AN UNUSUAL CITRATE SYNTHASE FROM MANGO FRUIT

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Summary

Citrate synthase from mango fruit differs from other aerobic citrate synthases in that it is inhibited by several sulfhydryl binding reagents. The enzyme is protected from inhibition by its substrates acetyl CoA and oxaloacetate. This enzyme is an "S-citrate synthase" with a molecular weight of 65,000.

Every living cell thus far examined for citrate synthase (citrate oxalacetate-lyase (CoA-acetylating) E.C.4.1.3.7.) has been found to contain it. A large number of citrate synthases have been studied, including enzymes from bacteria (1), plants (2), and animals (3 - 5). Only the enzyme from certain anaerobes (6, 7) has been shown to be affected by sulfhydryl reagents. It was surprising to us therefore, to find that the citrate synthase from mango fruit, an aerobic tissue, was inhibited by certain sulfhydryl reagents. Since the SH sensitive citrate synthases from anaerobic bacteria were unusual also in that they synthesized an "R-citrate" instead of "S-citrate", as do the citrate synthases from animals and other bacteria, we have determined the stereospecificity of the mango enzyme.

Materials and Methods

Mangos were obtained from J. Francis Williams, Perrine, Florida. Acetyl CoA was prepared as previously described (3). All other reagents were obtained from commercial sources. Citrate synthase was assayed by the coupled MDH method (3). Each assay contained 0.1 M Tris HCl pH 8.1, 0.01 M potassium malate, 3×10^{-4} M DPN, 10^{-4} M acetyl CoA and 25 μg of MDH (Boehringer) and enzyme in a total volume of 1.0 ml. Reactions were started with acetyl CoA and absorbance at 340 nm followed spectrophotometrically.

Enzyme Preparation

430 grams of mango fruit were homogenized in 1 L of solution containing 1 M Tris HCl pH 7.5, 0.1 M KCl, 10^{-3} M EDTA and 10^{-3} M mercaptoethanol. Homogenization was performed in a 2 gallon stainless steel Waring blendor. Eight homogenization periods of 30 seconds each were used with cooling periods between so that the homogenate temperature did not exceed 5°. All procedures were performed at temperatures of about 5°.

The homogenate was centrifuged at 10,000 g for 30 minutes. The precipitate was discarded. 320 gms of ammonium sulfate were added to the supernatant solution (1050 ml) (50% ammonium sulfate saturation). The mixture was stirred for 15 minutes and then centrifuged at 10,000 g for 30 minutes.

The precipitate was suspended in 0.1 M Tris HCl pH 7.4 containing 10^{-3} M EDTA and 10^{-3} M mercaptoethanol. Most of the original activity was in this fraction (S.A. 0.3 units/mg).

This fraction or fractions which had been passed over Bio-Gel A 1.5 M (S.A. 0.7) were used for our studies.

Molecular weight was estimated by the gel filtration method on Bio-Gel A 1.5 M.

Results and Discussion

The most striking difference between mango citrate synthase and other citrate synthases is its sensitivity to certain SH reagents. Table 1 shows that mango citrate synthase is inhibited by 5, 5'-dithio bis (2 nitrobenzoate) (DTNB) and that it can be protected against DTNB-inhibition by either of its substrates. In addition to DTNB both Hg^{++} and paramercuribenzoate (pMB) inhibit the enzyme (Table II). Slight inhibition is seen with N-ethyl-maleimide and about 20% stimulation was seen with iodoacetate and iodo-acetamide. Inhibition by Hg^{++} and pMB was completely reversed with dithio-threitol but only a slight reversal of the DTNB inhibited enzyme was found.

We have shown previously that neither pig heart (4) nor *E. coli* citrate synthase (1) is inhibited by DTNB. A variety of other citrate

TABLE I

INHIBITION BY DTNB

ADDITIONS TO ENZYME	10^{-3}M DTNB	ACTIVITY %
None	-	100
None	+	10
OAA (10^{-3}M)	+	60
AcCoA (10^{-3}M)	+	100

Enzyme in 0.1 M Tris HCl pH 8.1 was incubated with the additions shown above for 30 min. at room temperature. Aliquots were then assayed using the MDH assay.

