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Total Synthesis of Acetate from CO_2

II. Purification and Properties of Formyltetrahydrofolate Synthetase from *Clostridium thermoaceticum*

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Formyltetrahydrofolate synthetase from *Clostridium thermoaceticum* had optimal activity at 55 to 60 C. K_m values were: adenosine triphosphate (ATP), 6.25×10^{-4} M; formate, 1.67×10^{-3} M; and tetrahydrofolate, 1.00×10^{-3} M; at *p*H 8.0. There was a requirement for divalent metals and specifically for ATP.

The overall mechanism of the total synthesis of acetate from CO_2 by *Clostridium thermoaceticum* is gradually being defined (5, 7) and may occur as outlined in reactions 1 to 8, in which corrinoid-E is an enzyme-corrinoid complex, THF is tetrahydrofolate, ADP is adenosine diphosphate, P_i is inorganic phosphate, NADP is nicotinamide adenine dinucleotide phosphate, and NADPH is reduced nicotinamide adenine dinucleotide phosphate.

$$4\ \text{Pyruvate} + 4\ \text{ADP} + 4\ P_i + 4\ \text{NADP} \rightarrow 4\ \text{acetate} + 4\ CO_2 + 4\ \text{ATP} + 4\ \text{NADPH} + 4\ H^+ \quad (1)$$

$$CO_2 + \text{NADPH} + H^+ \rightleftarrows \text{HCOOH} + \text{NADP}^+ \quad (2)$$

$$\text{THF} + \text{HCOOH} + \text{ATP} \rightleftarrows \text{formyl-THF} + \text{ADP} + P_i \quad (3)$$

$$\text{Formyl-THF} + 2\ \text{NADPH} + 2\ H^+ \rightarrow\rightarrow\rightarrow CH_3\text{-THF} + 2\ \text{NADP}^+ \quad (4)$$

$$CH_3\text{-THF} + \text{corrinoid-E} \rightarrow \text{THF} + CH_3\text{-corrinoid-E} \quad (5)$$

$$CO_2 + CH_3\text{-corrinoid-E} + \text{ATP} \rightarrow \text{HOOC-}CH_2\text{-corrinoid-E} + \text{ADP} + P_i \quad (6)$$

$$\text{HOOC-}CH_2\text{-corrinoid-E} + \text{NADPH} + H^+ \rightarrow \text{acetate} + \text{corrinoid-E} + \text{NADP}^+ \quad (7)$$

$$\text{Sum: 4 pyruvate} + 2\ \text{ADP} + 2\ P_i \rightarrow 5\ \text{acetate} + 2\ CO_2 + 2\ \text{ATP}. \quad (8)$$

The most important evidence for this sequence is the following. CO_2 is converted to a methyl which is linked to the cobalt of corrinoids (1, 4), and this methyl is converted to the methyl of acetate (4, 6) in fermentations of pyruvate by extracts of *C. thermoaceticum*. Furthermore, $^{14}CH_3$-THF is converted to the methyl of acetate under similar conditions (5), and, in addition, carboxymethyl-B_{12} is reduced to acetate (4) (reactions 5 to 7). Nevertheless, none of the enzymes involved in these transformations has been isolated and purified extensively. Li et al. (3) purified an NADPH-dependent formate dehydrogenase from *C. thermoaceticum* and showed that this enzyme catalyzes an exchange of CO_2 with formate, but they were unable to demonstrate the net conversion of CO_2 to formate. We now wish to report on the purification of formyltetrahydrofolate synthetase (EC 6.3.4.3) from *C. thermoaceticum*. This enzyme, which catalyzes reaction 3, may play an important role in the synthesis of acetate by coupling with formate dehydrogenase (reaction 2) and may thus provide, through the energy of the adenosinetriphosphate (ATP), a system favorable for the net synthesis of formyltetrahydrofolate from CO_2, NADPH, ATP, and THF.

