Biochimica et Biophysica Acta, 526 (1978) 293—298

BBA Report

BBA 61341

EFFECT OF Fe^{2+} AND Mn^{2+} ON 3-MERCAPTOPICOLINATE INHIBITION OF CYTOSOLIC AND MITOCHONDRIAL PHOSPHO*ENOL*PYRUVATE CARBOXYKINASES OF FIVE SPECIES

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(Received May 9th, 1978)

Summary

Liver cytosolic or mitochondrial fractions of five species were incubated with 30 μM Fe^{2+} or with 100 μM Mn^{2+} prior to assaying for phospho*enol*-pyruvate carboxykinase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) activity in the presence of 3-mercaptopicolinate. Only the cytosolic carboxykinases were activated 3—4-fold by Fe^{2+} or Mn^{2+}. Fe^{2+} enhanced the inhibitory potency of 3-mercaptopicolinate 10—50-fold against the cytosolic and the mitochondrial carboxykinases, but Mn^{2+} was ineffective. Mn^{2+} interfered with Fe^{2+}-enhancement of inhibition by 3-mercaptopicolinate in a manner competitive with Fe^{2+}. It is hypothesized that Fe^{2+} and 3-mercaptopicolinate form a coordination complex that inhibits the carboxykinases and that 3-mercaptopicolinate does not bind to a carboxykinase containing Mn^{2+}.

Phospho*enol*pyruvate carboxykinase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) activity is distributed between the cytosolic and mitochondrial compartments in the liver of almost all mammalian and avian species except in the rat and mouse in which it is exclusively cytosolic. Previous information indicated that, unlike the cytosolic carboxykinases, the mitochondrial carboxykinases did not have a site that bound divalent transition metal ions not complexed to a nucleotide. The activity of the cytosolic carboxykinases from 6 animal species is enhanced much more than that of the mitochondrial carboxykinases by incubation with micromolar amounts of Fe^{2+} or Mn^{2+} prior to assaying for enzyme activity and ferro-activator, a protein that permits Fe^{2+} to activate purified phospho*enol*-pyruvate carboxykinase, is found almost exclusively in the cytosol of these species [1]. Fe^{2+} enhances the inhibition of rat liver cytosolic carboxykinase by the metal chelating agents, quinolinate or 3-mercaptopicolinate [2—5]. The

latter finding has prompted us to hypothesize that quinolinate and 3-mercaptopicolinate decrease the activity of the enzyme by forming complexes with Fe^{2+} that inhibit the enzyme [4, 5]. Robinson and Oei [10] found that the mitochondrial carboxykinase from guinea pig liver responded less than the cytosolic carboxykinase to the inhibitors quinolinate and 3-mercaptopicolinate when Fe^{2+} was not present. Because of the now widespread use of 3-mercaptopicolinate as an agent for studying gluconeogenesis, we have compared the effects of Fe^{2+} and Mn^{2+} (the transition metal ion previously believed to be essential for phospho*enol*pyruvate carboxykinase activity) on its inhibitory potency against the cytosolic and mitochondrial phospho*enol*-pyruvate carboxykinases of several species.

3-Mercaptopicolinic acid was a gift of Dr. Harry L. Saunders, Smith Kline Corp., Philadelphia Pa. Cytosol was the supernatant fraction obtained from centrifuging a 1 : 3 homogenate (w/v) of liver in 10 mM triethanolamine chloride pH 7.5, 0.25 M sucrose at 105 000 × *g* for 1 h. Mitochondria were isolated from the original homogenate of liver according to the method of Johnson and Lardy [7], except that the mitochondrial pellet was washed four to seven additional times to reduce contamination with cytosolic phospho*enol*-pyruvate carboxykinase. Mitochondrial fractions were the supernatant fractions obtained from centrifuging mitochondrial pellets at 30 000 × *g* for 30 min after they had been frozen/thawed 3 times on ethanol/solid CO_2 in a volume of 5 mM HEPES/NaOH at pH 7.5 corresponding to 0.4 of the volume of the liver.