TABLE II

EFFECT OF SULFHYDRYL REAGENTS ON MANGO CITRATE SYNTHASE

ADDITIONS	ACTIVITY	ACTIVITY AFTER 30 MIN. WITH DTT
10^{-4} M	% of Control	% of Control
0	100	112
DTNB	0	7
$HgCl_2$	0	112
PMB	0	92
N Ethyl maleimide	78	60
Iodoacetate	123	120
Iodoacetamide	123	111

Enzyme was incubated with 10^{-4} M reagent for 30 minutes at room temperature and an aliquot assayed for activity using the malate dehydrogenase coupled assay. After 30 minutes with the reagent, DTT was added to a final concentration of 10^{-3} M. Activity was assayed after 30 min. incubation with DTT.

synthases from animal and bacterial sources are also insensitive to DTNB. Nor is sulfhydryl sensitivity a general property of plant citrate synthases since neither spinach leaf nor lemon fruit citrate synthase are affected by DTNB.

In the case of the citrate synthases from certain anaerobes two different effects of SH reagents have been reported. O'Brien and Stern (6) have reported that pMB could inhibit the citrate synthase from Clostridium kluyveri about 40% and in so doing caused a change in stereospecificity of the enzyme from an R-citrate synthase to an S-citrate synthase. On the other hand, Gottschalk and Dittbrenner (7) showed that the citrate synthase from Clostridium acidi-urici could be completely inhibited by pMB and no change in stereospecificity could be observed. The enzyme from C. acidi-urici in contrast to the mango enzyme was not inhibited by DTNB but was inhibited by iodoacetamide. In addition the C. acidi-urici citrate synthase requires a metal ion (Mn^{+2} or Co^{+2}) for activity. Mango citrate synthase activity is not dependent on added metal ions nor can it be inhibited by the addition of ethylene diaminetetraacetate.

In order to test whether the mango enzyme had additional similarities to the anaerobic citrate synthase we tested the stereospecificity of the mango enzyme. 1-^{14}C-acetyl CoA was condensed with oxaloacetate in the presence of mango citrate synthase. The labeled citrate was isolated and cleaved with pure rat liver citrate cleavage enzyme in the presence of malate dehydrogenase and NADH to convert the oxaloacetate to malate. The resulting mixture was hydrolyzed (to convert acetyl CoA to acetate) and the acetate and malate isolated and their radioactivity determined. Figure 1 shows the results of such an experiment carried out in the presence of pMB, a condition reported by Stern (7) to affect the stereospecificity of the C. kluyveri enzyme. Similar results are obtained in the absence of pMB. Only the acetate contained ^{14}C indicating that the mango citrate synthase was an S-citrate synthase having the same stereospecificity of most other citrate synthases.

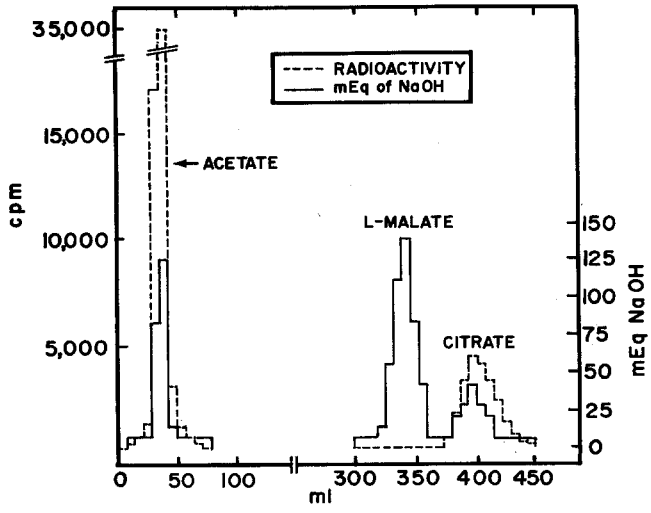

Figure 1. Stereospecificity of mango citrate synthase.

