THE ROLE OF COFACTORS AND ENZYME-BOUND BIOTIN IN THE CO_2-PYRUVATE EXCHANGE REACTIONS

C. W. Shuster[1] and F. Lynen

Max-Planck-Institut für Zellchemie, München, and Chemisches Laboratorium der Universität, München, Institut für Biochemie

Received September 14, 1960

Cell free extracts of several anaerobic bacteria catalyze a rapid exchange between $C^{14}O_2$ and the carboxyl group of pyruvic acid. Previous studies have shown that the exchange involves several reaction steps with Clostridium butylicum extracts and may be associated with the phosphoroclastic reaction producing molecular hydrogen, acetyl-phosphate and CO_2 from pyruvate (Wolf and O'Kane, 1955, 1953). A rapid incorporation of $C^{14}O_2$ into pyruvate is also observed with fractions from Clostridium kluyveri in the absence of either the phosphoroclastic reaction or acetoin formation. In view of the established role of enzyme-bound biotin in carboxylation (Lynen et al., 1959) and transcarboxylation reactions (Swick and Wood, 1960), experiments have been designed to determine a possible mechanism of CO_2 activation in anaerobic α-ketoacid metabolism.

The incorporation of $C^{14}O_2$ into pyruvate with extracts and fractions of C. kluyveri was measured by a method similar

1 Postdoctoral Fellow of the National Science Foundation. Present address: Department of Bacteriology, University of California, Berkeley, California.

to that described previously (Wolf and O'Kane, 1955). The activity observed in crude extracts varied greatly among different cell preparations, dried cell preparations possessing less than 20% of the activity observed with extracts of freshly harvested cells. A protein fraction was obtained by precipitation with acid ammonium sulfate after dialysis of the crude extract for 24 hours against 0.05 M K-maleate buffer, pH 6.9, containing 0.005 M Versene. The active proteins precipitated between 20 and 60% ammonium sulfate saturation. This fraction, after further dialysis against maleate-Versene buffer for 36 hours with several changes of solution, catalyzes no exchange activity in the absence of added cofactors. Activity is partially restored, 70 to 80% by the addition of thiamine-pyrophosphate (TPP), adenosine triphosphate (ATP) and magnesium ion (Table I). The enzyme fractions are fully saturated with respect to TPP at 10^{-5} M and with respect to ATP at 10^{-4} M.

Table I

CO_2-Pyruvate Exchange: Cofactor Effects

	C.P.M. in Pyruvate	% Exchange
Complete	4023	5.03
" - TPP	432	0.54
" - ATP	960	1.19
" - TPP - ADP	106	0.13
" - $MgCl_2$	265	0.33

Each tube contained (μ Moles/0.5 ml): Na-pyruvate, 50; $KHC^{14}O_3$, 50 (80,000 c.p.m.); TPP, 0.25; ATP, 1.0; $MgCl_2$, 5; Mg, K_2-Versenate, 5; Na_2S, 1; K-maleate buffer, pH 6.9, 100; C. kluyveri fraction, 1 mg. protein; 30 minutes at 35°.

Attempts to resolve protein fractions of TPP by alkaline treatment resulted in enzyme inactivation, but it was found that an absolute TPP requirement could be demonstrated after re-

peated dialysis against neutral Versene solutions. The role of TPP has been implicated previously in the exchange reaction with C. butylicum, but in contrast to the C. kluyveri system, no ATP requirement could be demonstrated (Mortlock, Valentine, and Wolf, 1959). An additional point of difference lies in the fact that the reaction catalyzed by C. kluyveri extracts is not stimulated by the additions of either orthophosphate or coenzyme A.

Enzyme fractions rapidly lost activity with time and during fractionation apparently due to exposure to atmospheric oxygen. Only partial reactivation was achieved by adding low concentrations of Na_2S (10^{-3} M) to the reaction mixture; other thiol reducing agents including coenzyme A were found to be inhibitory.

The role of enzyme-bound biotin in the exchange reaction is suggested by the fact that the enzyme is completely inhibited by avidin in the presence of saturating concentrations of co-factors. When trace amounts of biotin (0.1 μM) were pre-incubated with avidin, no inhibition is observed (Table II).

Table II

Inhibition of $C^{14}O_2$-Pyruvate Exchange by Avidin

	$C^{14}O_2$ incorporated into Pyruvate C. P. M	
		+ Biotin, 0.1 μM
Complete system	2974	2643
" " + Avidin 0.01 units	836	2654
" " + Avidin 0.03 units	434	2476
" " + Avidin 0.06 units	174	2200

Each tube contained (μ Moles/0.5 ml): Na-pyruvate, 50; $KHC^{14}O_3$, 50 (80,000 c.p.m.); TPP, 0.25; ATP, 1.0; $MgCl_2$, 5; Mg,K_2-Versenate, 5; Na_2S, 0.1; K-maleate buffer, pH 6.9, 100; C. kluyveri fraction, 1 mg. protein; 30 minutes at 35°.

The addition of biotin alone did not stimulate the exchange activity, and higher concentrations (10^{-3}M) proved to be inhibitory.

