Acyl-Coenzyme A Carboxylase of the Free-Living Nematode *Turbatrix aceti*. 2. Its Catalytic Properties and Activation by Monovalent Cations†

Haruko Meyer and Franz Meyer*

ABSTRACT: A highly purified acyl-CoA carboxylase from the nematode *Turbatrix aceti* catalyzes the ATP, Mg^{2+}, and HCO_3^- dependent α-carboxylation of acetyl-CoA, propionyl-CoA, and butyryl-CoA at the respective rates of 6.8, 39.7, and 9.1 μmol per min per mg. The enzyme is inhibited by avidin and sulfhydryl reagents. It is activated up to 30-fold by the monovalent cations K^+, Rb^+, Cs^+, or NH_4^+, with the apparent activation constants of 11.0, 4.1, 10.0, and 6.7 mM, respectively. In the presence of K^+, the apparent K_m for ATP increases 5-fold, and the K_m for HCO_3^- decreases 4-fold, whereas the K_m for propionyl-CoA remains constant. Of the partial reactions, the ATP-^{32}P exchange reaction and the carboxylation of free biotin have a nearly absolute requirement for K^+. By contrast, the [^{14}C]acetyl-CoA–malonyl-CoA exchange reaction proceeds without K^+ at 80% of its maximum rate. The data indicate that K^+ primarily stimulates the first half of the carboxylation reaction, i.e., the ATP-dependent carboxylation of the biotinyl residue.

In the accompanying paper (Meyer et al., 1978), we describe the isolation of a biotin-containing acyl-CoA carboxylase from the free-living nematode *Turbatrix aceti*, the purification to homogeneity of this enzyme, and certain of its physical characteristics. In this paper, we describe some of the kinetic properties of the enzyme.

A distinct feature of the *T. aceti* acyl-CoA carboxylase is that it has a composite of properties which are generally considered typical of either acetyl-CoA carboxylases or propionyl-CoA carboxylases. The enzyme has a relatively broad substrate specificity for short-chain acyl-CoA esters, depends on K^+ for full catalytic activity, and is apparently located within the cytoplasm of the cells.

Experimental Procedure

The commercial sources of all special reagents and the procedures for the purification of the *T. aceti* enzyme are the same as those described in the preceding paper (Meyer et al., 1978). When required, the enzyme was freed from K^+ by extensive dialysis against 0.1 M Tris-HCl, pH 7.5, containing 1 mM dithiothreitol and 1 mM EDTA; such preparations were used within a few days.

Assay of the Overall Carboxylation Reaction. Acyl-CoA carboxylase activity was determined by measuring the acetyl-, propionyl-, or butyryl-CoA dependent incorporation of $H^{14}CO_3^-$ into malonyl-CoA or its derivatives. The reaction mixture contained 80 mM Tris-HCl buffer, pH 8.0, 40 mM KCl, or 20 mM K_2HPO_4, pH 8.0, 10 mM $MgCl_2$, 1 mM ATP (Na salt), 1.5 mM acetyl-, propionyl-, or butyryl-CoA (Li salts), 20 mM $NaH^{14}CO_3$ (2×10^5 cpm/μmol), and 10 to 15 milliunits of enzyme in a final volume of 0.4 mL (pH 8.1). The reaction was run for 5 min at 30 °C and was stopped by the addition of 0.2 mL of 2 N perchloric acid. After centrifugation of the mixture, 0.2 mL of the clear supernatant was placed in a scintillation vial and incubated in a 70 °C water bath under a gentle stream of air until all unreacted $^{14}CO_2$ was expelled. The dry residue was then dissolved in 0.1 mL of water, blended with Aquasol (New England Nuclear), and counted in a liquid scintillation counter. One unit of enzyme activity was defined as the amount of enzyme (mg of protein) required to catalyze the fixation of 1 μmol of $H^{14}CO_3^-$ into malonyl-CoA (or its derivatives) per min under the given assay conditions.

Assay of the Carboxylation of Free Biotin. The reaction mixture (1.5 mL) containing 200 μg of K^+-free enzyme, 80 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 1 mM dithiothreitol, 20 mM (+)-biotin, 1 mM ATP, 10 mM $MgCl_2$, and 20 mM $NaH^{14}CO_3$ (7×10^6 cpm/μmol) was incubated both with and without 40 mM KCl at 30 °C. During incubation, 0.1-mL aliquots were withdrawn at given time intervals. Each aliquot was mixed with 0.8 mL of ice-cold water containing 1 drop of 1-octanol. The solution was gassed with CO_2 at 1 °C for 40 min, and the residual radioactivity in the form of *N*-carboxybiotin was counted in a liquid scintillation counter (Stoll et al., 1968).

