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Exchange of Methyl Protons of Acetyl Coenzyme A Catalyzed by Adenosine Triphosphate Citrate Lyase†

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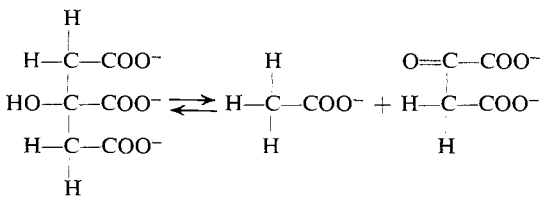
ABSTRACT: ATP citrate lyase requires the presence of oxalacetate in order to catalyze the exchange of the methyl protons of acetyl-CoA. This activity was measured by nuclear magnetic resonance spectroscopy by following exchange of deuterons of D_2O into acetyl-CoA, and by following the exchange of tritium from [^{3}H]acetyl-CoA into H_2O. The reaction requires sulfhydryl groups on the enzyme. Oxalacetate cannot be replaced by L-malate or α-ketoglutarate, substances which induce an enolase in citrate synthase.

There are three enzymes known to catalyze the same bond-making and -breaking reaction on citrate. These three



are citrate lyase (EC 4.1.3.6), citrate synthase (citrate lyase (CoA acetylating) EC 4.1.3.7), and ATP citrate lyase (citrate lyase (CoA acetylating and ATP dephosphorylating) EC 4.1.3.8). In order for citrate to be formed, a proton must first be removed from an acetyl unit in each case. Eggerer (1965) first demonstrated that such an enolization could be measured in citrate synthase only if the inducer substance L-malate was added. Srere (1967) was also able to demonstrate this activity of citrate synthase and showed α-ketoglutarate, too, could serve as an inducer for this enolization.

We have examined the ATP citrate lyase for the similar reaction measuring the exchange of deuterons from D_2O into acetyl-CoA by nuclear magnetic resonance spectroscopy and also by measuring the exchange of tritium from [^{3}H]acetyl-CoA into the water of the medium. The results of these experiments are presented in this paper.

Materials and Methods

Rat liver ATP citrate lyase (mol wt 500,000) was purified using the procedure of Srere (1959) for the chicken liver enzyme followed by chromatography on Bio-Gel A 1.5 m. Such preparations show single bands by disc gel electrophoresis and have specific activities of 3.5–4.0 μmoles of citrate cleaved per min per mg of protein. This preparation contains 2 munits of citrate synthase/mg and 3 of malate dehydrogenase/mg. The protein was estimated by the method of Lowry *et al.* (1951). Acetyl-CoA and deuterated acetyl-CoA were prepared using the method of Simon and Shemin (1953) and both preparations contained some free acetate. Control experiments showed that the presence of acetate did not effect the results. Nmr spectra were recorded with a Varian A-60-A nmr spectrometer and were performed at room temperature (27°).

[^{3}H]Acetyl-CoA was prepared by reacting with [^{3}H]acetic anhydride and CoASH in 0.1 M Tris base. [^{3}H]Acetyl-CoA was subsequently isolated by ion-exchange chromatography on a DEAE-cellulose column using the method of Moffatt and Khorana (1961). Tritium-exchange experiments were performed in 0.1 M Tris-Cl (pH 8.1) with enzyme, [^{3}H]acetyl-CoA and other substrates. The tritiated water was collected by a lyophilization technique described by Bloom (1958). The collected H_2O (10 μl) was placed in 10 ml of Bray's solution and the radioactivity determined in a Packard Tri-Carb scintillation counter.

Rates were calculated as suggested by Rose (1970) using the equation $v = 2.3nt^{-1}$[AcCoA] log (1-fraction reacted), where $n = 3$ since 3 equiv of protons are involved.

Results

Exchange of Protons of Acetyl-CoA with D_2O Nuclear Magnetic Resonance. The nmr spectrum of acetyl-CoA is shown in Figure 1. The line at −2.35 ppm (sodium 3-(trimethylsilyl)-1-propanesulfonate = 0) is due to the protons of the acetyl group of acetyl-CoA. The peaks at 0.72 and −0.85 ppm are due to the methyl groups of the pantothenic acid portion of the acetyl-CoA. The separation in resonances of the two methyl groups is due to the neighboring asymmetric carbon atom. Since the protons of methyl groups of the pantothenic acid portion of the molecule do not exchange, the amount of exchange of protons of the acetyl group with deuterons of the medium is estimated from the decrease in ratio of peak heights at −2.35 ppm to that of −0.85 ppm.