The enzyme has been purified as described in Table 1 and assayed with procedures similar to those of Rabinowitz and Pricer (8). The assay mixture contained 0.1 M triethanolamine buffer (*p*H 8), 0.2 M mecaptoethanol, 10 mM $MgCl_2$, 2.3 mM DL-tetrahydrofolate, 5 mM ATP, 10 mM formate, and the enzyme. After 5 min at 55 C, the reaction was stopped by addition of 2.0 ml of 2% perchloric acid; the mixture was centrifuged, and the absorbancy of the supernatant liquid was measured at 355 nm after 30 min at room temperature. The product, N^{10}-formyltetrahydrofolate, is converted in acid to N^5,N^{10}-methenyltetrahydrofolate, $\epsilon = 22 \times 10^3$ at 355 nm. The identity of the product from ^{14}C-formate was confirmed by chromatography and by the spectrum of the ^{14}C-compound. A unit of enzyme activity is expressed throughout as micromoles of product per minute, and specific activity is expressed as units per milligram of enzyme. On centrifugation (Fig. 1), the enzyme from step VI (Table 1) gave a single peak with an $S_{20,w}$ value of 10.2. The value is somewhat higher than the 9.25 found for the enzyme from other clostridia

TABLE 1. *Purification of formyltetrahydrofolate synthetase from C. thermoaceticum*[a]

Step no.	Procedure	Vol	Protein content	Total protein	Specific activity	Total activity	Recovery
		ml	*mg/ml*	*mg*	*units/mg*	*units*	%
I	Cell-free extract	68	25.7	1,750	3.48	6,000	100
II	Heat treatment	65.5	20	1,310	4.51	5,925	98.6
III	$(NH_4)_2SO_4$ fractionation (40–70%)	24	29	695	6.40	4,460	71.2
IV	TEAE column chromatography	10	8.40	84	22.1	1,850	30.8
V	First Sephadex G-100	14	1.1	15.1	80	1,210	22.2
VI	Second Sephadex G-100	4	1.8	7.2	108.5	730	13.0

[a] (I) The cells were grown for 4 days at 55 C as described by Li et al. (2), centrifuged (9.2 g, wet weight), and washed in 50 mM maleate-50 mM mercaptoethanol buffer (*p*H 7). The suspension (1 g of cells per 8 ml of this buffer) was passed twice through a French Press at 0 C and then centrifuged at 37,000 × *g*. (II) The solution was heated at 55 C for 15 min, cooled, and centrifuged. (III) The protein was fractionated by the addition of solid $(NH_4)_2SO_4$ and the fraction between 40 and 70% saturation was dissolved in the 50 mM maleate-50 mM mercaptoethanol buffer. (IV) The enzyme was dialyzed for 18 hr against 5 mM maleate-5 mM mercaptoethanol buffer (*p*H 7) at 4 C and placed on a column (4.5 by 48 cm) made up of an equal mixture of triethylaminoethyl (TEAE, type 63) and Celite no. 545. It was eluted with the same buffer and began to appear after 640 ml had passed through the column. The enzyme was precipitated with 70% saturated $(NH_4)_2SO_4$, and the precipitate was dissolved in 50 mM maleate-50 mM mercaptoethanol buffer, pH 7. (V) The enzyme was passed through a Sephadex G-100 column (2 by 40 cm) equilibrated with 5 mM maleate-5 mM mercaptoethanol buffer (*p*H 7). (VI) Fractions 5 and 6 (14 ml) were passed through a second Sephadex G-100 column (2.0 by 52 cm) and fractions 6 to 8 were concentrated with 3 volumes of saturated $(NH_4)_2SO_4$, *p*H 7, at 0 C. The precipitate was dissolved in 50 mM maleate buffer (*p*H 7).

