Phosphoenolpyruvate Carboxytransphosphorylase. IV. Requirement for Metal Cations*

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ABSTRACT: Phosphoenolpyruvate carboxytransphosphorylase from propionic acid bacteria requires a freely dissociable divalent metal such as Mg^{2+} but is inhibited by a variety of structurally unrelated metal chelators even in the presence of a large excess of Mg^{2+}. The results indicate that the enzyme has a requirement for two types of metal cations, one dissociable (type I) and one tightly bound (type II). In addition a third metal (a heavy metal) is stimulatory for the pyruvate reaction.

Both the oxalacetate and pyruvate reactions are inhibited to the same degree by almost identical concentrations of these chelators suggesting that the type II metal is required for both reactions. No evidence was found of a requirement for a specific monovalent ion by carboxytransphosphorylase. EDTA-treated and dialyzed enzyme is inactive with Mg^{2+} alone but addition of Co^{2+} along with Mg^{2+} activates the enzyme for the oxalacetate reaction and Cu^{2+} is effective for the pyruvate reaction. $^{60}Co^{2+}$ binding experiments proved that the reactivation was not due to binding of $^{60}Co^{2+}$ to an apoenzyme. Tests of EDTA-treated and dialyzed enzyme in combination with untreated enzyme showed that the EDTA-treated enzyme was inhibitory apparently because it retained bound EDTA which inhibited the untreated enzyme. It is proposed that Co^{2+} activates by removing the EDTA from the type II metal. The inhibition of the oxalacetate reaction by *o*-phenanthroline is shown to be of two types, an instantaneous inhibition which is reversible and a time-dependent inhibition which is irreversible; there is little evidence for the time-dependent, irreversible inhibition of the pyruvate reaction. These results have been interpreted with reference to and appear consistent with the present hypothesis that two enzyme forms of carboxytransphosphorylase exist, one catalyzing the oxalacetate reaction and the other, containing a heavy metal, catalyzing the pyruvate reaction.

Previous studies (Lochmüller *et al.*, 1966; Wood *et al.*, 1969a; Davis *et al.*, 1969) have shown that phosphoenolpyruvate carboxytransphosphorylase (pyrophosphate oxalacetate carboxy-lyase (phosphorylating), EC 4.1.1.38) obtained from propionic acid bacteria catalyzes fixation of CO_2 with phosphoenolpyruvate and P_i to form oxalacetate and PP_i and in the absence of CO_2, pyruvate and PP_i are the products. A mechanism for the two reactions is presented in the following paper by Wood *et al.* (1969b).

Davis *et al.* (1969) have shown that the reactions of carboxytransphosphorylase are inhibited by EDTA. Immediate inhibition was obtained only after the enzyme was previously incubated with a thiol compound, such as β-mercaptoethanol. On prolonged incubation with EDTA, there was inhibition in the absence of thiol and activity was restored in the forward oxalacetate reaction by addition of Co^{2+} in excess of the EDTA but not by Cu^{2+}; whereas the activity was restored in the pyruvate reaction by excess Cu^{2+} but not by Co^{2+}. In these experiments Mg^{2+} was required and was present in large excess of the EDTA and therefore the inhibition was not due to the removal of Mg^{2+}. It was proposed that the EDTA inhibited by complexing a second required metal which did not dissociate from the enzyme. The catalytic activities of carboxytransphorphorylase thus were considered to involve at least two metal sites which have been designated type I (dissociable metal) and type II (tightly bound metal). Furthermore, it was postulated that two forms of carboxytransphosphorylase exist, one which catalyzes the oxalacetate reaction and the other the pyruvate reaction. The form which catalyzes the pyruvate reaction appears to require for maximum activity in addition to the two metals (type I and type II) a heavy metal while the form catalyzing the oxalacetate reaction requires only type I and type II metals. The present report is concerned with verification of the requirement of the type II metal.

Materials and Methods

Highly purified phosphoenolpyruvate carboxytransphosphorylase was prepared from *Propionibacterium shermanii* as previously described (Lochmüller *et al.*, 1966; Wood *et al.*, 1969a). For most of the experiments crystalline carboxytransphosphorylase was used which originally had a specific activity of about 20 but in some experiments had lost activity during storage. β-Mercaptoethanol (distilled before use), α,α′-dipyridyl, tetramethylammonium hydroxide, and sodium diethyldithiocarbamate were obtained from Eastman Kodak Co.; 1,10-phenanthroline and EDTA from Sigma Chemicals, 8-hydroxyquinoline from K & K Chemicals; Sephadex G-50 from Pharmacia; and Chelex-100 (50–100 mesh) from Bio-Rad Laboratories. $^{60}Co^{2+}$ was obtained from New England Nuclear Corp., and radioactivity was determined on a Packard Auto-Gamma spectrophotometer attached to the electronics of a Packard Tri-Carb liquid scintillation spectrophotometer. The metals and other reagents were of analytical grade obtained