Assay of the ATP-^{32}P Exchange Reaction. The reaction mixtures, each containing in 0.5 mL, 13 μg of K^+-free enzyme, 80 mM Tris-HCl, pH 7.1, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, 1.5 mM ADP, 10 mM $MgCl_2$, 20 mM $NaHCO_3$, and 2 mM ^{32}P (sodium salt, 1×10^6 cpm/μmol), were incubated both with and without 40 mM KCl at 30 °C. The reactions were terminated at various time intervals by the addition of 0.4 mL of ice-cold 10% trichloroacetic acid, 0.5 mL of 6 N H_2SO_4, and 1 mL of 5% ammonium molybdate. The inorganic form of ^{32}P was removed from the reaction mixture by extracting it twice with 3-mL portions of isobutyl alcohol and once with 3 mL of ethyl ether. The organic ^{32}P, left in the aqueous phase in the form of ATP, was thereafter measured for radioactivity (Gregolin et al., 1968).

Assay of the [^{14}C]Acetyl-CoA–Malonyl-CoA Exchange Reaction. The reaction mixtures, each containing in 0.5 mL, 6 μg of K^+-free enzyme, 80 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 1 mM dithiothreitol, 0.2 mM malonyl-CoA, and 0.2 mM [1-^{14}C]acetyl-CoA (7×10^6 cpm/μmol) were incubated both with and without 40 mM KCl at 30 °C. The reactions were terminated at various time intervals by heating the assay mixtures at 65 °C for 2 min. Excess [^{14}C]acetyl-CoA was deacylated by arsenolysis and thereafter evaporated as [^{14}C]acetic acid. The radioactivity in the acid stable malonyl-CoA was counted (Gregolin et al., 1968).

† From the Department of Microbiology, State University of New York, Upstate Medical Center, Syracuse, New York 13210. *Received October 27, 1977.* This work was supported by Grant AI-0748610 from the National Institutes of Health, United States Public Health Service.

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Analysis of the Carboxylation Products. To identify the product of the carboxylation reaction, large batches of the purified enzyme were incubated with $H^{14}CO_3^-$ and with either acetyl-CoA or propionyl-CoA as substrate. The ^{14}C-labeled reaction products were analyzed both in the form of their thioesters and as free acids by paper chromatography (Vagelos, 1960). Radioactive spots were detected by autoradiography, and free acids were also located by spraying the chromatograms with bromocresol green.

Subcellular Localization of the Enzyme. T. aceti, grown in 200 mL of Stock Culture Medium, was harvested and washed as described in the preceding paper. The washed organisms were suspended in 3 volumes of 0.02 M Tris-HCl, pH 8.0, containing 0.05 M sucrose, 0.2 M mannitol, 0.01 mM EDTA, and 0.5 mM dithiothreitol. They were divided into three equal portions and homogenized in a chilled glass tissue grinder (40-mL capacity) with ten up-down strokes. Immediately thereafter, an equal volume of 0.02 M potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose, 0.01 mM EDTA, and 0.5 mM dithiothreitol was added to the homogenate. The homogenate was then subjected to a series of centrifugations: 480*g* for 10 min (twice), 750*g* for 10 min, 17 000*g* for 10 min, 30 000*g* for 20 min, and 70 000*g* for 60 min. The sediment from each run was resuspended in the same phosphate-sucrose buffer. The 17 000*g* sediment, which presumably contains the mitochondrial fraction (Rothstein et al., 1970), was washed once with buffer by subjecting it to a cycle of low- and high-speed centrifugation. All sediments were then homogenized once more in a power-driven Teflon pestle homogenizer to release particle-bound enzymes.

Results

Enzyme Requirements. Like most other biotin-containing carboxylases (Moss & Lane, 1971), the nematode enzyme requires HCO_3^-, ATP, and Mg^{2+} for the carboxylation of acetyl-CoA, propionyl-CoA, or butyryl-CoA. Unlike the acetyl-CoA carboxylases of higher animals, the nematode enzyme does not require citrate or isocitrate for activation. Thus, when the enzyme was preincubated with 5 to 20 mM of citrate or isocitrate, no change in the catalytic activity was observed.

During the purification of the carboxylase, we noticed that the enzyme requires potassium phosphate buffer for maximum activity. When we examined K^+ and HPO_4^{2-} separately, it became clear that K^+ and not HPO_4^{2-} activated the enzyme. Enzyme preparations freed from K^+ by extensive dialysis against 0.1 M Tris-HCl buffer, pH 7.5, were stimulated up to 30-fold in their catalytic activities by K^+. Currently, we do not know whether the residual activity associated with the dialyzed enzyme is attributable to an intrinsic property of the enzyme or to small amounts of K^+ which are still bound to the enzyme.

The activation of the enzyme by K^+ was instantaneous. When two enzyme batches, one containing K^+ (40 mM KCl) and the other free of K^+, were preincubated at 30 °C for 30 min and, thereafter, assayed in the presence of K^+, both enzyme batches carboxylated the substrate at nearly identical initial rates. Time-course studies likewise demonstrated that K^+ increases the carboxylation rate without a lag phase.