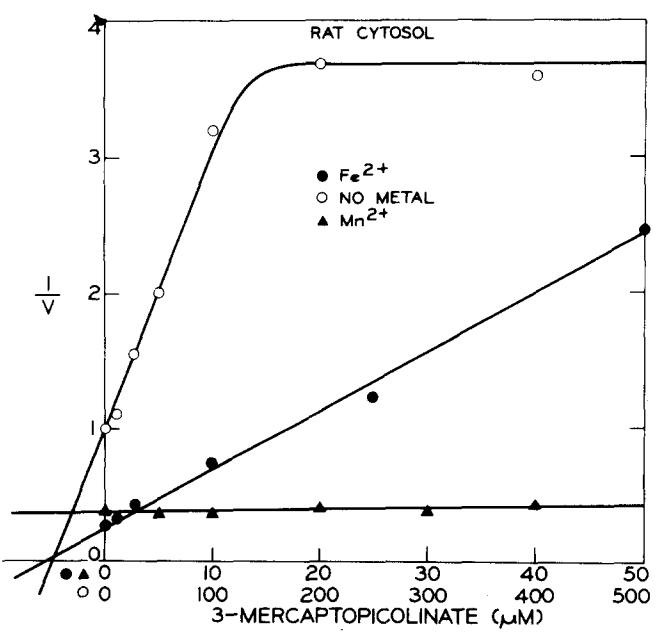

Fig. 1. Dixon plot of 3-mercaptopicolinate inhibition of rat liver cytosolic phospho*enol*pyruvate carboxykinase. Cytosol was incubated with 30 μM $FeCl_2$, 100 μM $MnCl_2$ or without an added transition metal salt prior to assaying for enzyme activity in the presence of 3-mercaptopicolinate. Each point is the mean of 4 or more determinations. The symbols beneath the abcissa indicate the scale of 3-mercaptopicolinate concentrations applicable to data points with the same symbol.

Phospho*enol*pyruvate carboxykinase activity was assayed in the direction of phospho*enol*pyruvate formation by the method of Seubert and Huth [8] in a final volume of 1.0 ml of 1.5 mM oxaloacetate, 2 mM Na_3ITP, 3 mM $MgCl_2$, 50 mM HEPES/NaOH pH 7.5 and the desired concentration of 3-mercaptopicolinate at 25°C as previously described [9]. The reaction was initiated by adding the incubation mixture described below and terminated by the addition of 10 mg KBH_4. A standard reaction time of 5 min was used. After the reaction mixture was deproteinized with $HClO_4$ and neutralized with KOH, phospho*enol*pyruvate was determined by a standard enzymatic assay. Prior to enzymatic assay metal salts and 10 to 40 µl of subcellular fractions were incubated at 0°C for 10 min in a final volume of 0.2 ml 0.8 mM dithiothreitol, 4.2 mM HEPES/NaOH pH 7.5 as previously described [4, 9]. The incubation was initiated by addition of metal salt and terminated by addition of 0.1 ml incubation mixture to 0.9 ml enzyme reaction mixture.

Fig. 1 shows that although both Fe^{2+} and Mn^{2+} increase the maximal velocity of rat liver cytosolic phospho*enol*pyruvate carboxykinase, only Fe^{2+} significantly enhances inhibition of the enzyme by 3-mercaptopicolinate.

