BOUND PYRIDINE NUCLEOTIDE OF MALIC-LACTIC TRANSHYDROGENASE*

M. I. Dolin, E. F. Phares and M. V. Long

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Received October 8, 1965

The microorganism Micrococcus lactilyticus derives its energy from the fermentation of lactate to propionate, acetate and CO_2. One of the steps in this fermentation, the conversion of lactate to malate, is catalyzed by a malic-lactic transhydrogenase (reaction 1).

$$\text{malate} + \text{pyruvate} \rightleftharpoons \text{oxalacetate} + \text{lactate} \qquad (1)$$

This enzyme was described by Phares and Long (1956). The enzyme has no malic or lactic dehydrogenase activity and does not require exogenous cofactors. None of the artificial electron acceptors tested, including phenazine methosulfate, will function as oxidant. The enzyme was identified as a highly fluorescent pyridinoprotein (Dolin, Phares and Long, 1964) containing firmly bound NAD^+ and NADH in a molar ratio of 0.3/1. The fluorescence and spectral properties of the enzyme were briefly described. These data will be reported in more detail in the present communication. A malic-lactic transhydrogenase has also been isolated by Allen and Galivan (1965).

EXPERIMENTAL

Isolation and molecular properties — Cultures of M. lactilyticus were grown and extracts prepared as previously described (Delwiche, Phares and Carson, 1956). Briefly, the purification consists of chromatography on DEAE-cellulose, equilibrated with 0.01 M

* Research sponsored by the U. S. Atomic Energy Commission under contract with the Union Carbide Corporation.

Tris-acetate, pH 7.6. After elution of the enzyme with an approximately linear gradient of sodium acetate (0.02 M to 1 M, pH 7.6), the active eluates are pooled, fractionated with ammonium sulfate and the fraction precipitating between 0.53 and 0.60 saturation is rechromatographed on Sephadex G-75, equilibrated with 0.5 M ammonium acetate, pH 6.7. Elution is carried out with the latter buffer. Active fractions are pooled, dialyzed against 0.01 M Tris-acetate, pH 7.5, and placed on a column of DEAE-Sephadex A-50, equilibrated with 0.02 M Tris-acetate, pH 7.5. A linear acetate gradient is used for elution (0.05 M Tris-acetate, pH 7.5 to 0.2 M ammonium acetate, pH 6.7). The active fractions (approximately 20 times purified over the original extract) appear homogeneous in velocity sedimentation at protein concentrations of 0.2 to 2.5 mg/ml. Preliminary results obtained by short-column equilibrium sedimentation (Yphantis, 1964) indicate a molecular weight of 115,000 at 3° (assuming a $\bar{V}$ of 0.75), with dissociation to a lower molecular weight at higher temperatures. Molecular weight determinations were carried out in 0.01 M Tris-Cl (pH 7.4), containing 0.1 M NaCl, at a protein concentration of 1 mg/ml. Based on the total pyridine nucleotide content, estimates of the minimum molecular weight range between 30,000 and 40,000.

Assays — Enzyme-bound NADH is liberated by bringing the enzyme to pH 10 to 11 with KOH and heating for 2 min at 100°. The liberated NADH is assayed fluorimetrically (Lowry *et al.*, 1957) with either alcohol or lactic dehydrogenase. Oxidized NAD is assayed as follows. The enzyme is brought to pH 1.5 with HCl. This treatment immediately and irreversibly destroys the fluorescence of the enzyme, as would be expected if all the fluorescence were attributable to NADH. The NAD^+ is then assayed fluorimetrically by the strong-alkali method (Lowry *et al.*, 1957). A Neurospora NADase control establishes the specificity of the assay for NAD^+. Bound pyruvate is assayed with lactic dehydrogenase on the same heated fraction used for the NADH determination.

The enzyme assays, as well as the spectral and fluorimetric determinations reported

in this paper, were carried out under conditions in which the enzyme has maximal activity (0.1 M Tris-Cl buffer, pH 7.9). In the standard assay (2×10^{-2} M substrates) the formation of oxalacetate (reaction 1) is followed spectrophotometrically at 280 mμ. (K_s for malate, $\sim 3 \times 10^{-3}$ M; K_s for pyruvate, $\sim 7 \times 10^{-3}$ M). The turnover is approximately 13,000 moles per min per mole enzyme-bound pyridine nucleotide.