With the implication of a biotin-linked reaction step, a correlation exists between cofactor effects of the CO_2-pyruvate exchange reaction and the established mechanism of biotin action in the carboxylation of methylcrotonyl CoA (Lynen et al., 1959). In view of these observations, the overall incorporation of $C^{14}O_2$ into pyruvate can be visualized by the following reaction mechanism:

1. Pyruvate + TPP $\rightleftharpoons$ "α-TPP-lactic acid"[1]
2. "α-TPP-lactic acid" + Biotin-Enzyme $\rightleftharpoons$ "α-hydroxyethal-TPP" + CO_2-Biotin-Enzyme
3. CO_2-Biotin-Enzyme + ADP + Pi $\rightleftharpoons$ ATP + Biotin-Enzyme + CO_2

---

4. Sum: Pyruvate + TPP + ADP + Pi $\rightleftharpoons$ "α-hydroxyethyl-TPP" + CO_2 + ATP

The intermediate formation of the thiamine compounds in Reactions 1 and 2 can only be presumed. These compounds, "α-TPP-lactic acid" and "α-hydroxyethyl-TPP", have been proposed and isolated as intermediates in thiamine-linked cleavage reactions of pyruvate (Breslow, 1957, 1958; Krampitz et al., 1958; Holzer, 1959). The proposed structure of the "α-TPP-lactic acid" intermediate is electronically analogous to a ß-keto acid (Metzler, 1960) rendering the carboxyl group susceptible to nucleophilic attack by biotin. The CO_2 acceptor function of biotin in Reaction 2 is entirely analogous to its role in the transformations of ß-methylglutaconyl CoA (Lynen et al., 1959), oxalacetate (Swick and Wood, 1959), and methylmalonyl CoA (Stadtman et al., 1960; Kaziro, Leone, and Ochoa, 1960). The function of ATP in CO_2 activation, Reaction 3, is entirely similar to that in the carboxylation of methylcrotonyl CoA

1 α-Pyrophosphoryl-thiaminyl-lactic acid

and propionyl CoA.

In addition to the properties described, some evidence was obtained for an alternate mechanism replacing Reaction 3. It was found that trace amounts of caproyl CoA (10^{-6} M) together with acetyl CoA (10^{-5} M) enhance the rate of exchange in the absence of either ATP or inorganic phosphate, Table III.

Table III

The Effects of Caproyl CoA and Acetyl CoA on the CO_2-Pyruvate Exchange

	$C^{14}O_2$ incorporated into Pyruvate C. P. M.
Complete	1900
" - TPP	542
" - Caproyl CoA	321
" - Acetyl CoA	470
" - Caproyl CoA - Acetyl CoA	127

Each tube contained (μ Moles/0.5 ml): Na-Pyruvate, 50; $K_2HC^{14}O_3$, 50 (67,000 c.p.m.); TPP, 0.25; Caproyl CoA, 2 X 10^{-3}; Acetyl CoA, 0.02; $MgCl_2$, 5; Mg,K_2-Versenate, 5; K-Maleate buffer, pH 6.9, 100; C. kluyveri fraction, 1 mg. protein; 60 minutes at 35°.

Although the extent of the stimulation is minor in comparison with the ATP effect, the data suggest the presence of a still undisclosed mechanism, possibly involving a transcarboxylation sequence, for the activation of CO_2 and carboxylation of the biotin enzyme. It is perhaps significant that caproyl CoA is also required for the exchange between the carboxyl group of malonyl CoA and CO_2 observed in extracts from the same organism (Vagelos, 1959).

References

Wolf, R. S., and O'Kane, D. J. (1955) J. Biol. Chem. 215, 637.

Wolf, R. S., and O'Kane, D. J. (1953) J. Biol. Chem. 205, 755.

Lynen, F., Knappe, J., Lorch, E., Jütting, G., and Ringelmann, E. (1959) Angew. Chem. 71, 481.

Swick, R. W., and Wood, H. G. (1960) Proc. Natl. Acad. Sci. U.S. 46, 28.

Mortlock, R. P., Valentine, R. C., and Wolf, R. S. (1959), J. Biol. Chem. 234, 1653.

Breslow, R. (1957) Chemistry and Industry, 892; (1958) J. Am. Chem. Soc. 80, 3719.

Krampitz, L. O., Greull, G., Miller, C. S., Bicking, J. B., Skeggs, H. R., and Sprague, J. M. (1958) J. Am. Chem. Soc. 80, 5893.

Holzer, H., and Beaucamp, K. (1959) Angew. Chem. 71, 776.

Metzler, D. E., in The Enzymes, II, (Boyer, P. D., Lardy, H., and Myrbäck, K., eds.), Academic Press Inc., New York 1960. p. 330.

Stadtman, E. R., Overath, P., Eggerer, H., and Lynen, F. (1960) Biochem. Biophys. Res. Comm., 2, 1.

Kaziro, Y., Leone, E., and Ochoa, S., Abstracts of the 137th Meeting of the American Chemical Society, Cleveland, 1960, p. 39 C.

Vagelos, P. R. (1959) J. Am. Chem. Soc. 81, 4119.