Effects of pH and Ionic Strength. The effect of pH on the carboxylation rate was studied between pH 6.0 and 9.0 using standard assay conditions. In the low pH range, imidazole hydrochloride and, in the high pH range, Tris-HCl were used as buffers. The pH activity curves for the carboxylation of propionyl-CoA and acetyl-CoA were very similar. With both substrates, the carboxylase activity was low in the acidic pH range and reached its maximum between pH 7.8 and 8.6. Omission of K^+ from the reaction mixture flattened the pH–activity curve.

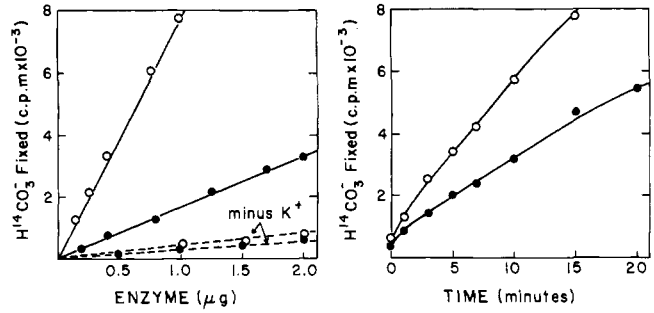

FIGURE 1: (Left) Carboxylation rate of acetyl-CoA (●) or propionyl-CoA (○) as a function of enzyme concentration determined with and without K^+. (Right) Carboxylation rate of acetyl-CoA (●) or propionyl-CoA (○) as a function of time. Assay conditions were as described in Table I.

To determine the effect of ionic strength on the carboxylation rate, reactions were carried out at various Tris-HCl concentrations at a constant pH (8.1) and a constant KCl concentration (40 mM). The carboxylation rates were nearly identical at Tris concentrations between 20 and 100 mM; they decreased when the Tris concentration exceeded 100 mM.

Initial Velocities. The initial velocity of the HCO_3^- incorporation into acid-stable material (malonyl-CoA derivatives) was determined in the presence of ATP, Mg^{2+}, and either acetyl-CoA or propionyl-CoA. All assays were conducted under standard conditions with each component present at saturating concentrations. As shown in Figure 1, the amount of HCO_3^- fixed was proportional to the enzyme concentration over a limited range (left panel) and was nearly linear during the first 5 to 10 min of the incubation period (right panel).

Substrate Specificity. Our original aim was to isolate acetyl-CoA carboxylase from the nematode. We had, therefore, followed acetyl-CoA carboxylase activity during the isolation and purification of the enzyme. When we later tested the purified enzyme for its substrate specificity, we found that it carboxylates not only acetyl-CoA but also propionyl-CoA and butyryl-CoA. The relative carboxylation rates for these substrates were 100% for propionyl-CoA, 23% for butyryl-CoA, and 17% for acetyl-CoA.

CoA esters with acyl chains longer than four carbon atoms, such as valeryl-CoA, hexanoyl-CoA, heptanoyl-CoA, or palmitoyl-CoA, were not carboxylated by the enzyme. Moreover, CoA esters with branched acyl chains seemed to be poor substrates. Isobutyryl-CoA, for example, was carboxylated at a rate 50 times slower than that of propionyl-CoA. Not carboxylated at all by the enzyme was β-methylcrotonyl-CoA, which is the natural substrate of another class of acyl-CoA carboxylases (Moss & Lane, 1971). Taken together, these results establish that the nematode enzyme is specific for a group of straight-chain acyl-CoA esters ranging in chain length from two to four carbon atoms.

Kinetic Constants for Acyl-CoA Esters. When the apparent K_m and V_{max} values for acyl-CoA esters were determined (Table I), a plot of the initial carboxylation velocities against varied concentrations of each substrate gave a hyperbolic saturation curve. Substrate inhibition was not observed until high substrate concentrations were used. The apparent K_m values for acetyl-, propionyl-, and butyryl-CoA ranged between 0.2 and 0.4 mM. These K_m values compare well with those reported on mammalian propionyl-CoA carboxylases (Halenz et al., 1962) but are ten times higher than the values reported

TABLE I: Kinetic Constants of the *T. aceti* Acyl-CoA Carboxylase.[a]

Reactant analyzed	Assay condition	K_m (mM)	V_{max} (nmol of CO_2 fixed min^{-1} μg^{-1})
Acetyl-CoA	Standard	0.42	6.8
Propionyl-CoA	Standard	0.23	39.7
Butyryl-CoA	Standard	0.33	9.1
Isobutyryl-CoA	Standard	0.82	0.7
HCO_3^-	Standard, with propionyl-CoA	2.30	
ATP	Standard, with propionyl-CoA	0.25	
Propionyl-CoA	Minus K^+	0.21	1.7
HCO_3^-	Minus K^+, with propionyl-CoA	9.10	
ATP	Minus K^+, with propionyl-CoA	0.054	

[a] Apparent K_m and V_{max} values were determined from Lineweaver-Burk plots with all reactants at saturating levels except for the reactant being analyzed. The standard reaction mixture consisted of 80 mM Tris-HCl, pH 8.0, 40 mM KCl, 20 mM $NaH^{14}CO_3$, 1 mM ATP, 10 mM $MgCl_2$, 1.5 mM acyl-CoA, and enzyme. The amounts of enzyme used were 0.42 μg with propionyl-CoA, 3 μg with propionyl-CoA minus K^+, 1.25 μg with acetyl-CoA as well as butyryl-CoA, and 3 μg with isobutyryl-CoA. The reactions were run for 5 min.