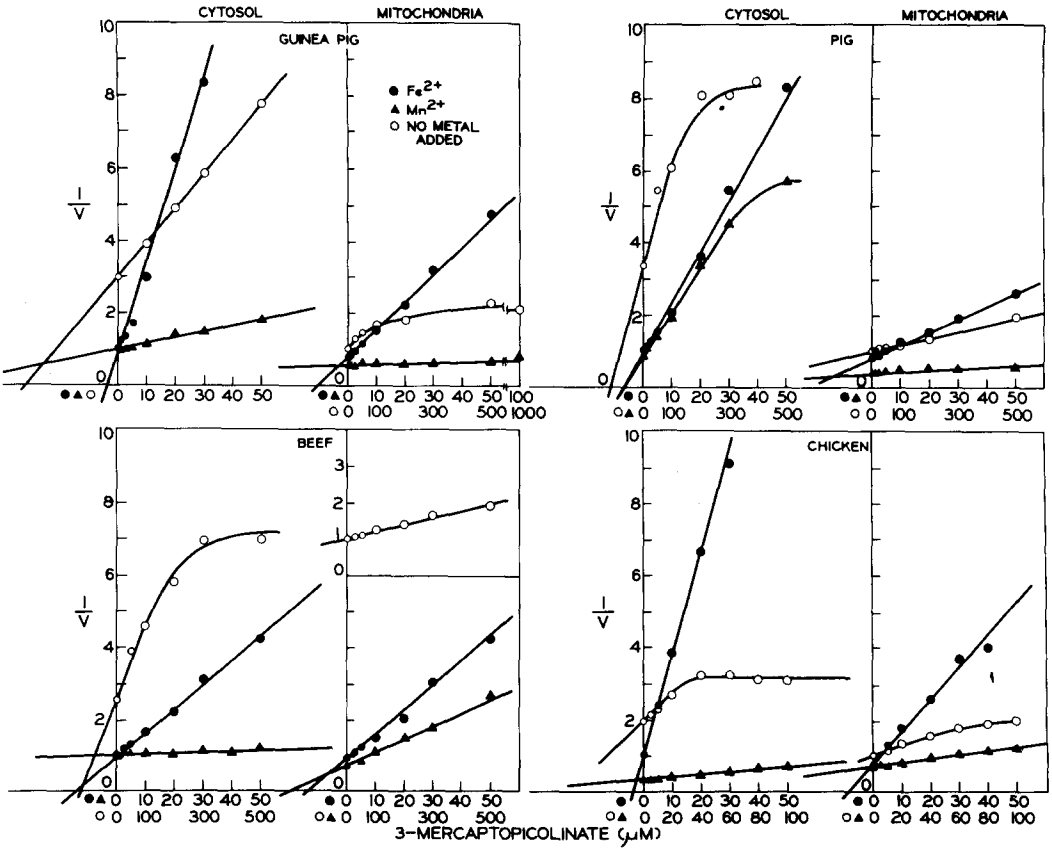

Fig. 2. Dixon plots of 3-mercaptopicolinate inhibition of liver cytosolic and mitochondrial phospho*enol*pyruvate carboxykinases of four species. Conditions of experiments and plotting of data are the same as in Fig. 1. The symbols beneath the abcissa indicate the scale of 3-mercaptopicolinate concentrations applicable to data points with the same symbol.

Essentially identical results were obtained with the carboxykinases in the cytosolic and mitochondrial fractions from 4 other animal species except that Fe^{2+}, especially, and also Mn^{2+}, did not significantly activate the mitochondrial carboxykinases (Fig. 2). The lack of strong inhibition of the cytosolic enzymes activated by Mn^{2+} and the strong inhibition of the mitochondrial enzymes in the presence of Fe^{2+} indicate that activation in itself is not necessary for inhibition by 3-mercaptopicolinate. Inhibition constants of the enzymes that had been incubated with 30 μM Fe^{2+} prior to assaying for activity were 1/10 to 1/50 of those when the enzymes were incubated with 100 μM Mn^{2+} or without an added transition metal ion. In the absence of added Fe^{2+}, inhibition was in many instances incomplete. The K_i values of the Fe^{2+}-treated cytosol enzymes were similar or slightly lower than those of the Fe^{2+}-treated mitochondrial enzymes (Table I). In the absence of added Fe^{2+}, inhibition of the cytosolic enzymes tended to be greater than that of the mitochondrial enzymes. This might have been due to higher concentrations of certain transition metals in some cytosol fractions that could permit 3-mercaptopicolinate to inhibit the carboxykinase. Incubation of guinea pig cytosolic or mitochondrial fractions with 5, 30 or 300 μM Mn^{2+} instead of 100 μM Mn^{2+} did not enhance inhibition by 3-mercaptopicolinate (data not shown).

When rat liver cytosol is incubated with Mn^{2+} in addition to 30 μM Fe^{2+}, the K_i of 3-mercaptopicolinate for phospho*enol*pyruvate carboxykinase increases as the concentration of Mn^{2+} is increased (Fig. 3). The pattern demonstrates that Mn^{2+} interferes with but does not prevent inhibition [10] of the enzyme by 3-mercaptopicolinate-Fe^{2+}. Mn^{2+} (3 mM) did not decrease whatsoever the absorption at 625 nm due to a complex formed by 0.15 mM 3-mercaptopicolinate and 0.15 mM Fe^{2+} at pH 7.5. Complexes of Mn^{2+} and 3-mercaptopicolinate do not absorb at this wavelength (data not shown). Thus, the effect of Mn^{2+} on 3-mercaptopicolinate inhibition must have been due to an interaction with the carboxykinase rather than to a disruption of a

TABLE I

EFFECT OF Fe^{2+}, Mn^{2+} AND NO ADDED TRANSITION METAL ION ON 3-MERCAPTOPICOLINATE INHIBITION CONSTANTS (μM) FOR LIVER CYTOSOLIC AND MITOCHONDRIAL PHOSPHO*ENOL*PYRUVATE CARBOXYKINASES (PEPCK) OF FIVE ANIMALS

Experiments were performed as in Fig. 1. K_i values were the horizontal intercepts on Dixon plots for linear patterns. For non-linear patterns, the K_i was the 3-mercaptopicolinate concentration determined graphically which gave 50% inhibition. In most cases, 2 or more experiments besides those in Fig. 2 were performed with concentrations of inhibitor higher and lower than its K_i.

Animal	K_i (μM)					
	Cytosolic PEPCK with added:			Mitochondrial PEPCK with added:		
	Fe^{2+}	Mn^{2+}	None	Fe^{2+}	Mn^{2+}	None
Rat	5	217	47			
Guinea pig	3	61	30	10	198	400
Cow	14	140	195	13	178	630
Pig	6	94	168	21	328	500
Chicken	4	82	*	8	123	100

*Maximum inhibition was 35%.