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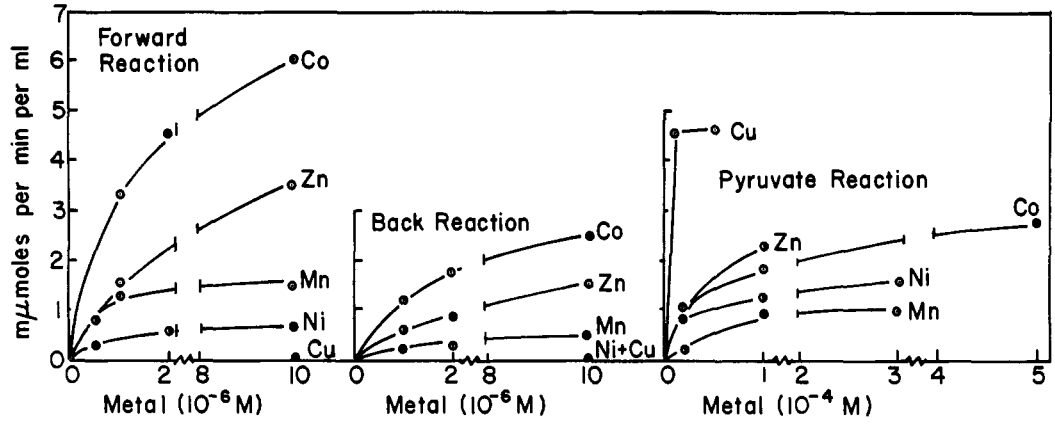

FIGURE 1: Effect of concentration of various divalent metals in the presence of Mg^{2+} on the reactivation of carboxytransphosphorylase treated with EDTA. The carboxytransphosphorylase had been incubated with EDTA and then dialyzed (see Methods) and the assays of the forward and oxalacetate back-reactions and of the pyruvate reactions were as described previously (Davis *et al.*, 1969). It was not possible to test some metals at higher concentrations because they precipitate as phosphates; 1.36 μg of carboxytransphosphorylase (specific activity 14) was used per ml for forward reaction, while 8.2 μg was used per ml for the pyruvate and back-reactions.

from commercial sources. Malate dehydrogenase was purified from propionic acid bacteria (Allen *et al.*, 1964). Sephadex was washed repeatedly by suspending in triple-distilled water, filtered on a sintered-glass funnel, and finally suspended in Chelex-treated potassium phosphate buffer.

Preparation of Chelex and Treatment of Reagents with Chelex. The Chelex was prepared in the following manner. About 10 g of Chelex was suspended in 100 ml of NH_4OH and held for 1 hr. It was then washed ten times on a sintered-glass funnel by repeated suspension in 150 ml of distilled water followed by filtration with suction. The Chelex was then suspended in 300 ml of 0.1 N HCl. The HCl was removed by filtration. The resin was washed again with water and then repeatedly with 50 mM potassium phosphate buffer (pH 6.9). Since phosphate inhibits the back-reaction, the Chelex was washed briefly with distilled water and stored as a concentrated suspension in water. For removal of metals, one volume of gravity-packed Chelex was added to five volumes of the solution to be treated and the mixture was stirred in an ice bath for 30 min. It was then centrifuged and the supernatant solution was used in the assay. The combined mixture for the assays including the linking enzymes in the back-reaction were treated with Chelex.

Assay of Carboxytransphosphorylase. Assays of the oxalacetate reaction, both forward and back, and of the pyruvate reaction were as described previously (Wood *et al.*, 1969a; Davis *et al.*, 1969). Protein was determined by the ultraviolet absorption method of Warburg and Christian (1941). A unit of enzyme activity is expressed as micromoles of product formed per minute at 25° and was measured in the oxalacetate forward reaction with 0.1 mM $CoCl_2$ added to the assay mixture. The specific activity is given as units per milligram at 25°.

Preparation of EDTA-Treated and Dialyzed Carboxytransphosphorylase. Carboxytransphosphorylase (360 μg; specific activity ~15.0) in 1 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM EDTA and 1 mM β-mercaptoethanol was incubated for 48 hr or longer at 0°. EDTA was then removed by dialysis of the 1 ml in Visking tubing (0.5-cm diameter) which had been previously boiled twice for 15 min in 0.1 mM EDTA. The dialysis was for 6–8 hr at 0° against 100 ml of 1 mM β-mercaptoethanol and 50 mM phosphate buffer (pH 6.9) which had been treated with Chelex-100. The buffer was changed each hour. When the pyruvate reaction was studied, β-mercaptoethanol was deleted from the incubation and dialysis.

Results

Attempts to Prepare Apoenzyme by Treatment with EDTA. The fact that the inhibition by EDTA could be reversed by addition of Co^{2+} to the oxalacetate reaction and by Cu^{2+} to the pyruvate reaction (Davis *et al.*, 1969) posed the possibility that the EDTA removed a bound metal and thus formed an apoenzyme and that a holoenzyme was reconstituted for the oxalacetate reaction by Co^{2+} and for the pyruvate reaction by Cu^{2+}. Others have observed that replacement of metals may change the specificity of enzymes. For example, Vallee (1961) showed that the peptidase activity of carboxypeptidase can be increased without decrease in esterase activity by replacing Zn^{2+} of the enzyme with Co^{2+}. Likewise Malmstrom *et al.* (1964) found that substitution of Co^{2+} for Zn^{2+} had exactly opposite effects on the rate of hydration of CO_2 and the hydrolysis of the ester, *p*-nitrophenyl acetate, by carbonic anhydrase.

