# EXISTENCE OF TWO MALIC ENZYMES IN ESCHERICHIA COLI

H. Katsuki, K. Takeo, K. Kameda and S. Tanaka

Department of Chemistry, Faculty of Science
Kyoto University, Kyoto, Japan

Received March 27, 1967

Malic enzyme is known to be widely distributed in living organisms. It is also established that the enzymes from animal and plant require TPN as coenzyme. With microorganisms including E. coli, many papers have appeared demonstrating TPN-requiring enzyme (Parvin et al., 1964; Ashworth et al., 1965). On the other hand, the DPN-requiring enzyme was found only in some lactic acid bacteria grown on malate medium (Korkes and Ochoa, 1948; Duerre and Lichstein, 1961) and in Shizosaccharomyces pombe grown on grape juice medium (Temperli et al., 1965). Although Saz and Hubbard (1957) reported that the enzyme from Ascaris lumbricoides responded to TPN as well as DPN, it was due to the low specificity of the enzyme in its coenzyme requirement. No paper seems to have appeared demonstrating the existence of two types of the enzyme in one organism.

The present paper describes the evidence which shows that E. coli W has two malic enzymes requiring DPN and TPN, respectively, and also that both activities are differently affected by the growth conditions of the cells.

DPN-linked malic enzyme: The cells were grown with shaking at 30°C on the "enriched medium" (see Table III). The cells, harvested at late logarithmic growth phase, were suspended in two

volumes of 0.25M Tris-HCl buffer, pH 7.4. They were disrupted by sonic disintegration at 10K cycles for 10 min., and the mixture was centrifuged at 24,000 x G for 20 min. The "crude extract" obtained was fractionally precipitated with ammonium sulfate (33-44%). The precipitate was suspended in 0.05M Tris-HCl buffer, pH 7.4, and it was dialyzed overnight at 4°C against 0.01M Tris-HCl buffer, pH 7.4 (= 1st A. S. fraction). After treatment with 1% protamine sulfate (0.1mg/mg of protein), the supernatant obtained was again fractionally precipitated with ammonium sulfate (36-41%). The precipitate was dissolved in Tris-HCl buffer and dialyzed as described before (= 2nd A. S. fraction). After adjusting to pH 6.0, it was treated with calcium phosphate gel (1.5mg/mg of protein) and after the gel was collected by centrifugation it was washed with 0.01M phosphate buffer, pH 6.0, 6.5 and 7.0, successively. Malic enzyme was eluted with 0.05M phosphate buffer, pH 7.0 (= gel fraction). Specific activities of DPN- and TPN-linked malic enzymes, and of malic dehydrogenase and their ratios to each other throughout these steps are shown in Table I.

DPN-linked malic enzyme was purified about 28 fold. Although the enzyme preparation in the last step still had slight activities of malic dehydrogenase and of TPN-linked malic enzyme, it is evident that the activity of DPN-linked malic enzyme was due to neither of them.

The DPN-linked decarboxylation of malate was found to proceed stoichiometrically. By incubating the reaction mixture for 12 min. at 30°C , 0.24 μmole of pyruvate was formed under the simultaneous reduction of 0.19 μmole of DPN. By another incubation for 40 min., malate gave rise to 2.31 μmoles of pyruvate and 2.01 μmoles of carbon dioxide. The confirmation of pyruvate

as the reaction product was carried out by cochromatography of its 2,4-dinitrophenylhydrazone derivative with the authentic sample on paper.

Table I Purification of DPN-linked Malic Enzyme

| Fraction | Specific activity (units/mg of protein) | | | Protein | (D)/(M) | (D)/(T) |
|---|---|---|---|---|---|---|
| | (D) DPN-linked malic enzyme | (M) Malic dehydrogenase | (T) TPN-linked malic enzyme | mg | | |
| Crude extract* | 21.1 | 1930 | 95.8 | 5250 | 0.01 | 0.48 |
| 1st A. S. fraction | 79.8 | 958 | 45.3 | 1430 | 0.08 | 0.83 |
| 2nd A. S. fraction | 288 | 103 | | 204 | 2.8 | |
| Gel fraction | 590 | 6 | 63 | 15.8 | 100 | 9.2 |

The enzyme activities of malic enzymes were determined by the usual spectrophotometric method. The reaction mixture for the assay of malic enzymes contained the following constituents ($\mu$moles in 3 ml): potassium L-malate, 15; $MnSO_4$, 3; DPN or TPN, 0.2; Tris-HCl buffer, 250; and the enzyme. Malic dehydrogenase was assayed by the usual method (Ochoa, S., 1955). The reactions were carried out at 30°C and one enzyme unit was defined as the amount of enzyme which caused an increase (or decrease) of 0.001 per min. in optical density at 340 m$\mu$.