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TABLE I: Requirements for Exchange between Acetyl-CoA and D_2O.[a]

Ac-CoA (μ-moles)	OAA (μ-mole)	Mg^{2+} (μmole)	ADP (μmole)	P_i (μ-mole)	Enzyme (nmole)	V_H (μmoles/min per mg)
16					2.0	N[b]
14	0.2				0.6	3.3
10		0.050	0.05	0.5	2.0	N
14	0.2	0.05	0.05	0.5	0.6	2.62
16	1				0.001[c]	N
10	1				1.0[d]	N
8					0.2[e]	N
8					2.0[f]	N

[a] Incubations were carried out in 0.1 M Tris-Cl (pH 8.1) and 2 mM dithiothreitol in a total volume of 0.5 ml (D_2O) at 30°. Addition of buffer, oxaloacetate (OAA), and enzyme reduced 1 M D_2O content to 80%. This exchange includes both forward and condensation directions. [b] N denotes exchange is less than 0.01 μmole/min per mg. [c] Pig heart citrate synthase. [d] Iodoacetamide-inactivated citrate cleavage enzyme. [e] In presence of 20 μmoles of L-malate. [f] In presence of 20 μmoles of α-ketoglutarate.

Since no change in line width occurs in the first part of the reaction the decrease in peak height is proportional to decrease in area of the peak and therefore to decrease in concentration of protons. This also normalizes the data for variations due to instrumental changes. When acetyl-CoA and enzyme are incubated together, no change in the peak height of the −2.35-ppm resonance is seen but when oxalacetate is added, the protons are exchanged for deuterons of the medium and the peak at −2.35ppm decreases. This exchange is not affected by the remaining substrates of the citrate ATP lyase reaction either when added singly or in various combinations. L-Malate, D-malate, or α-ketoglutarate cannot replace oxaloacetate in the exchange reaction nor can enzyme inhibited by iodoacetamide catalyze the exchange. ATP citrate lyase, though pure, still contains traces of citrate synthase activity. We have therefore measured the exchange catalyzed by pig heart citrate synthase under our conditions using about ten times the amount of synthase as was present in our cleavage preparation. No exchange was detectable at these low citrate synthase and oxaloacetate concentrations and short time periods (Table I).

Exchange of ^3H from [^3H]Acetyl-CoA with H_2O. The exchange of the tritium of tritiated acetyl-CoA with the protons of water was also dependent on the presence of both enzyme and oxalacetate. As in the case of the deuterium-exchange experiments, little effect is observed with combinations of the remaining substrates (Table II). The capacity of the enzyme to catalyze the exchange reaction is inhibited by iodoacetamide treatment of the enzyme. Little exchange occurred when oxalacetate was replaced with L-malate, D-malate, or α-ketoglutarate. Citrate synthase at levels much higher than found in any enzyme preparation did not catalyze a significant tritium exchange (Table III). The rate of exchange was proportional to the amount of enzyme added. The exchange rate is constant for 30 min, and is dependent on both

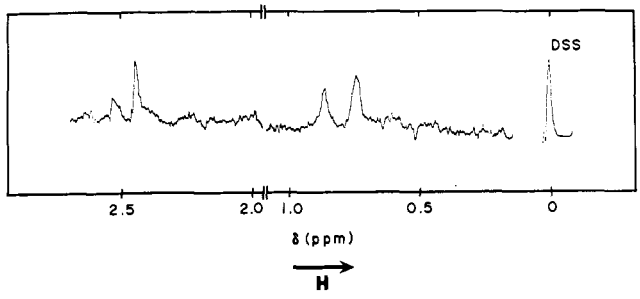

FIGURE 1: Nmr spectrum (60 MHz) of acetyl-CoA (10 μmoles) in 0.5 ml of D_2O with sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal standard.

the concentration of acetyl-CoA and oxaloacetate (Figures 2 and 3). Apparent K_m values for acetyl-CoA and oxalacetate were 7×10^{-4} and 2×10^{-5} M, respectively.