Carboxytransphosphorylase was incubated with EDTA and then dialyzed to remove the excess EDTA as described under Methods. The preparation was then tested for the effect of metals on the activity of the enzyme. It was found (Figure 1) that the EDTA-treated enzyme was completely inactive in the presence of 12 mM $MgCl_2$ but when supplemented with Co^{2+}, Zn^{2+}, Mn^{2+}, or Ni^{2+} the forward oxalacetate reaction was reactivated, while the pyruvate reaction was restored to varying extents by Cu^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, or Ni^{2+}.[1] With 4 mM $MgCl_2$ present in the assay, the back-reaction was reactivated by Co^{2+}, Zn^{2+}, or Mn^{2+} similar to the forward reaction. Cu^{2+} was the most effective activator for the pyruvate reaction while

[1] The following metals were tested in the forward reaction with Mg^{2+} at 12 mM and were found inactive: $AgNO_3$ (0.01 mM), $BaCl_2$ (1 mM), $CaCl_2$ (1 mM), $Fe((NH_4)SO_4)_2$ (0.01 mM), $Pb(NO_3)_2$ (0.1 mM), $SrCl_2$ (1 mM), $HgCl_2$ (0.01 mM), $(NH_4)HCO_3$ (1 mM), and $(NH_4)_6MoO_{24}$ (1 mM)

TABLE I: Binding of ^{60}Co by EDTA-Treated Carboxytransphosphorylase.[a]

Expt	Treatment of Enzyme (units/mg)	Effect of Co^{2+} before Treatment with ^{60}Co: Act. of Enzyme with Co^{2+}	Effect of Co^{2+} before Treatment with ^{60}Co: Ratio of Enzyme Act. without Co^{2+} to That with Co^{2+}	Condns for Binding of ^{60}C: Time (min)	Condns for Binding of ^{60}C: ^{60}Co (mM)	Incorp of ^{60}Co after Sephadex Filtration (moles of Co/mole of enzyme): Second	Incorp of ^{60}Co after Sephadex Filtration (moles of Co/mole of enzyme): Third	Ratio of Enzyme Act. without Co^{2+} to That with Co^{2+} after ^{60}Co Treatment
1	EDTA treated	12.0	0.08	25	0.05	0	0	0.82
2	Untreated	8.0	0.62	45	0.01	1.6	2.6	1.0
3	Untreated	9.1	1.0	25	0.5	44.0	40.5	1.0
4	EDTA treated	12.0	0.36	25	0.5	25.0	25.0	0.98

[a] The enzyme in all experiments was diluted in 50 mM phosphate (pH 6.8) and 1 mM β-mercaptoethanol. All incubations with ^{60}Co were in the same buffer and the columns of Sephadex G-50 were equilibrated with this buffer. Assays were done as described by Davis *et al.* (1969). Experiment 1: 91 μg of the EDTA-treated and dialyzed enzyme in 2 ml was incubated at 0° for 25 min with 0.05 mM ^{60}Co of specific activity of 2.9×10^8 cpm/μmole Co^{2+}; 1.8 ml was passed over a 68×1.1 cm Sephadex column, then 1.0 ml containing the peak of the enzyme activity was passed over a second Sephadex column of 43×1.1 cm. The enzyme activity was again determined with and without Co^{2+} in the assay. The remaining experiments were done in a similar fashion. Experiment 2: 73 μg of five-times-crystallized enzyme was incubated in 1.52 ml containing 0.01 mM ^{60}Co of specific activity 4.1×10^8 cpm/μmole; 0.8 ml of the peak from the second Sephadex column was mixed with 1 mM EDTA and then passed through a third 32×1.1 cm column. Experiment 3: 89 μg of five-times-crystallized enzyme was incubated with 0.5 mM ^{60}Co of specific activity 3.7×10^7 cpm/μmole and treated as in expt 2. Experiment 4: 91 μg of EDTA-treated and dialyzed enzyme was treated as in expt 3. The moles of Co^{2+} bound per mole of enzyme has been calculated as follows, where 4.3×10^5 is the molecular weight of carboxytransphosphorylase (Lochmüller *et al.*, 1966): cpm per ml/cpm per mg of ^{60}Co = mg of Co per ml, mg of Co per ml/59 = mmoles of Co per ml, mmoles of Co^{2+} per ml/mg of protein per ml = mmoles of Co per mg of protein, and mmoles of Co/mg of protein $\times$ 430,000 = moles of Co per mole of enzyme. The recovery of carboxytranscarboxylase was nearly 100% and the milligrams of protein per milliliter was calculated from the units per milliliter divided by the original specific activity of the enzyme.

exhibiting no reactivation of the forward or back-reactions. It is seen in Figure 1 that about 50% of the maximal velocities of the forward and back-reactions were achieved with 1×10^{-6} M concentrations of activating metals; while the pyruvate reaction was activated at similar concentrations by Cu^{2+}, but the other metals were required at a fivefold greater concentration. The fact that there was activity in the absence of Cu^{2+} may indicate heavy metals were not completely removed or there may not be an absolute requirement for heavy metals.

Binding of $^{60}Co^{2+}$ by EDTA-Treated Carboxytransphosphorylase. Tests with $^{60}Co^{2+}$ were undertaken to determine if there was binding of $^{60}Co^{2+}$ as would be expected if apoenzyme were being converted into holoenzyme. The results of these experiments indicated that an apoenzyme was not formed during the EDTA treatment.