TABLE II: Carboxylation Rates for Single and Mixed Acyl-CoA Esters.[a]

Substrate	Velocity (nmol min^{-1} μg^{-1}) Experimental	Theoretical[b]
Acetyl-CoA	5.1	
Butyryl-CoA	6.4	
Propionyl-CoA	30.9	
Acetyl-CoA + propionyl-CoA	27.0	23.6
Acetyl-CoA + butyryl-CoA	6.6	6.8
Butyryl-CoA + propionyl-CoA	24.7	22.8

[a] The reaction mixtures contained 80 mM Tris-HCl, pH 8.0, 40 mM KCl, 20 mM $NaH^{14}CO_3$, 1 mM ATP, 10 mM $MgCl_2$, 0.66 μg of enzyme, and 1.5 mM of each acyl-CoA ester listed in the table. [b] The theoretical velocities were calculated according to Webb & Morrow (1959) employing the K_m values for the substrates listed in Table I.

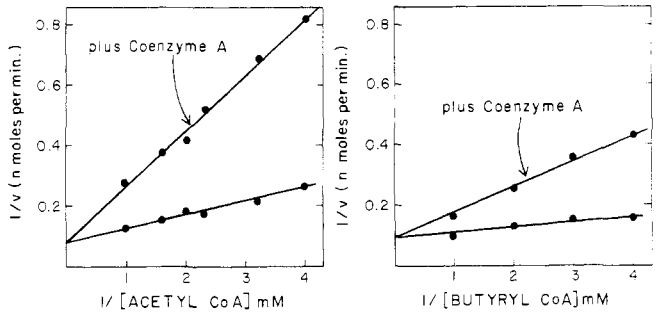

FIGURE 2: Lineweaver-Burk plots for acetyl-CoA (left) and for butyryl-CoA (right) in the presence and absence of 5 mM coenzyme A. Assay conditions were as described in Table I.

on mammalian and avian acetyl-CoA carboxylases activated by citrate (Moss & Lane, 1971).

The chain length of the substrate's acyl group seems to affect largely the maximum velocity of the carboxylation reaction (V_{max}) and only moderately affect the affinity of the substrate for the enzyme ($1/K_m$). The basis of this assumption is that the difference between the V_{max} values for acetyl-CoA and propionyl-CoA is more than fivefold, whereas the difference between the corresponding K_m values is less than twofold.

Substrate Binding Site. To determine whether a single site on the enzyme accounts for the carboxylation of the various acyl-CoA substrates, we conducted two types of experiments. First, we examined the enzyme by the "mixed substrate method" of Webb & Morrow (1959). We measured CO_2 fixation with each acetyl-, propionyl-, and butyryl-CoA alone and with two of these substrates combined. The results of these experiments revealed (Table II) that the carboxylation velocity obtained with two substrates combined is lower than the sum of the carboxylation velocities obtained with each substrate alone. The experimental values are in reasonably good agreement with the calculated values arrived at on the assumption that a single substrate binding site on the enzyme is involved.

In the second type of experiment, we studied the carboxylation of each acetyl-CoA and butyryl-CoA in the presence of coenzyme A, which gives competitive inhibition with either substrate. If the two substrates bind to the same enzyme site, as the first experiment suggests, then the inhibition constant (K_i) for coenzyme A should be the same with acetyl-CoA as with butyryl-CoA. The results corroborate this. The K_i for coenzyme A was 1.59 mM with acetyl-CoA and 1.47 mM with butyryl-CoA (Figure 2). A similar K_i value was obtained with propionyl-CoA as the substrate.

Kinetic Constants for HCO_3^-, ATP, and Mg^{2+} with and without K^+. The apparent K_m values for HCO_3^- and ATP, with propionyl-CoA as substrate, were determined from Lineweaver-Burk plots (Table I). The K_m for HCO_3^- was 2.3 mM with K^+ present. Although this value is rather high when compared with the K_m values for the other reactants, it is typical of biotin-containing carboxylases (Moss & Lane, 1971). The K_m for ATP was 0.25 mM in the presence of 10 mM $MgCl_2$ and K^+. When the ATP concentration was varied from 0.03 to 1.0 mM, the accompanying change in the velocity of the carboxylation reaction followed a hyperbolic saturation curve. At ATP concentrations above 1 mM, a Lineweaver-Burk plot curved slightly upward indicating that at these levels ATP became inhibitory for the enzyme. An increase in the ATP concentration from 1 to 10 mM caused a 10% reduction in the reaction rate.