Effect of pH on ^3H Exchange. The effect of pH on the rate of exchange of tritium from [^3H] acetyl-CoA with the protons of the medium is illustrated in Figure 4. The exchange does not seem to occur below pH 6. The rate then increases sharply and reaches maximum in the pH range 6–7.4, and is unaffected with the further increase in pH. The rate decreases near pH 10.

Effect of Temperature on ^3H Exchange. The rate of exchange is dependent on temperature, increasing almost threefold in the temperature range 12–42°. An activation energy of 8 kcal/mole has been calculated from an Arrhenius plot. Results are given in Table IV.

Effect of [$^2H_3$]-Acetyl-CoA on the Rate of the Reverse Citrate ATP Lyase Reaction. The substitution of deuterioacetyl-CoA for acetyl-CoA has no effect on the rate of the reverse citrate ATP lyase reaction (Table V).

Measurement of Kinetic Isotope Effect (K_H/K_T). The isotope effect (K_H/K_T) was measured by incubating [^3H]acetyl-CoA

TABLE II: Requirements for Exchange of [^3H]Acetyl-CoA with H_2O.[a]

Additions	10^4 cpm/10 μl in Recovd H_2O	V_T (μmoles/min per mg)
None	0.09	0.026
Mg^{2+} + ADP + P_i	0.06	0.09
OAA	3.3	0.9
OAA + Mg^{2+}	4.3	1.2
OAA + ADP	4.5	1.3
OAA + P_i	4.7	1.3
OAA + Mg^{2+} + ADP	3.6	1.0
OAA + ADP + P_i	3.5	0.9
OAA + Mg^{2+} + P_i	4.0	1.1
OAA + Mg^{2+} + ADP + P_i	3.9	1.1

[a] Incubations were for 10 min at room temperature each containing 100 μg of enzyme, 0.5 μmole of [^3H]acetyl-CoA (6.5×10^6 cpm) in 0.1 M Tris-Cl (pH 8.1) and containing as indicated the following: 0.05 μmole of OAA, 0.5 μmole of $MgCl_2$, 0.05 μmole of ADP, and 0.5 μmole of P_i in a final volume of 0.5 ml. The reaction is stopped by 35 μl of 1 N KOH.

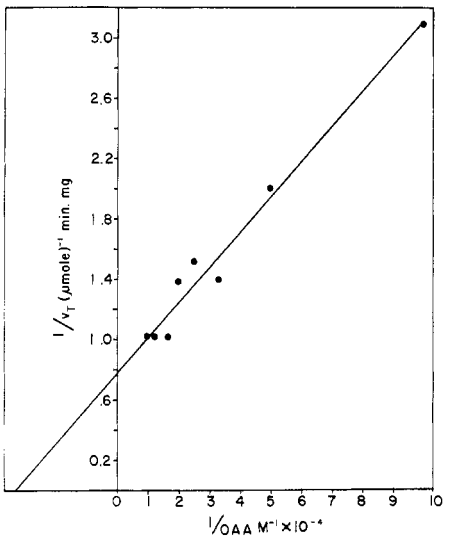

FIGURE 2: Effect of oxalacetate concentration on proton exchange. ATP citrate lyase (100 μg) was incubated with 0.5 μmole of [^{3}H]-acetyl-CoA (6.5×10^5 cpm) with varying amount of oxalacetate in 0.1 M Tris-Cl (pH 8.1) at room temperature for 10 min. The final reaction was stopped by adding 35 μl of 1 N KOH.

and acetyl-CoA together with enzyme in D_2O. The rate of proton release was measured by following the disappearance of the peak (02.35 ppm) as described earlier in the nmr section. Tritium release was measured by stopping the reaction with 1 N KOH and distilling the volatile counts. An average value of K_H/K_T of 6.6 in four sets of determination was obtained.