The EDTA-treated and dialyzed enzyme preparation was incubated with $^{60}Co^{2+}$ and then passed through a Sephadex column to remove unbound $^{60}Co^{2+}$ cations. Fractions containing enzyme were assayed in the forward reaction and the radioactivity of each fraction was determined. To ensure complete removal of uncomplexed $^{60}Co^{2+}$, the enzyme peak from the first Sephadex column was passed through a second Sephadex column and the enzymic activity and radioactivity of the eluents were determined again. Usually the Sephadex treatment was repeated a third time. The moles of Co^{2+} bound per mole of enzyme was calculated from the radioactivity based on a molecular weight of carboxytransphosphorylase of 4.3×10^5 (Lochmüller *et al.*, 1966). The effect of cobalt on the enzymic activity was determined before and after treatment of the enzyme with $^{60}Co^{2+}$ and filtration through Sephadex. If apoenzyme had been formed there should be little activity in the absence of cobalt prior to treatment with $^{60}Co^{2+}$. If the apoenzyme was reconstituted to holoenzyme during the treatment with $^{60}Co,^{2+}$ it should bind $^{60}Co^{2+}$ and not require cobalt for activation.

It is observed (Table I, expt 1) that after treatment with $^{60}Co^{2+}$ and Sephadex filtration, that the requirement for Co^{2+} by EDTA-treated enzyme had largely disappeared, the ratio of activity without and with Co^{2+} being 0.82 as shown in the last column of Table I. Nevertheless no radioactivity was associated with the protein and thus no cobalt was bound to the enzyme. Clearly reactivation was not due to reconstitution of a holoenzyme from an apoenzyme by binding of Co^{2+}.

Some enzyme preparations which have not been treated with EDTA are activated by Co^{2+} even in the presence of 12 mM Mg^{2+} (Wood *et al.*, 1969a; Davis *et al.*, 1969). This stimulating effect occurred with 0.1 mM and even at 0.01 mM Co^{2+} and thus did not appear to be due to its function as a type I metal. Co^{2+} does serve as a type I metal in place of Mg^{2+} but the K_m is 0.5 mM (Lochmüller *et al.*, 1966). Tests for binding of Co^{2+} were therefore undertaken with this type of preparation to determine if this cobalt activation was accompanied by binding of Co^{2+}. As seen in column 4 (expt 2), the enzyme was only 62% as active without Co^{2+} as with Co^{2+} (0.01 mM).

TABLE II: The Inhibitory Effect of EDTA-Treated and Dialyzed Enzyme upon the Enzymatic Activity of Untreated Carboxytransphosphorylase.[a]

Trial	Amt Untreated Enzyme (ml)	Amt of EDTA-Treated and Dialyzed Enzyme (ml)	Enzyme Act. (ΔOD/min)	Inhibn of Untreated Enzyme (%)
1	0.04		0.085	
2		0.04	0.005[b]	
3	0.04	0.04	0.025	72
4	0.04	0.02	0.060	34

[a] Crystalline carboxytransphosphorylase (52 μg/ml; specific activity ~ 23.0) was treated with EDTA and dialyzed with repeated changes against 50 mM potassium phosphate (pH 6.8) and 1 mM β-mercaptoethanol (see Methods). Before use the dialyzed preparation was diluted an additional four times with the same phosphate buffer containing β-mercaptoethanol yielding a preparation containing 13 μg/ml. Untreated enzyme was diluted to a similar concentration with the same phosphate buffer. The amounts of enzyme used per 0.33 ml of assay mixture are shown in the table. Assays were as described previously (Davis *et al.*, 1969). [b] When 0.01 mM $CoCl_2$ was added to this assay, the ΔOD was increased to 0.083.

Following incubation with $^{60}Co^{2+}$ and removal of excess $^{60}Co^{2+}$, radioactivity remained associated with the enzyme and about 2 moles of Co^{2+} was estimated to be bound per mole of enzyme. It was observed that the enzyme was now completely active without addition of Co^{2+} to the assay (last column) suggesting the bound $^{60}Co^{2+}$ was activating the enzyme.

Tests were then undertaken with an enzyme preparation which was not activated by Co^{2+} (expt 3, ratio equal 1 in column 4). Presumably this preparation would not contain any apoenzyme and might not bind $^{60}Co^{2+}$. It is seen that there was binding of $^{60}Co^{2+}$ but there was about 40 moles of Co^{2+} bound per mole of enzyme. The concentration of Co^{2+} was 0.01 mM in expt 2 and 0.5 mM in expt 3. Thus at higher concentrations of Co^{2+} there appears to be nonspecific binding of $^{60}Co^{2+}$ but the cobalt was tightly bound since it was not removed even though the enzyme was treated with EDTA and passed through Sephadex a third time. When the dialyzed, EDTA-treated enzyme was tested with higher concentrations of $^{60}Co^{2+}$ (expt 4), there likewise was apparent nonspecific binding of Co^{2+}. The fact that there was nonspecific binding at higher concentrations of Co^{2+} makes it difficult to evaluate the significance of binding observed at lower concentrations of Co^{2+}.