The K_m for Mg^{2+} could not be determined since a Lineweaver-Burk plot for Mg^{2+} produced a curved line. The optimal Mg^{2+} concentration was 5 to 10 mM in the presence of 1 mM ATP and K^+. Mn^{2+} at 1 to 2 mM could substitute for Mg^{2+}, but the maximum velocity obtained with this ion was about 20% lower than that obtained with Mg^{2+}. Both metal ions inhibited the enzyme at high concentrations.

In the absence of K^+, the rate of propionyl-CoA carboxylation was reduced 30-fold (Table III). To learn more about the K^+ effect, we also determined the K_m values for propionyl-CoA, HCO_3^-, and ATP without K^+ (Table I). We found that K^+ has no effect on the K_m for propionyl-CoA. K^+, however, decreased the K_m for HCO_3^- 4-fold and increased the K_m for ATP 5-fold at both high (10 mM) and low (2.5 mM) Mg^{2+} concentrations.

Parallel experiments performed with acetyl-CoA as substrate in place of propionyl-CoA gave similar K_m values for HCO_3^- and ATP. With K^+ present, these values were comparable with the values reported for citrate-activated animal

TABLE III: Kinetic Constants of Activating Cations.[a]

Cation	Apparent K_A (mM)	V_{max} (nmol min^{-1} μg^{-1})
K^+	11.0	38.7
Rb^+	4.1	36.1
Cs^+	10.0	25.3
NH_4^+	6.7	25.0
None		1.2

[a] The apparent activation constant (K_A) and maximum velocity (V_{max}) for the carboxylation of propionyl-CoA were calculated from a double reciprocal plot of the cation concentration vs. the reaction velocity. In calculating K_A, corrections were made for the small activity observed in the absence of cations (Edwards & Keech, 1968).

acetyl-CoA carboxylases and for animal propionyl-CoA carboxylases (Moss & Lane, 1971).

Activation Constants for K^+ and Other Monovalent Cations. Since K^+-requiring enzymes are generally also activated by certain other monovalent cations (Suelter, 1970), the cation effect on the nematode enzyme was further investigated. The rate of propionyl-CoA carboxylation increased nearly hyperbolically with increasing concentrations of any one of the following cations: K^+, Rb^+, Cs^+, or NH_4^+. The maximum velocities attained by the addition of K^+ or Rb^+ were almost equal and were 30% higher than those attained by the addition of Cs^+ or NH_4^+. The cation concentration required to yield half maximum velocity (K_A) ranged from 4 to 11 mM. In terms of their affinities for the enzyme, the order of the cations was $Rb^+ > NH_4^+ > Cs^+ > K^+$ (Table III). Neither Li^+ nor Na^+ affected the enzymatic activity when present at a concentration of 40 mM, but both caused a 5–10% inhibition at 100 mM. Although the cation effect was studied in greater detail with propionyl-CoA as substrate, a similar effect was observed with acetyl-CoA and butyryl-CoA.

The specificity of K^+-requiring enzymes for monovalent cations seems to be partially governed by the cation's ionic radius (Mildvan, 1970). Cations with a larger ionic radius than K^+, such as Rb^+, Cs^+, and NH_4^+, are activators, whereas cations with a smaller ionic radius, such as Na^+ and Li^+, are not. The activation constants of the enzymes for monovalent cations are usually high; an average value of 10 mM has been reported (Suelter, 1970). Since the *T. acetic* enzyme also requires cations of a specific size and at high concentrations for activation, it fits into the group of K^+-requiring enzymes.

The Effect of K^+ on the Partial Reactions. It has been shown with carboxylases from several different sources that the carboxylation of acetyl-CoA and propionyl-CoA is a two step reaction:

$$ATP + HCO_3^- + \text{biotin-Enz} \overset{Mg^{2+}}{\rightleftharpoons} CO_2\text{-biotin-Enz} + ADP + P_i \quad (1)$$

$$CO_2\text{-biotin-Enz} + \text{acyl-CoA} \rightleftharpoons \text{biotin-Enz} + \text{carboxylated acyl-CoA} \quad (2)$$

To establish whether the *T. aceti* enzyme is similar in this respect, we examined the partial reactions involved in each step and also determined which of the two half reactions is activated by K^+. The assay conditions for the partial reactions were similar to those employed with other acyl-CoA carboxylases. All reactions were conducted at pH 8.0, except for the ATP-^{32}P exchange reaction where the pH was lowered to 7.0 in order to bring it closer to the pH optimum of this reaction (Figure 3). These experiments showed that in the presence of K^+ the nematode enzyme catalyzes the carboxylation of free biotin