0.5 units of mango citrate synthase were incubated with 2×10^{-3} M, 1-^{14}C-acetyl CoA 4×10^{-3} M oxaloacetate, 4×10^{-2} M PCMB 0.1 M Tris-HCl pH 8.1 for 2 hours at room temperature. The reaction was stopped by heating to 70° for 6 min. The ^{14}C citrate was isolated on silicic acid as described by Varner (13). The ^{14}C citrate was cleaved by pure rat liver citrate cleavage enzyme and the organic acids isolated as described previously (14).

Mango citrate synthase is considerably smaller than any other citrate synthase thus far examined. Enzymes from rat liver (5), rat heart (5), pig heart (13, 14), moth flight muscle (4) and pigeon breast muscle (4) are about 100,000, while the enzyme from E. coli is about 200,000 (1) and that from Azotobacter is about 500,000. The partially purified mango enzyme appears to have a molecular weight of 65,000 (Figure 2). It is possible that the crude enzyme behaves differently on gel filtration than would a purified one, although such behaviour is not usually observed.

A number of substances have been reported to affect citrate synthase activity and have been proposed to act physiologically as modifiers of this enzyme. We have tested three of these ATP, DPNH, and α ketoglutarate at a concentration of 5 mM they inhibited the mango citrate synthase 26%, 37%, and 32% respectively. The effect of ATP can be reversed by increasing acetyl CoA concentration.

Although citrate synthase has been reported in a number of plant tissues

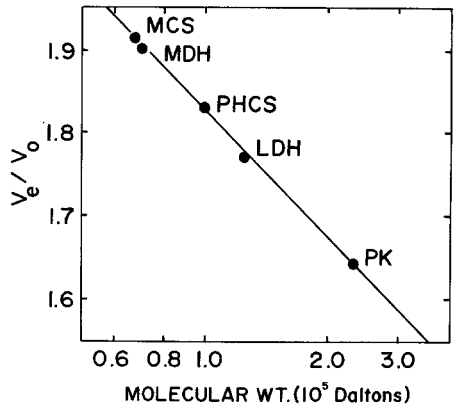

Figure 2. Gel Filtration

A column of Bio-Gel A 1.5M 0.9 cm x 105 cm was used. The equilibrating and eluting fluid was 0.1 M Tris-HCl pH 7.4 containing 0.01 M mercaptoethanol. 0.9 ml samples were collected. The void volume was 63 ml determined with blue dextran ($MW > 2 \times 10^6$). The molecular weight markers used were malate dehydrogenase (MDH) M.W. = 7×10^4; pig heart citrate synthase (PHCS) M.W. = 1×10^5; lactate dehydrogenase (LDH) M.W. = 1.4×10^5; and pyruvate kinase (PK) M.W. = 2.3×10^5.

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not one of these has been obtained in pure form. Partial purification of the enzyme from Garcinia leaves (8) and from lemon fruit (9) has been reported. In addition Sarkissian (10) has indicated that he has achieved partial purification of citrate synthase from bean seedling and cauliflower buds. Sarkissian also clamied to have observed stimulation of these citrate synthases by indole acetate, accompanied by binding of indole acetate to the protein and by an increase in molecular size of the enzyme. There have been two reports in which no effect of indole acetate on plant citrate synthase (11, 12) was observed. We have assayed mango citrate synthase in the presence of indole acetate at 10^{-3} M and 10^{-6} M and have not observed any stimulation of its activity.

The citrate synthase from mango fruit is clearly an unusual one, differing from other S-citrate synthases in its SH sensitivity and molecular weight, and differing from the SH sensitive citrate synthase of certain anaerobes in its stereospecificity and in its response to DTNB and iodoacetamide.

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