Evidence That EDTA Remains Bound to EDTA-Treated and Dialyzed Enzyme. The fact that EDTA-treated and dialyzed enzyme was reactivated by cobalt (Table I, expt 1) but did not bind $^{60}Co^{2+}$ suggested that the Co^{2+} in low concentration acted in part at least by complexing and removing enzyme-bound EDTA and thus reactivated the enzyme. If this were the case, it seemed likely that the EDTA-treated and dialyzed enzyme might, when mixed with untreated enzyme, release sufficent EDTA to inhibit the activity of untreated enzyme. It is seen (Table II) that when equal amounts of untreated and EDTA-treated and dialyzed enzyme were mixed there was 72% inhibition of their combined activity. Addition of one-half the amount of treated enzyme caused a 34% inhibition. All inhibition was relieved by the addition of 0.1 mM Co^{2+} to the assay. It will be shown in Table III that 0.003 mM EDTA causes about 50% inhibition. Since 0.04 ml of the EDTA-treated and dialyzed enzyme preparation in 0.33 ml of assay mixture caused more than 50% inhibition, the EDTA concentration in the enzyme preparation was probably greater than 0.03 mM. Clearly the stimulating effect of Co^{2+} on this preparation can be explained by its complexing the inhibitory EDTA.

Attempts to remove the EDTA by longer dialysis than 10 hr were not successful because the enzyme lost most of its activity for either the pyruvate or oxalacetate reactions on prolonged dialysis (30 hr). EDTA-treated enzyme which was passed through a Sephadex column did regain a considerable part of its activity without a requirement for Co^{2+}. Apparently apoenzyme was not formed by this procedure.

Although it cannot be categorically stated that an apoenzyme is not formed by treatment with EDTA and dialysis, which is activated by Co^{2+} for the oxalacetate reaction and Cu^{2+} for the pyruvate reaction, the present evidence indicates that these metals stimulate by removing EDTA from the enzyme.

Effect of Various Metal Chelators on Carboxytransphosphorylase. Structurally EDTA has certain similarities to the products of the forward and pyruvate reaction. Since it is already known that malic and maleic acids are effective inhibitors of both reactions (Lochmüller *et al.*, 1966; Davis *et al.*, 1969), it seemed possible that EDTA might not be acting as a metal binding agent but instead as a dead-end inhibitor. Therefore a variety of metal chelators were tested. If these structurally unrelated chelators likewise were inhibitory, the hypothesis that inhibition by EDTA is due to complexing an enzyme-bound metal would be strengthened. The results are given in Table III. The assays were conducted with enzyme diluted in both the presence and absence of β-mercaptoethanol (the former condition is optimal for the forward reaction and the latter optimal for the pyruvate reaction (Davis *et al.*, 1969)). The chelator was present in the complete assay mixture and the reaction was started by addition of the enzyme. The values have been calculated from initial rates and are expressed as micromoles product formed per minute per milligram of protein.

It is seen (Table III) that in the presence of thiol both reactions were inhibited by all the chelators. *m*-Phenanthroline is not a chelator and it is significant that it was without effect, thereby suggesting that *o*-phenanthroline is acting as a chelating agent and not through some effect due to the aromatic properties of the compound.

With the exceptions of diethyldithiocarbamate and 8-hydroxyquinoline, the inhibition in both reactions was much lower in the absence of than in the presence of thiols. These results are in agreement with the previous observations that immediate inhibition with EDTA occurs only in the presence of thiols (Davis *et al.*, 1969). The inhibition by diethyldithiocarbamate in the absence of thiol is under-

TABLE III: Effect of Chelators on Carboxytransphosphorylase Reactions.[a]

Chelator	Concn of Chelator (mM)	Forward Reaction: With β-Mercaptoethanol Rate[b]	Forward Reaction: With β-Mercaptoethanol Inhibn (%)	Forward Reaction: Without β-Mercaptoethanol Rate[b]	Forward Reaction: Without β-Mercaptoethanol Inhibn (%)	Pyruvate Reaction: With β-Mercaptoethanol Rate[c]	Pyruvate Reaction: With β-Mercaptoethanol Inhibn (%)	Pyruvate Reaction: Without β-Mercaptoethanol Rate[c]	Pyruvate Reaction: Without β-Mercaptoethanol Inhibn (%)
None		6.5		3.6		1.0		3.1	
EDTA	0.1	1.42	88	3.24	10			3.1	0
	0.03	1.67	74	3.24	10				
	0.01					0.2	80	3.1	0
	0.003	2.82	57			0.4	60	3.1	0
o-Phenanthroline	0.5			2.6	28			2.9	6
	0.1	1.67	74	3.1	14	0.29	71	2.9	6
	0.03	2.1	68	3.1	14				
	0.01					0.65	35		
α,α′-Dipyridyl	1.0	3.34	49			0.32	68		
	0.5			2.6	28			3.5	13 activation
	0.1	4.8	26	4.05	13 activation	0.73	27	3.5	13 activation
Diethyldithiocarbamate	5.0	1.9	71						
	1.0					0.20	80		
	0.5			1.45	60			0.16	95
	0.1	4.6	30	2.6	28	0.57	43	0.65	79
8-Hydroxyquinoline	1.0	2.3	65						
	0.5			1.63	55	0.41	59	1.3	58
	0.1	5.2	20	4.2	17 activation	0.57	43	2.9	6
NaCN	1.0	2.82	57						
m-Phenanthroline	0.1	6.5	0						