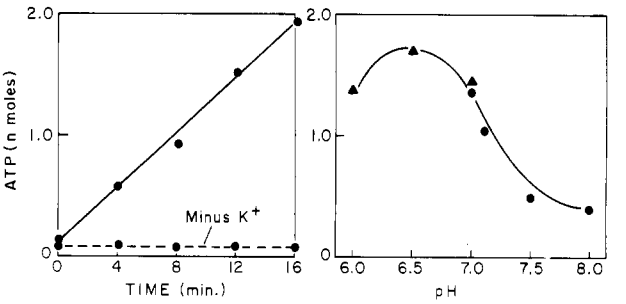

FIGURE 3: The stimulatory effect of K^+ on the ATP-^{32}P exchange reaction (left). The effect of pH on the rate of the ATP-^{32}P exchange reaction (right); the buffer used in the high pH range was Tris-HCl (●) and in the low pH range was imidazole-HCl (▲).

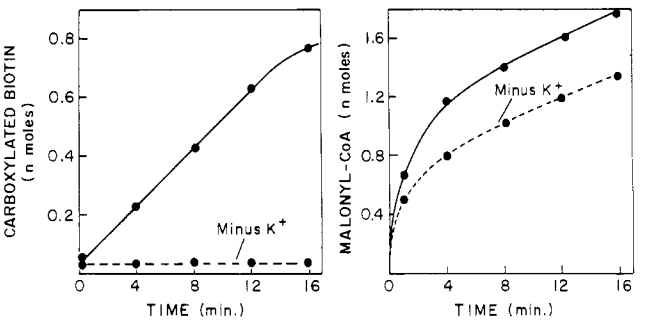

FIGURE 4: The stimulatory effect of K^+ on the carboxylation of free biotin (left) and on the [^{14}C]acetyl-CoA–malonyl-CoA exchange reaction (right). Assay conditions were as described under Experimental Procedure.

plus the ATP-^{32}P exchange reaction as part of the first half reaction, and the [^{14}C]acetyl-CoA–malonyl-CoA exchange reaction as part of the second half reaction (Figures 3 and 4). The carboxylation rate of free biotin observed with the nematode enzyme (about 4 nmol min^{-1} mg^{-1}) is comparable to the carboxylation rate of free biotin reported for avian acetyl-CoA carboxylase (Gregolin et al., 1968). But the rates of the ATP-^{32}P exchange reaction (10 nmol min^{-1} mg^{-1}) and of the [^{14}C]acetyl-CoA–malonyl-CoA exchange reaction (50 nmol min^{-1} mg^{-1}) obtained with the nematode enzyme are considerably lower than those reported for other acetyl-CoA or propionyl-CoA carboxylases (Gregolin et al., 1968; Giorgio & Plaut, 1967). One possible explanation for this discrepancy is that in our experiments an excess of enzyme had to be used to obtain measurable catalytic rates with the K^+-free controls.

In the absence of K^+, neither the ATP-^{32}P exchange reaction nor the carboxylation of free biotin proceeded at measurable rates, whereas the [^{14}C]acetyl-CoA–malonyl-CoA exchange reaction proceeded at 80% of the rate attainable in the presence of K^+. Thus K^+ primarily increases the rate of the first half of the carboxylation reaction.

Inhibition by Avidin and Sulfhydryl Reagents. Avidin inactivates biotin-containing enzymes by forming a tight complex with the biotinyl residue. Avidin also inactivated the *T. aceti* enzyme (Table IV). It inhibited, at equal concentrations, both the ATP-^{32}P exchange reaction and the [^{14}C]acetyl-CoA–malonyl-CoA exchange reaction (Table IV). This inhibition pattern is in accordance with biotin's role as a CO_2 carrier in both reactions.

Sulfhydryl reagents are also known to inhibit many acyl-CoA carboxylases. Both *N*-ethylmaleimide (MalNEt)[1] and

[1] Abbreviations used: MalNEt, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate.

TABLE IV: Enzyme Inhibition by Avidin and Sulfhydryl Reagents.[a]

Additions to preincubation mixture (per μg of enzyme)	Enzyme act. after preincubation (%) Propionyl-CoA carboxylation	ATP-^{32}P exchange	[^{14}C]Acetyl-CoA-malonyl-CoA exchange
None	100	100	100
Avidin[b]			
0.5 μg	80	48	52
10.0 μg	0	0	0
PCMB			
3 nmol	44	85	93
5 nmol	6	42	74
30 nmol	0	1	1
MalNEt			
120 nmol	39	27	34
240 nmol	12	14	9
500 nmol	2	3	2
120 nmol, propionyl-CoA	65		
120 nmol, ATP, Mg^{2+}	24		
120 nmol, ATP, Mg^{2+}, HCO_3^-	16		

[a] The buffered enzyme solutions containing 40 mM KCl were preincubated at 22 °C for 20 min with the compounds listed in the table and were then assayed for the remaining enzyme activity. In those experiments where propionyl-CoA, ATP, Mg^{2+}, or HCO_3^- was added to the preincubation mixtures, the inhibitor, MalNEt, was added last; the concentrations of the substrate and cofactors in the preincubation mixtures were those required for the final assay. [b] One milligram of avidin contained 12.8 units.

