A MAGNETIC RESONANCE STUDY OF THE CITRATE SYNTHASE REACTION[1]

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During the reaction catalyzed by citrate synthase, one proton is removed from the methyl carbon of the acetyl group of acetyl CoA. Bové et al. (1959) with THO, and Marcus and Vennesland (1958) with D_2O, showed that the enzyme incubated with acetyl CoA alone does not catalyze an exchange between the protons of the acetyl group and those of the medium. Kosicki and Srere (1961) showed a decreased rate of the citrate-synthase-catalyzed reaction when deutero-acetyl-CoA was used as substrate; these data were interpreted as showing that the proton removal from the acetyl group was probably a rate-determining step, in agreement with conclusions of Bové et al. (1959). Eggerer (1965) recently reported the important observation that L-malate induced an acetyl-CoA enolase activity in citrate synthase, as measured by the incorporation of tritium from THO into the acetyl group of acetyl CoA; D-malate and a number of other compounds tested did not induce this activity.

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Oxalacetate forms a stable binary complex with citrate synthase (1966). Formation of the complex was associated with changes in the UV spectrum which were interpreted as resulting from altered protein conformation. The binary complex, unlike the enzyme, was not denatured by urea, and certain organic acid anions also could partially protect against urea-induced unfolding. These results were interpreted as indicating binding of certain anions to the active site. Some of the dicarboxylate anions tested for binding to the active site had also been tested by Eggerer for exchange; L-malate, the only one so tested that induced exchange, was by the protection criterion the most tightly bound compound of his series (Table 1). It was tempting to speculate that the protein conformation necessary to catalyze proton removal is formed in the presence of oxalacetate and that L-malate binds sufficiently well to cause a partial conformation change, enabling the enzyme to catalyze the very slow exchange reaction.

Table 1

Comparison of Binding to the Ability to Induce Exchange

Compound	Binding* (Srere, 1966)	Exchange (Eggerer, 1965)
Maleate	0.90	No
D-malate	0.89	No
Pyruvate	0.77	No
Dimethyl glutarate	0.75	No
Succinate	0.74	No
L-malate	0.58	Yes

*This value is the ratio of the rate of unfolding of enzyme in urea in the presence of an organic anion to the rate of unfolding in urea alone. A value of 1.0 indicates no binding and 0.00 indicates very tight binding. No linear relation can be assumed between strength of binding and this value.

To examine the hypothesis that binding of the organic anion to the enzyme induced a structural change that resulted in enolase activity, I inves-

tigated the induction of this exchange reaction using the nuclear magnetic resonance (NMR) technique to follow the incorporation of D from D_2O medium into the CH_3 group of acetyl CoA. The pertinent parts of the spectra for acetyl CoA and the control compounds are shown in Fig. 1. The line at -2.35 ppm (tetramethyl silane = 0) is due to the protons of the acetyl group of acetyl CoA. The peaks at -0.72 ppm and -0.85 ppm in both CoA and acetyl CoA represent the protons on the two methyl carbons of the pantothenate portion of CoA. Similar peaks are seen at -0.85 ppm and -0.90 ppm in D-pantoate and -0.87 ppm and -0.90 ppm in D-pantothenate. The separation of resonances of the protons of the two methyl carbons is due to the neighboring

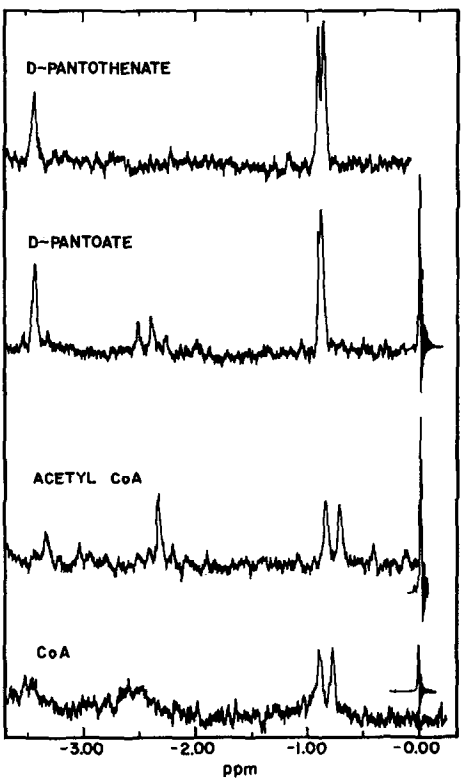

Figure 1. NMR spectra for potassium D-pantoate 0.1 M in D_2O, potassium D-pantothenate 0.1 M in D_2O, lithium acetyl CoA 0.02 M in 0.05 M potassium phosphate pD 7.7 in D_2O, and CoA in 0.02 M potassium phosphate pD 7.7 in D_2O. All spectra were obtained on a Varian A56/60 Spectrometer at room temperature. Tetramethyl silane (TMS) was used as an external standard at 0.00 ppm. All chemical shifts are reported in relation to TMS.

asymmetric carbon. The spectra reflect differences in the chemical shifts of methyl protons in the various rotational isomers.

The resonance peak at -2.35 ppm due to the methyl protons of the acetyl group of acetyl CoA corresponds well with the proton resonance of -2.40 ppm reported for thiolacetic acid (1962). A small peak at -2.10 ppm, variable in size with different preparations of acetyl CoA, is probably due to protons of contaminating acetic acid (reported at -2.10 ppm (1962)).