[a] Crystalline enzyme was used throughout. In the absence of β-mercaptoethanol 1.65 μg of enzyme was used per 0.33 ml of assay mixture for both the forward and pyruvate reactions; while with β-mercaptoethanol 0.66 μg was used for the forward reaction and 3.3 μg for the pyruvate reaction. The enzyme used for test of the forward reaction with β-mercaptoethanol present had a specific activity of 6.5; while that used for the forward reaction without the thiol and for the pyruvate reaction both with and without thiol had about twice the specific activity, *i.e.*, 12.0. The per cent inhibition or activation was determined from the reactivities in the presence and absence of inhibitors, *i.e.*, specific activity with specified chelator concentration/specific activity without chelator × 100 = per cent inhibiton. Conditions for the assays were as described previously (Davis *et al.*, 1969). [b] In micromoles of oxalacetate formed per minute per milligram. [c] In micromoles of pyruvate formed per minute per milligram.

standable since it is a thiol compound as well as a chelator. The inhibition of the pyruvate reaction by 8-hydroxyquinoline in the absence of thiols may be a reflection of its greater affinity for Cu^{2+} (Sillén and Martell, 1964). Thus it may not be inhibiting by chelating type II metal but rather by removal of the heavy metal which is believed to be required for the pyruvate reaction (Davis *et al.*, 1969). Likewise the activation of the oxalacetate reaction at low concentration may be because of binding of heavy metals which inhibit the oxalacetate reaction. There is no obvious explanation of the activation of the pyruvate reaction by α,α′-dipyridyl in the absence of thiols.

All the chelators exhibit some inhibition in the absence of thiols if the assay is continued for several minutes. Thiol compounds enhance but are not absolutely required for inhibition.

The results of Table III support the view that a type II metal in addition to Mg^{2+} is required for the carboxytransphosphorylase reaction. Inhibition by complexing of Mg^{2+} can be eliminated since Mg^{2+} is present in large excess of the chelators. Furthermore, o-phenanthroline and α,α′-dipyridyl have only slight affinities for Mg^{2+} (Sillén and Martell, 1964). It is considered significant that in the presence of thiols both reactions appear inhibited to a similar degree by these chelators and that in the absence of thiols both reactions are inhibited on prolonged incubation. These observations are in agreement with the suggestion that the enzyme-bound metal (type II) has a function in both reactions. The functions of the type I and type II metals are considered by Wood *et al.* (1969b).

Reversible and Nonreversible Inhibition of Carboxytrans-

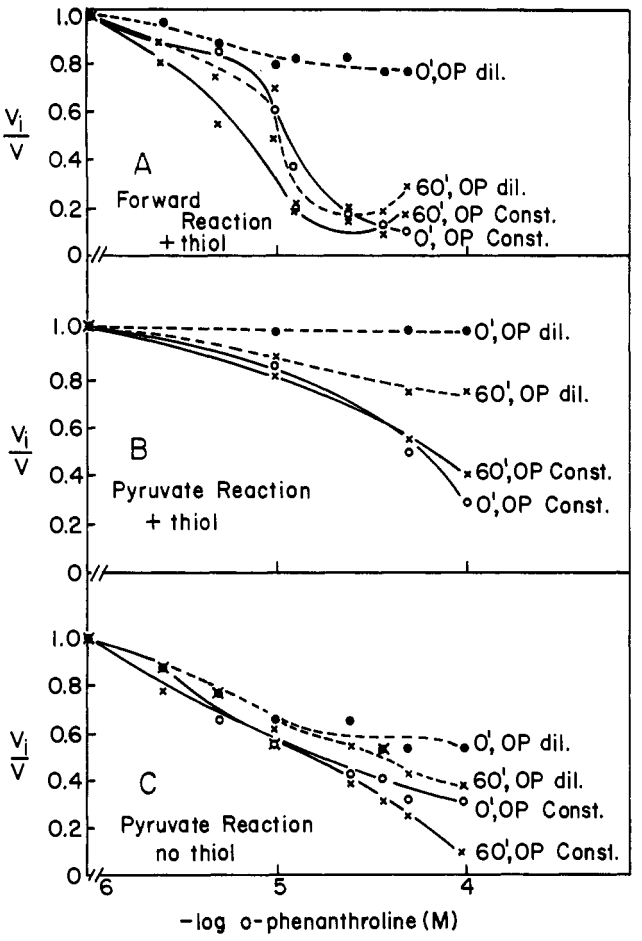

FIGURE 2: Determination of the instantaneous and time-dependent inhibition of carboxytransphosphorylase by *o*-phenanthroline (OP) and the reversibility of the inhibitions. The reagents were treated with chelex in the NH_4^+ form to remove contaminating ions. Assays were done as described previously (Davis *et al.*, 1969). In the experiments of part A 36 μg of enzyme/ml in 50 mM phosphate and 1 mM β-mercaptoethanol buffer (pH 6.8) were mixed with the indicated concentration of *o*-phenanthroline and immediately 2.16 μg of this protein was used per ml in the assay of the forward reaction without addition of *o*-phenanthroline to the assay (●---●) and also with the indicated concentration of *o*-phenanthroline maintained in the assay (O—O). The same type of experiments was done after 60-min incubation in the chelator without addition of *o*-phenanthroline to the assay mixture (x---x) and with the indicated concentration maintained in the assay (x—x). The activity of the control (no *o*-phenanthroline) expressed as micromoles of oxalacetate formed per minute per milligram of protein was 7.4 before and after the 60-min incubation. In the experiments of part 2B, the procedure was the same except 107.6 μg of enzyme was used per ml and 8.6 μg/ml was used in the assay of the pyruvate reaction. The activity of the untreated enzyme expressed as micromoles of pyruvate formed per minute per milligram of protein was 1.2 before incubation and 1.0 after 60-min incubation. In the experiments of part C, 53.8 μg of enzyme/ml in 50 mM phosphate (pH 6.8) (no thiol) was mixed with the indicated concentration of *o*-phenanthroline and 3.2 μg of protein was used per ml in the assay of the pyruvate reaction. The activity of the untreated enzyme was 2.2 before and after the 60-min incubation. The results are expressed as ratio of the velocity of the reaction with enzyme treated with inhibitor, V_i, to that of the enzyme not treated with inhibitor, V.