TABLE V: Subcellular Distribution of Acyl-CoA Carboxylase in *T. aceti*.[a]

Cell fraction	Distribution of enzyme act. (%) Succinate dehydrogenase[b]	Acetyl-CoA carboxylase	Propionyl-CoA carboxylase
Nuclei + cell debris (750*g*)	6	8	7
Mitochondria (17 000*g*)	78	4	3
Microsomes (30 000*g*)	3	4	3
Microsomes (70 000*g*)	3	10	8
Cytosol (supernatant)	10	74	72

[a] Freshly harvested organisms in isotonic solution were homogenized in a glass tissue grinder and subjected to differential centrifugation (for details, see Experimental Procedure). [b] Determined by measuring the reduction of $K_3Fe(CN)_6$ (Bonner, 1955).

p-chloromercuribenzoate (PCMB) inactivated the *T. aceti* enzyme at levels similar to those reported to be inhibitory for other acetyl- and propionyl-CoA carboxylases (Moss & Lane, 1971). Preincubation of the nematode enzyme with substrate (propionyl-CoA) partially protected the enzyme against the action of sulfhydryl reagents (Table IV). On the other hand, preincubation with ATP, Mg^{2+} and HCO_3^- promoted inactivation by these reagents (Table IV). The same pattern of protection by propionyl-CoA was observed by other investigators in studies of bovine and porcine propionyl-CoA carboxylases (Hegre & Lane, 1966; Edwards & Keech, 1968) and was taken as an indication that at least one essential sulfhydryl group is located at or near the substrate binding site of the enzyme. In the case of the nematode enzyme, however, both the transcarboxylation reaction and the ATP-^{32}P exchange reaction were equally susceptible to sulfhydryl reagents (Table IV). This could imply that two sulfhydryl groups are involved in the overall carboxylation reaction, one in the first half reaction and the other in the second half reaction. Another possibility is that only one sulfhydryl group participates in the overall reaction, but that it lies close to both the substrate binding site and the biotinyl prosthetic group of the enzyme. Consequently, any chemical alteration of this group would affect both partial reactions.

Carboxylation Products. The carboxylation products of acetyl-CoA and propionyl-CoA, labeled by $H^{14}CO_3^-$ fixation, cochromatographed with authentic malonyl-CoA and methylmalonyl-CoA, respectively, in the solvent systems: ethanol/0.1 M potassium acetate, pH 4.5 (1:1), and butanol/acetic acid/H_2O (5:2:3) at 4 °C. The two free acids obtained by alkaline hydrolysis of the corresponding thioesters cochromatographed with authentic malonic acid and methylmalonic acid respectively in the solvent systems: ethanol/NH_4OH/H_2O (8:1:1) and ethyl ether/acetic acid/H_2O (13:3:1) (Denison & Phares, 1952). Malonic acid and methylmalonic acid were the only radioactive spots detected on the chromatograms. We conclude from these results that the nematode enzyme fixes HCO_3^- to the α-carbon atom of acyl thioesters to form the corresponding malonyl-CoA derivatives.

Intracellular Localization of the Enzyme. In accordance with their physiological functions, mammalian acyl-CoA carboxylases are associated with specific subcellular fractions. Acetyl-CoA carboxylase, for example, is located in the cytosol, whereas propionyl-CoA carboxylase is located in the mitochondria (Halenz et al., 1962; Kaziro & Ochoa, 1964). In view of this and the relatively broad substrate specificity of the nematode carboxylase, it seemed desirable to investigate the intracellular location of the enzyme in *T. aceti*.

The tissue fractions of *T. aceti* were prepared according to established procedures (Rothstein et al., 1970). After the organisms were broken up under osmotically controlled conditions, the homogenate was resolved by differential centrifugation into a mitochondrial, a microsomal, and a cytoplasmic fraction. Each subcellular fraction was then assayed for the presence of carboxylase activity using both acetyl-CoA and propionyl-CoA as substrate. As a check for the extent of cross contamination, each fraction was, in addition, assayed for the presence of the mitochondrial marker enzyme succinate dehydrogenase.

As illustrated in Table V, the carboxylase activity was highest in the cytoplasmic fraction and lowest in the mitochondrial fraction, regardless of whether the activity was assayed with acetyl-CoA or propionyl-CoA as substrate. These preliminary findings strongly indicate that the acyl-CoA carboxylase of *T. aceti* is located in the cytoplasm and not in the mitochondria.