Discussion

The three different citrate lyase enzymes have been shown by Eggerer *et al.* (1970), Retey *et al.* (1970), and Klinman and Rose (1971) to have the same stereochemistry for the protons on the methyl carbon of the acetyl groups. Their results show that an inversion of configuration of these protons occurs and rule out the mechanism of enolization proposed by Eggerer (1965) for citrate synthase in which a carboxylate group of oxalacetate acts as an internal base aiding

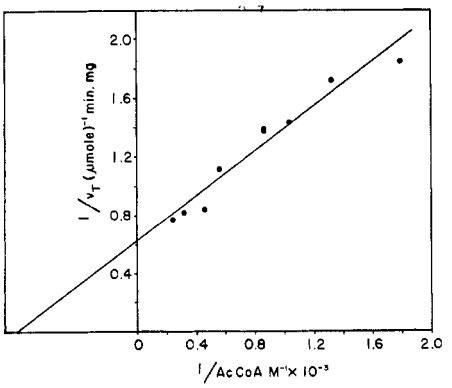

FIGURE 3: Effect of acetyl-CoA concentration on proton exchange. ATP citrate lyase (100 μg) was incubated with varying amount of [^{3}H]acetyl-CoA (2.8×10^6 cpm) with 0.05 μmole of oxalacetate at room temperature in 0.1 M Tris-Cl (pH 8.1) for 10 min. The final reaction volume was 0.5 ml. The reaction was stopped by adding 35 μl of 1 N KOH.

TABLE III: Effect of Various Additions on Exchange from [^{3}H]Acetyl-CoA.[a]

Additions	V_T (μmole/min per mg)
L-Malate (30 μmole)	0.11
L-Malate (1 μmole)	0.05
D-Malate (50 μmole)	0.04
Iodoacetamide enzyme + 0.05 μmole of OAA	0.18
Pig heart citrate synthase (0.01 μg) + 0.05 μmole of OAA	0.01
Pig heart citrate synthase (100 μg) + 0.05 μmole of OAA	0.01
None	0.03
Denatured enzyme + 0.05 μmole of OAA	0.01
α-Ketoglutarate (20 μmole)	0.07

[a] The incubations are carried out in total volume (0.5 ml) 10 min at room temperature containing the following: 100 μg of enzyme (except where synthase is used), 0.1 M Tris-Cl (pH 8.1), 0.5 μmole of [^{3}H]acetyl-CoA (6.5×10^5 cpm), and the additions as shown in a final volume of 0.5 ml. Reactions were stopped by addition of 35 μl of 1 N KOH.

in the enolization reaction. Since ATP citrate lyase alone cannot catalyze the exchange of protons, the role of oxalacetate in the enolization could be visualized in other ways. The oxalacetate in forming a binary complex with the enzyme might cause a conformation change in the enzyme so that an amino acid residue is brought next to the methyl group resulting in the hydrogen exchange. The second way oxalacetate could be involved could be in the reversible formation of citryl-CoA, thus affecting an exchange. It is possible that oxalacetate causes a conformation change to allow the exchange of a sequestered proton. It is also possible that the enzyme catalyzes the proton removal by itself but the re-

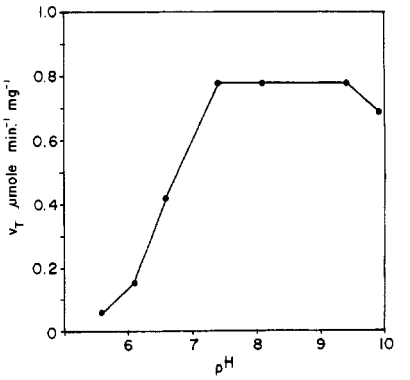

FIGURE 4: pH dependence of ^{3}H-exchange reaction. Each tube contained 100 μg of enzyme in 0.1 M buffer and 0.5 μmole of [^{3}H]acetyl-CoA (1.6×10^6 cpm) in a total volume of 0.5 ml. In the pH range 5.5–7.4 potassium phosphate bufler was used, in the range 8.1–9.4 Tris-HCl was used. Each tube also contained 0.05 μmole of oxalacetate. Incubation time was 10 min. Control tubes without oxalacetate at each pH were also analyzed and values comparable to controls in Table III were obtained.