phosphorylase with Chelators. The time dependence of the inhibition by chelators as well as the reversibility of the inhibition has been investigated to obtain further information concerning the type of binding between the chelator and enzyme-bound metal. Vallee and coworkers (Hoch *et al.*, 1958; Williams *et al.*, 1958) have observed an instantaneous, reversible inhibition and also a time-dependent, irreversible inhibition of alcohol dehydrogenase from yeast. They consider that the instantaneous, reversible inhibition results from coordination of the chelator with the metalloenzyme but without disruption of the enzyme–metal ligands, whereas the time-dependent, irreversible inhibition results from disruption of the enzyme–metal ligands. Catalysis of both reactions of carboxytransphosphorylase has been investigated using *o*-phenanthroline (Figure 2). The chelator was added to the enzyme solution and a portion was removed at zero time and again after 60-min incubation at 25°. The enzyme was assayed in the presence and absence of a concentration of inhibitor equal to that present during the incubation. Assay in the absence of added inhibitor is a test of reversibility since the inhibitor is diluted beyond its inhibitory concentration.

It is seen (Figure 2A) that V_i/V for the oxalacetate reaction was about 0.2 at concentrations of *o*-phenanthroline greater than 10^{-5} M at 60 min whether or not the *o*-phenanthroline was diluted in the assay. Thus the inhibition was irreversible after 60 min. At zero time, however, the inhibition at 10^{-5}–10^{-4} M *o*-phenanthroline was largely removed (V_i/V about 0.8) if the chelator was diluted in the assay. Thus the inhibition of the oxalacetate reaction is of two types, instantaneous, reversible and time dependent, irreversible.

The situation differs in the pyruvate reaction. In this case (Figure 2B) the inhibition in the presence of thiol is somewhat reversible even after incubation for 60 min at 10^{-4} M *o*-phenanthroline, V_i/V being 0.76 at 60 min when the *o*-phenanthroline was diluted and 0.4 when it was not.

The results with the pyruvate reaction when the enzyme was not treated with thiol (Figure 2C) gave only partial reversibility even at zero time, V_i/V being less than 0.6; the inhibition at 60 min was even less reversible. The results of these studies, therefore, show that only in the oxalacetate reaction is there clear evidence of two types of inhibition, *i.e.*, instantaneous, reversible inhibition and a time-dependent, irreversible inhibition. It should be noted that with thiol present the rate of the pyruvate reaction is slow since it is inhibited by thiols and more enzyme is used in the assay. Thus the inhibition of the two reactions may not be quantitatively comparable.

Tests for the Requirement of Monovalent Metals by Carboxytransphosphorylase. Webster (1965) has shown that acetyl-CoA synthetase has a requirement for two types of divalent metals and recently he has found (Webster, 1966) that a monovalent metal is required in addition. Webster (1965) found that one of the divalent metals is required for one partial reaction and both metals for a second partial reaction of the over-all reaction. The carboxytransphosphorylase reaction is similar in that only the type I metal is required for exchange of CO_2 into oxalacetate but both type I and type II metals are required for the exchange of $^{32}P_i$ into phosphoenolpyruvate (Wood *et al.*, 1969b). Because of the similarity of the divalent metal requirements and since both reactions involve PP_i, it seemed worthwhile to determine whether carboxytransphosphorylase has any requirement for monovalent metals. The procedures were as described by Webster (1966). All reagents except $MgCl_2$ and $CoCl_2$ were treated with Dowex 50W (50–100 mesh) in the tetramethylammonium form to convert them into the tetramethylammonium salts. The enzyme had a specific

activity of 23 when assayed with the usual reagents and there was no significant loss of activity when the reagents were used in which K^+ and Na^+ ions were replaced by tetraethylammonium ions (Table IV). Thus there was no evidence that K^+, Na^+, or other monovalent metals are required by carboxytransphosphorylase.