Discussion

It has been shown in these studies that the *T. aceti* acyl-CoA carboxylase requires K^+ for full activity. Propionyl-CoA carboxylases from rat, cow, and pig also require K^+ for full activity (Moss & Lane, 1971). Microbial propionyl-CoA carboxylases, by contrast, do not seem to require K^+ (Alberts & Vegelos, 1972). Likewise, all acetyl-CoA carboxylases, with the exception of one plant enzyme (Nielsen & Stumpf, 1976), do not require K^+ for activation. The modes by which K^+ activates both the mammalin propionyl-CoA carboxylases and the nematode enzyme seem to be comparable. This can, in part, be inferred from similarities in their kinetic properties. With the porcine enzyme, K^+ decreases the K_m for HCO_3^- (Ed-

wards & Keech, 1968) and with the bovine enzyme, K^+ increases the rate of the ATP-^{32}P exchange reaction (Giorgio & Plaut, 1967).

Apart from the mammalian propionyl-CoA carboxylases, several other classes of enzymes catalyzing different types of reactions are activated by monovalent cations (Suelter, 1970; Mildvan, 1970). The chemical basis of this activation is still unknown. For some time, it has been thought that K^+ induces a fairly broad conformational change in the enzyme (Evance & Sorger, 1966). More recent experiments, however, with enzymes catalyzing transphosphorylation reactions, such as pyruvate kinase (Nowark, 1976) and pyruvate carboxylase (McClure et al., 1971; Scrutton, 1974), have provided evidence that K^+ plays a more direct role in catalysis. In these cases, the monovalent cation appears to bind within the catalytic center of the enzyme, where it presumably forms a ternary complex with the enzyme and the substrate. Pyruvate carboxylase is of particular interest here, because it has several features in common with the nematode enzyme. Both enzymes catalyze the K^+-dependent ATP-^{32}P exchange reaction. Moreover, in both cases, K^+ alters the apparent K_m values for HCO_3^- and ATP. All these data suggest that K^+ also plays a direct role in the reactions catalyzed by the nematode enzyme by changing either the enzyme's conformation or charge distribution at the site where the biotinyl residue is being carboxylated.

Although the nematode enzyme has almost the same affinities for acetyl-CoA, propionyl-CoA and butyryl-CoA, it carboxylates these substrates at different rates (acetyl-CoA 17%, propionyl-CoA 100%, and butyryl-CoA 23%). The finding that the enzyme is most active with propionyl-CoA as substrate was somewhat unexpected because it was acetyl-CoA carboxylase activity that we had followed throughout the isolation and purification process. It is unlikely that the relatively broad substrate specificity of the enzyme is due to its contamination by other carboxylases. The nematode enzyme is in a highly purified state; furthermore studies on the binding sites for the three substrates have demonstrated that all three substrates compete for the same site on the enzyme.

As to the physiological role of the carboxylase in *T. aceti*—in particular the question of whether the enzyme functions as propionyl-CoA carboxylase, acetyl-CoA carboxylase or both—we can presently only make conjectures. One of the difficulties is that the nematode enzyme embodies features which are in part typical of propionyl-CoA carboxylases and in part typical of acetyl-CoA carboxylases. For instance, the enzyme's preference for propionyl-CoA as substrate and its requirement for K^+ are typical properties of animal propionyl-CoA carboxylases (Edwards & Keech, 1968; Giorgio & Plaut, 1967), whereas the enzyme's extramitochondrial location is characteristic of acetyl-CoA carboxylases. The finding that the enzyme is more active with propionyl-CoA as the substrate does not necessarily negate the possibility that it also functions as acetyl-CoA carboxylase, especially since its level of activity as acetyl-CoA carboxylase in crude nematode extracts (312 units per 100 g of wet worms) is as high as that of acetyl-CoA carboxylase in rat liver extracts (252 units per 100 g of liver tissue; Inoue & Lowenstein, 1972).

Additional clues about the enzyme's role in *T. aceti* can be found in the organism's metabolic activities. *T. acetic* synthesizes long-chain fatty acids de novo from acetate (Rothstein & Götz, 1968) and, therefore, must have the enzyme to generate malonyl-CoA.[2] Gluconogenic and anaplerotic reactions are other metabolic activities in which the acyl-CoA carboxylase may be involved. *T. acet* lives in an environment rich in short-chain fatty acids and, accordingly, has all the enzymes of the glyoxylate cycle for the utilization of acetate (Rothstein & Mayoh, 1966). To be able to utilize propionate, the organism most likely also has an enzyme system which converts propionyl-CoA to succinyl-CoA via methylmalonyl-CoA. Such a system occurs widely in nature and plays an important role in the energy metabolism of ruminants (Kaziro & Ochoa, 1964).

In light of these inferences, the enzyme's broad substrate specificity may not be an accidental trait, but rather an essential quality enabling the enzyme to participate in several metabolic processes. If this idea is valid, then it must be assumed that stringent control mechanisms exist for maintaining the proper flow of the different substrates. With the purified enzyme in hand and its basic properties worked out, inquiries into some of these problems have now become practical.

[2] We have looked for, but not found, any other enzyme which has acetyl-CoA carboxylase activity. Nevertheless, we cannot fully rule out the possibility that such an enzyme exists in a form so unstable as to escape detection.

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