe^{2+} ratio.

The results of a previous study with more than thirty aromatic compounds, including structural isomers of quinolinate, 3-mercaptopicolinate and 3-aminopicolinate, indicate that for a compound to activate or noncompetively inhibit the carboxykinase it must be a picolinate derivative capable of bonding to Fe^{2+} by its α-carboxyl group and its ring nitrogen (8). In addition, our unpublished NMR work shows that adding small quantities of Fe^{2+} to solutions of quinolinate and 3-aminopicolinate at pH 7.5, causes more broadening and/or more of a shift in the signal of the proton at position 6 than of the protons at positions 4 and 5, indicating that Fe^{2+} is probably bonding to the ring nitrogen of these compounds. The present study shows that the biologically active complexes are probably composed of two molecules of pyridine carboxylate and one of Fe^{2+}. The bonding of a transition metal by the α-carboxyl group and ring nitrogens of two molecules of picolinate derivative is a common structure for a coordination complex between a picolinate derivative and a transition metal (22,23). The group at position 3 of the picolinate derivatives is necessary for biologic activity and determines the action (inhibition or activation) the compound will have on the carboxykinase and its potency (8).

Why only certain pyridine carboxylates can act as inhibitors or activators of phosphoenolpyruvate carboxykinase is not known. It is interesting to speculate that the structures of their Fe^{2+} coordination complexes resemble the active site of naturally-occurring effectors of the carboxykinase, such as ferroactivator protein, or lower molecular weight ferroactivators, that permit Fe^{2+} to activate the enzyme (6,24,25).

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