* The activity in the "crude extract" was obtained by summing up the activities in three fractions obtained by ammonium sulfate fractionation (22-33%, 33-44% and 44-90%).

TPN-linked malic enzyme: The TPN-linked enzyme was extracted from the cells grown on the "minimal malate medium" (see Table III) and purified as follows. The crude extract was fractionally precipitated with ammonium sulfate (44-90%). The precipitate was dissolved in 0.05M Tris-HCl buffer, pH 7.4, and it was dialyzed as described before (= "A. S. fraction"). The "A. S. fraction"

contained no activity of DPN-linked malic enzyme and of TPN-linked malate dehydrogenase as shown in Table II. It is evident that a TPN-linked malic enzyme exists in this organism. This was also reported by Ashworth et al. (1965). The evidence that the oxidative decarboxylation of malate, linked with TPN, proceeds stoichiometrically was shown using the "A. S. fraction". Under the evolution of 1.42 μmoles of $CO_2$, 1.59 μmoles of pyruvate was formed as the reaction products.

Table II Purification of TPN-linked Malic Enzyme

| | Specific activity (units/mg of protein) | | | | Protein |
|---|---|---|---|---|---|
| | TPN-linked malic enzyme | DPN-linked malic enzyme | Malic dehydrogenase DPN-linked | Malic dehydrogenase TPN-linked | mg |
| Crude extract | 206 | 42.1 | 1720 | | 480 |
| A. S. fraction | 329 | 0 | 4470 | 0 | 174 |

The reaction conditions were the same as described in Table I.

Behaviors of the two malic enzymes: The two malic enzymes seem to be differently influenced by some metabolites and inhibitors from each other. For instance, oxaloacetate inhibited strongly the TPN-linked enzyme (95%) at $6.7 \times 10^{-4}$M, but the DPN-linked enzyme was inhibited only slightly (37%). These results together with the data shown above, support the existence of two malic enzymes in E. coli.

The data in Table III show the activity variation of two malic enzymes depending on the media on which the organism was grown. The activity of TPN-linked malic enzyme as well as that

Table III Activity Variation of Two Malic Enzymes

| | Exp. | Specific activity (units/mg of protein) | | |
|---|---|---|---|---|
| | | DPN-linked malic enzyme | TPN-linked malic enzyme | Malic dehydrogenase |
| Minimal glucose medium | 1 | 31.6 | 20.8 | 117 |
| | 2 | 10.6 | 59.4 | 672 |
| Minimal malate medium | 1 | 42.1 | 206 | 1720 |
| | 2 | 23.1 | 226 | 2570 |

The "minimum glucose medium" contained the following constituents (g. per liter): glucose, 4; $K_2HPO_4$, 7; $KH_2PO_4$, 3; $(NH_4)_2HPO_4$, 1; $(NH_4)_2SO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1. The "minimum malate medium" contained the same constituents as those described above except 14g. of potassium DL-malate instead of glucose. The "enriched medium" contained 10g. of polypeptone, 10g. of beef extract, 5g. of yeast extract, 28g. of potassium DL-malate, and 10g. of glycerol besides inorganic salts described above.

of malic dehydrogenase increased remarkably on the malate medium, while that of DPN-linked malic enzyme showed no increment. As to the metabolic function of malic enzyme, its role in $CO_2$ fixation with pyruvate to form malate was almost excluded (Ashworth *et al.*, 1965). No conclusion seems to have been drawn to this problem, though its role in $TPNH_2$ generation or in the formation of acetyl CoA is assumed (Jacobson *et al.*, 1966). At least it may be said that such will not be the case with DPN-linked malic enzyme. The studies on the metabolic function of the DPN-linked malic enzyme in *E. coli* are now in progress.

REFERENCES

Ashworth, J. M., Kornberg, H. L. and Ward, R. L., Biochem. J., 94, 28p (1965).

Duerre, J. A. and Lichstein, H. C., Can. J. Microbiol., 7, 217 (1961).
Jacobson, L. A., Bartholomans, R. C. and Gunsalus, I. C., Biochem. Biophys. Res. Comm., 24, 955 (1966).
Korkes, S. and Ochoa, S., J. Biol. Chem., 174, 463 (1948).
Ochoa, S., in Method in Enzymology, S. P. Colowick and N. O. Kaplan, Vol. 1, p. 753, Academic Press, New York, (1955).
Parvin, R., Pande, S. V. and Venditasubramanian, T. A., Biochim. Biophys. Acta, 92, 260 (1964).
Saz, H. J. and Hubbard, J. A., J. Biol. Chem., 225, 921 (1957).
Temperli, A., Künsch, U., Mayer, K. and Busch, I., Biochim. Biophys. Acta, 110, 630 (1965).