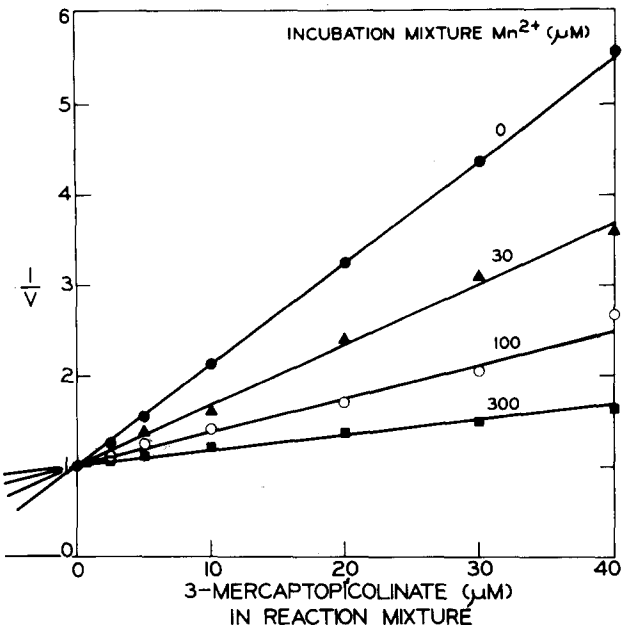

Fig. 3. Effect of Mn^{2+} on inhibition of phospho*enol*pyruvate carboxykinase by 3-mercaptopicolinate · Fe^{2+}. Rat liver cytosol was incubated with 30 μM $FeCl_2$ and various concentrations of Mn^{2+} prior to assaying for enzyme activity in the presence of 3-mercaptopicolinate. Each point is the mean of 2 or more determinations.

3-mercaptopicolinate-Fe^{2+} complex. This is assuming that the complex that absorbs at 625 nm is inhibiting the cytosolic enzyme and we have evidence for this (unpublished data). Results qualitatively similar to the above were obtained when the same concentrations of Mn^{2+} as in Fig. 3 and 5 μM or 100 μM Fe^{2+} were incubated with rat liver cytosol prior to assaying for activity (data not shown).

The present study shows that the liver mitochondrial phospho*enol*-pyruvate carboxykinases of four species are as sensitive as the cytosolic carboxykinases to inhibition by 3-mercaptopicolinate when Fe^{2+} is present. This was unexpected because we had previously assumed that mitochondrial carboxykinases did not bind transition metals not complexed to a nucleotide as Fe^{2+} and Mn^{2+} did not activate the liver mitochondrial carboxykinases of the same species used in the current study [1] and because of the findings of Robinson and Oei mentioned previously. This unresponsiveness was not due to the absence in mitochondria of ferroactivator, a cytosolic protein which permits Fe^{2+} to activate the cytosolic carboxykinases [1, 11, 12] for the mitochondrial carboxykinases were not activated when cytosol from the same species or pure rat liver ferroactivator were added to the mitochondrial fractions [1].

Since iron is about 200 times more plentiful than manganese in the liver of most animals [13, 14], inhibition of the mitochondrial phospho*enol*-pyruvate carboxykinase as well as the cytosolic enzyme, will have to be considered likely when 3-mercaptopicolinate is used to study gluconeogenesis. Robinson and Oei [6] have presented evidence that 3-mercaptopicolinate can

penetrate mitochondria. Our unpublished data indicate that complexes of 3-mercaptopicolinate and Fe^{2+} are charge neutral so these might also readily penetrate the mitochondrial inner membrane.

Speculation: The abilities of Fe^{2+} and Mn^{2+} to activate rat liver cytosolic phospho*enol*pyruvate carboxykinase are similar [1, 2, 4, 12] (Figs. 1 and 2) so the affinities of the two metals for the enzyme must be similar. Thus when rat liver cytosol was incubated with 30 μM Fe^{2+} and 30 to 300 μM Mn^{2+} in the absence of 3-mercaptopicolinate and activity stayed maximal (Fig. 3) some of the Fe^{2+} bound to the enzyme was probably displaced by Mn^{2+} as its concentration was increased. That Mn^{2+} interferes with but does not prevent inhibition of phospho*enol*pyruvate carboxykinase by 3-mercaptopicolinate · Fe^{2+} (Fig. 3) indicates that Mn^{2+} must compete with 3-mercaptopicolinate and/or Fe^{2+} for a common site on, or the same form of, the enzyme. In other words, the enzyme is not as strongly inhibited when it contains Mn^{2+}, because 3-mercaptopicolinate does not bind as tightly to Mn^{2+} on the enzyme as it does to Fe^{2+}.

The author is grateful to Professor Henry A. Lardy for his interest throughout the course of this work. The skillful technical assistance of Judith Kowalchyk is gratefully acknowledged. This work was supported by grants from the Juvenile Diabetes Foundation and from the American Diabetes Foundation.

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