TABLE IV: Effect of Monovalent Cations on the Oxalacetate Reaction of Carboxytransphosphorylase.[a]

Addition to Assay (mM)	Enzyme Not Treated with Dowex (μmoles/min per mg of protein)	Enzyme Treated with Dowex (μmoles/min per mg of protein)
$CoCl_2$ (0.1)	20.5	20.5
$CoCl_2$ (0.1) + KCl (9)	21.8	20.5
$CoCl_2$ (0.1) + KCl (18)	23.0	18.8
$CoCl_2$ (0.1) + NaCl (9)	23.0	19.6
$CoCl_2$ (0.1) + KCl (18)	21.8	19.6

[a] Constituents of the reaction mixture were freed of extraneous monovalent ions as follows: tetramethylammonium phosphoenolpyruvate was prepared by stirring 3 ml of the sodium salt for 30 min with 1 ml of wet Dowex 50W tetramethylammonium$^+$ form and was centrifuged to obtain the phosphoenolpyruvate solution; and tetramethylammonium bicarbonate was prepared by passing CO_2 through tetramethylammonium hydroxide until the pH was 7.4. Malate dehydrogenase and NADH each diluted in 5 mM tetramethylammonium phosphate (prepared by neutralizing phosphoric acid with tetramethylammonium hydroxide) were treated with Dowex resin as described above. The carboxytransphosphorylase had a specific activity of 23.0 with Co^{2+} in assays conducted without removal of specific monovalent ions. The carboxytransphosphorylase (10.4 μg/ml) in 50 mM tetramethylammonium phosphate (pH 6.8) and 1 mM β-mercaptoethanol was treated with Dowex 50W, tetramethylammonium$^+$ form. The supernatant fluid (containing enzyme) was decanted and 0.62 μg of enzyme was employed per ml of assay. The conditions of assay were as described by Davis *et al.* (1969).

Discussion

The evidence from these studies verifies and strengthens the proposal that there are at least two types of metals which function in carboxytransphosphorylase reactions, and in addition a heavy metal is stimulatory in the pyruvate reaction. One requirement is filled by dissociable divalent metals such as Mg^{2+} and is designated type I and the other by a tightly bound metal designated type II which has not been identified as yet. The most convincing evidence for the occurrence of the two metals is the demonstration (Table III) that a wide variety of metal chelators in the presence of a large excess of the type I metal (Mg^{2+}) inhibit carboxytransphosphorylase. It is extremely unlikely that all of these chelators would act nonspecifically, *i.e.*, in some manner not involving binding of the metal. It is not likely that the chelators act by binding Mg^{2+} since Mg^{2+} is present in a 1000-fold excess of the chelators and also some of the chelators, α,α′-dipridyl and *o*-phenanthroline, have only slight affinities for Mg^{2+}. Furthermore, since both reactions are inhibited to the same degree by the various chelators it appears a metal common to both the oxalacetate and the pyruvate reaction is involved. This evidence, together with the demonstration that Mg^{2+} is required for one partial reaction of carboxytransphosphorylase while both Mg^{2+} and the type II metal are required for another (Wood *et al.*, 1969b), makes the argument quite convincing for the role of two types of metals.

Thus far it has not been possible to isolate the apoenzyme. It appeared from the results of Figure 1 that an apoenzyme had been formed but subsequent experiments showed that carboxytransphosphorylase has an unusually high affinity for both EDTA and Co^{2+} (Tables I and II). These properties make it difficult to assess whether an apoenzyme has been formed or not, but all the results are in accord with the view that there is a metal requirement in addition to Mg^{2+}.

The instantaneous and time-dependent inhibitions observed with *o*-phenanthroline in the oxalacetate reaction (Figure 2A) are very similar to those observed with alcohol dehydrogenase which is a zinc metalloprotein (Hoch *et al.*, 1958; Williams *et al.*, 1958; Drum *et al.*, 1967). These results lend considerable support to the conclusion that carboxytransphosphorylase is a metalloprotein. The time-dependent, irreversible inhibition is considered by Vallee and collaborators to be the result of the binding of additional chelate molecules to the metal with the probable disruption of the enzyme–zinc ligands. However, direct proof that this type of cleavage is occurring with carboxytransphosphorylase is lacking at present.

The interpretation of similar tests with the pyruvate reaction is complicated by the fact that another form of the enzyme is believed to catalyze this reaction which involves both type I and type II metals and also a heavy metal (Davis *et al.*, 1969). There was very little evidence of the time-dependent, irreversible inhibition of this activity (Figure 2B,C) using enzyme with or without mercaptoethanol. Further study will be required to permit explanation of the difference in inhibition by *o*-phenanthroline of the two reactions catalyzed by carboxytransphosphorylase.

In addition to the effect of Co^{2+} on enzyme which has been treated with chelator, Co^{2+} has been found to have a stimulating effect on enzyme which has not been treated with chelator, see Table I. A similar stimulation of the pyruvate reaction by Cu^{2+} has not been observed with untreated enzyme. The explanation of the Co^{2+} effect with untreated enzyme is not certain. Co^{2+} may aid in the removal of heavy metals from the enzyme which inhibit catalysis of the oxalacetate reaction but stimulate the pyruvate reaction (Davis *et al.*, 1969). It has been observed (Wood *et al.*, 1969a, and unpublished results by M. E. Haberland, J. M. Willard, and H. G. Wood) that carboxytransphosphorylase dissociates to an enzymatically active subunit with a sedimentation constant of 7.4 S. This subunit has about one-third the specific activity in both the oxalacetate and pyruvate reactions as does the crystalline enzyme which has a sedimentation constant of 15.2 S. It is possible that the cobalt in some manner increases the proportion of 15.2 S with respect to the 7.4S form and thus increases the activity of enzymes which have lost activity due to partial dissociation to a 7.4S form.

The unequivocal demonstration that carboxytransphosphorylase is a metalloprotein must await the results of metal analyses, which have thus far been unsuccessful because it has been possible to prepare only a limited amount of enzyme.

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