TABLE IV: Effect of Temperature on Exchange of [^{3}H]Acetyl-CoA with H_2O.[a]

Temp (°C)	V_T (μmole/min per mg)
12	0.19
18	0.32
30	0.48
42	0.70

[a] ATP citrate lyase (100 μg) was incubated for 10 min with 0.5 μmole of [^{3}H]acetyl-CoA (1.9×10^6 cpm) and 0.5 μmole of OAA in 0.1 M Tris-Cl (pH 8.1). Total volume 0.5 ml. The reaction was stopped by 35 μl of 1 N KOH.

TABLE V: Effect of Deuterated Acetyl-CoA on Rate of the Reverse Citrate ATP Lyase Reaction.[a]

Enzyme (μg)	Acetyl-CoA (mM)	[$^2H_3$]-Acetyl-CoA (mM)	$-\Delta A$ at 232 mμ/min
59	0.1		0.013
59		0.1	0.012
118	0.1		0.030
118		0.1	0.032

[a] Incubation mixtures contain the following: KCl, 250 μmoles; ADP, 1.5 μmoles; $MgAc_2$, 0.5 μmole; AcCoA, 0.1 μmole; OAA, 0.1 μmole; and P_i, 10 μmoles in 0.1 N Tris-Cl (pH 8.1). Total volume 1 ml. The reaction was followed at 232 mμ as described by Plowman and Cleland (1967).

moved proton is kept sequestered in a hydrophobic region of the enzyme so that it is not exchangeable with the medium.

In the case of citrate synthase, no exchange can be detected in the presence of acetyl-CoA and oxalacetate if the reverse reaction is blocked. Presumably, formation of the citrate proceeds much more rapidly than the exchange of protons with the medium. The hydrogen exchange can be detected only in the presence of nonsubstrate inducers as L-malate or α-ketoglutarate.

Like citrate synthase, ATP citrate lyase alone does not catalyze a detectable hydrogen exchange of the methyl protons of acetyl-CoA. The addition of oxalacetate in the absence of components necessary to complete the net overall reaction does result in a rapid exchange of protons catalyzed by the enzyme. The rate of hydrogen exchange measured in D_2O was about 3.3 μmoles/min per mg, while simultaneous measurement of tritium exchange was about 0.5 μmole/min per mg. The V_{max} of the reverse reaction of ATP citrate lyase is about 0.5 μmole/min per mg (Plowman and Cleland, 1967). Addition of the remaining substrates to complete the reaction does not affect the exchange rate. Unlike citrate synthase, the enzyme does not respond well to the inducers L-malate or α-ketoglutarate.

Our data do not distinguish between the mechanisms of citryl-CoA formation, or the mechanism of citryl enzyme formation as postulated by Inoue *et al.* (1969), since both these mechanisms would result in an exchange of the proton with the medium.

The pH dependence of the exchange reaction is somewhat different from that of the overall forward reaction. Thus, at pH 7.4 and 9.5 the overall enzymatic activity is 50% of the maximum activity observed at pH 8.7, but there is no available data on the pH dependence of the reverse citrate ATP lyase reaction for proper comparison of these data. The exchange does require SH groups on the enzyme since iodoacetamide-treated enzyme is inactive. Iodoacetamide-treated enzyme, though inactive in the overall reaction, is phosphorylated by ATP as readily as active enzyme. The apparent pK of a group involved in the exchange is 6.5 and it is possible that an S^- group on the enzyme aids in the enolization of the proton.

Klinman and Rose (1971) reported that when ATP citrate lyase (in the presence of DPNH and malate dehydrogenase) was allowed to act on (3*S*,4*S*)-[4-^{3}H]citrate, 3% of the total counts were found in the H_2O. Since the oxalacetate is being removed as it is formed in their experiments their exchange is much less than we have observed. The failure to detect any isotope effect in the assay system (Table VI) is consistent with the fact that the rate of proton exchange that would be calculated is at least three to four times higher than the rate of synthesis of citrate.

Preliminary experiments with the third citrate enzyme, citrate lyase (*Klebsiella aerogenes*), indicates that the exchange (enolization) does not occur with the methyl protons of the acetate in the presence of enzyme alone. The instability of this enzyme and its inhibition by oxalacetate have made interpretation of other experiments difficult, but we are still pursuing the studies.

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