Substitution of the acetyl protons by deuterons results in a diminished resonance at -2.35 ppm. The amount of exchange was calculated as the ratio of the peak height at -2.35 ppm to that at -0.85 ppm. Since no exchange occurs at the methyl carbons on CoA, this method normalizes the data for variations in peak height due to instrumental changes. It would be more accurate to compare the integrated areas of the two peaks, but such elaborate calculation is not needed for what is demonstrated here. Prolonged incubations of enzyme and acetyl CoA alone show no change in this peak-height ratio. When substrate amounts of oxalacetate (OAA) are added, the resonance at -2.35 ppm disappears, since the methyl carbon of the acetyl group becomes a methylene carbon in citrate and the resonance of the protons on such a carbon appears elsewhere in the spectrum. This peak disappears when less than substrate amounts of OAA are added, since the protons are exchanged for deuterons by reversal of the reaction (termed equilibrium exchange by Eggerer).

My report that the SH groups on citrate synthase were not required for activity (1965) was confirmed by Eggerer who reported the enzyme to be fully active in ferricyanide (1965). By adding ferricyanide to the reaction mixture and oxidizing the released CoASH, he could inhibit the equilibrium exchange caused by OAA without affecting the exchange due to L-malate. This eliminated the possibility of exchange through formation of oxalacetate from L-malate and contaminating trace quantities of malate dehydrogenase and NAD. I have confirmed this observation using the NMR technique.

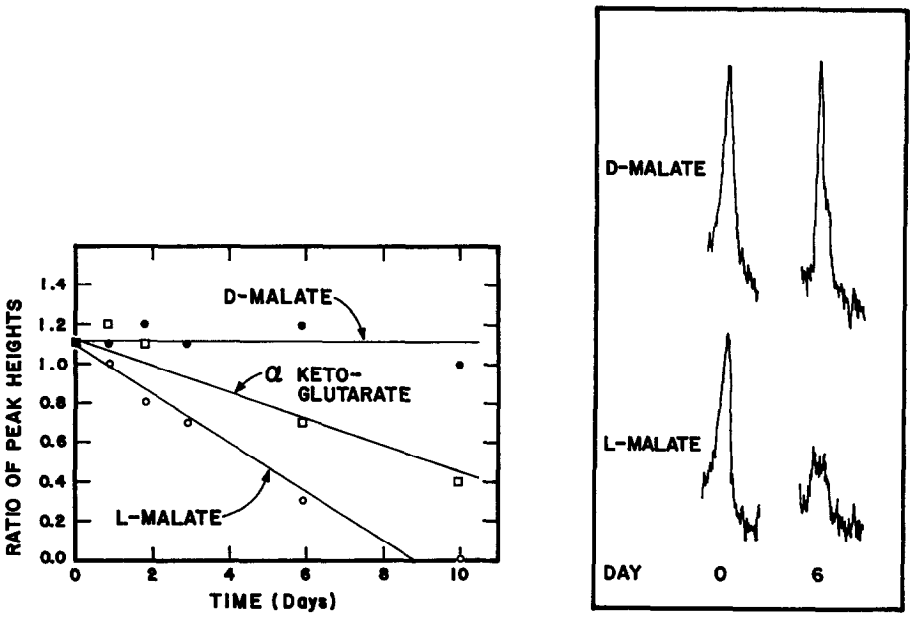

Figure 2. Each tube contained potassium phosphate buffer pD 7.7, 85 μmoles; lithium acetyl CoA, 15 μmoles; citrate synthase, 3 mg (~30 mμmoles); potassium ferricyanide, 50 μmoles; inducer, 50 μmoles; final D_2O excess 97% (determined by NMR), in a total volume of 0.45 ml. Incubations were at room temperature.

Left: resonances at -2.35 ppm for L-malate and D-malate at the beginning of the experiment and at 6 days. No hydrolysis of acetyl CoA was detected at the end of the experiment, and the enzyme was fully active.

Right: effects of L- and D-malate and α-ketoglutarate on the time course of exchange: The ratio of peak height at -2.35 ppm to that at -0.85 ppm is plotted vs time.

Figure 2 demonstrates that L-malate can induce the exchange of protons between the methyl carbon of the acetyl group of acetyl CoA and deuterons of the medium. D-malate does not induce an exchange; α-ketoglutarate induces it at a lower rate. Within experimental error, tricarballylate, D,L-isocitrate and transaconitate were inactive in inducing exchange. In similar experiments but without added ferricyanide, D,L-dimethyl malate (a gift of Dr. E. E. Snell, University of California (Berkeley)) induced exchange about one-fifth as fast as did L-malate. α-Ketoglutarate and oxalate are more effective than L-malate in binding to citrate synthase, as judged by their ability to protect the enzyme against denaturation by urea. Oxalate did not

induce enolase activity in the enzyme and α-ketoglutarate was less effective than L-malate. The results indicate that protection against urea unfolding can be achieved without changing the enzyme to the active conformation. This is in agreement with our observations that citrate can protect against urea unfolding with little change in UV spectrum (1966).

Eggerer (1965) interpreted his results as consistent with a mechanism proposed by Bové et al. (1959) in which the carboxyl group of oxalacetate aids in the removal of the methyl proton. My results (1966) indicated that both carboxyl groups, as well as the keto group of oxalacetate, were involved in binding to the enzyme and would consequently be unavailable for aiding the catalytic reaction.

It seems likely, therefore, that the proton removal is aided by a change in enzyme conformation where one group is brought into proper position upon binding of oxalacetate to the active site of the enzyme. A partial conformation change probably takes place with malate, and the slow exchange reported by Eggerer and also demonstrated here occurs.

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