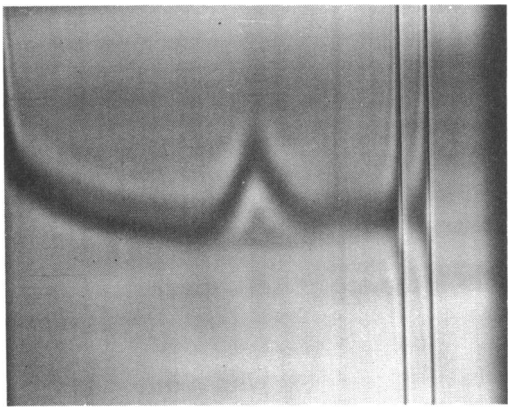

FIG. 1. *Sedimentation pattern of formyltetrahydrofolate synthetase. The sedimentation measurements were made with 1.8 mg of protein per ml in 0.005 M maleate buffer at pH 7.0 (with 0.005 M 2-mercaptoethanol), at a rotor speed of 59,780 rev/min, an average temperature of 5.1 C, and a bar angle of 35°. A single peak was indicated throughout the centrifugation. The exposure shown was at 44 min. The* $S_{20,w}$ = *10.2.*

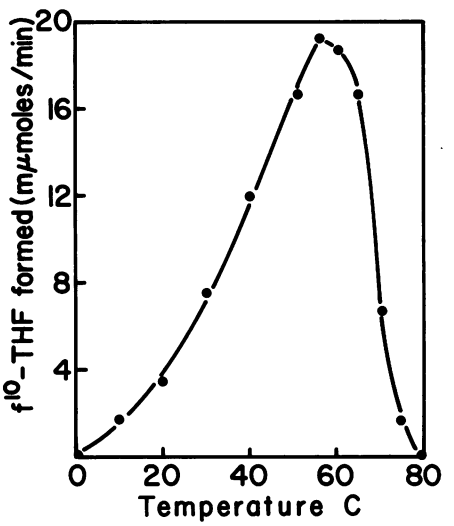

FIG. 2. *Effect of temperature on the initial rate of enzyme activity of formyltetrahydrofolate synthetase from C. thermoaceticum. Conditions were those used for assaying the enzyme except that the temperature was varied.*

(2). On electrophoresis on cellulose acetate, it moved as a single band, 1.2 cm toward the cathode at *p*H 6.2 (50 mM phosphate) and 30 volts cm^{-1} for 1 hr and towards the anode 4.2 cm at *p*H 9 [50 mM tris(hydroxymethyl)aminomethane (Tris) hydrochloride] and 33 volts cm^{-1} for 30 min. The enzyme, like formate dehydrogenase (3) and the acetate synthesis enzymes (4), is "thermophilic" and is most active at about 55 C (Fig. 2).

The enzyme is active over a wide range of *p*H (7.0 to 9.5), having somewhat higher activity at about *p*H 8.0. The activity was higher with triethanolamine buffer than with Tris buffer; phosphate was inhibitory. The enzyme was unstable

at low ionic strength. The apparent K_m values at 55 C and *p*H 8 were 6.25×10^{-4} M for ATP, 1.67×10^{-3} M for formate, and 10^{-3} M for tetrahydrofolate. A divalent ion is required; at 5 mM, Mg^{2+} was the most active, Mn^{2+} and Ca^{2+} were less effective, and Zn^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+} had little or no effect. Ethylenediamine tetraacetic acid (10 mM) caused a 94% inhibition under conditions of the usual assay. Uridine triphosphate, guanosine triphosphate, ADP, and adenosine monophosphate would not substitute for ATP. Similar properties have been reported for the synthetase from other clostridia (2). However, the enzyme from *C. thermoaceticum* has a higher temperature optimum and a lower apparent K_m for formate. The latter property may make it feasible for the enzyme to couple with formate dehydrogenase to form formyltetrahydrofolate from CO_2 in the synthesis of acetate. The specific activity (micromoles per min per mg of protein) of the enzyme preparation from *C. thermoaceticum* at 55 C was 108 compared to values of about 400 at 37 C for the enzyme from other clostridia (8). L. Ljungdahl (*unpublished data*) recently obtained values of about 400 for the enzyme from *C. thermoaceticum*.

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