Fluorescence was determined with a Farrand fluorometer (thiamin filters). Excitation and emission spectra were obtained with an Aminco-Bowman spectrofluorometer.

RESULTS

Spectrum — The spectrum of the transhydrogenase is shown in Fig. 1. There is a peak at 275 mμ and a broad absorption band which has its maximum at 350 mμ. This spectrum was taken manually. In a recording spectrophotometer, the ultraviolet side of

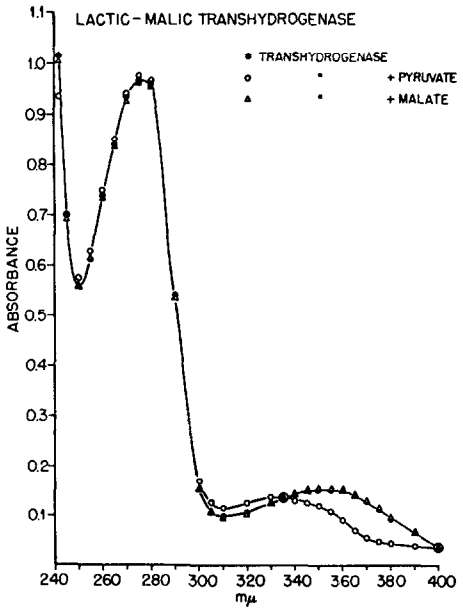

Fig. 1. Spectrum of the transhydrogenase in 0.1 M Tris-Cl, 0.1 M, pH 7.9. Pyrocell micro-cuvettes were used (0.2 ml volume, 1 cm light path). ●, transhydrogenase; O, transhydrogenase + potassium pyruvate, 10 μmoles per ml; Δ, transhydrogenase + potassium malate, 10 μmoles per ml. Spectra are corrected for absorbance of reagents and for dilution.

the protein peak shows four inflection points, which correspond with the fine-structure of phenylalanine. On the addition of excess pyruvate, the absorbance in the region from 340 to 390 mμ decreases, leaving a band at 330 mμ. This spectral change is accompanied by complete quenching of NADH fluorescence. At all degrees of partial quenching of fluorescence by pyruvate, the excitation peak remains at 350 mμ, i.e. the 330 mμ absorption band does not contribute to the excitation spectrum. The 330 mμ band can be made to disappear if the ionic strength is raised to 0.45 by the addition of KCl. Under these conditions, the bleaching caused by pyruvate extends from 310 to 400 mμ. Spectral changes similar to those elicited by pyruvate are also caused by oxalacetate. The addition of malate alone to the enzyme causes no change in the spectrum or fluorescence intensity. It is interesting that the 330 mμ band of the transhydrogenase resembles the absorption band of the nonfluorescent enzyme·NAD^+·fluoropyruvate complex of lactic dehydrogenase (Winer, 1965).

Reversal of complex formation — The changes caused by pyruvate addition to native enzyme are reversed by dialysis. Table I illustrates the effect of pyruvate addition and dialysis on the pyridine nucleotide content and fluorescence of the transhydrogenase. The native enzyme is 15 times as fluorescent as free NADH. On addition of pyruvate, the fluorescence is completely quenched and no NADH is detectable. After dialysis for 27 hr (line 3) 83% of the initial fluorescence returns and the NAD^+/NADH ratio is 1.1. (compared to 0.28 for the original enzyme). The total pyridine nucleotide content, however, has not changed. Enzyme-bound pyruvate was not determined, but the spectrum at this stage is that of an enzyme in which pyruvate-induced bleaching at 370 mμ has proceeded to one-half the extent shown in Fig. 1. The absorption peak is now at 340 mμ. Addition of excess pyruvate causes the bleaching to proceed to the full extent shown in Fig. 1; conversely, addition of malate produces the spectrum of the original enzyme.

The fluorescence-to-NADH ratio of 22 may be in error. If the enzyme at this

TABLE I

PYRIDINE NUCLEOTIDE CONTENT AND FLUORESCENCE OF MALIC-LACTIC TRANSHYDROGENASE

Additions	μmole/ml enzyme				Relative		
	NADH	NAD^+	NAD^+ + NADH	pyruvate	NAD^+/NADH	fluorescence	Fluorescence/NADH
1. *Transhydrogenase*	0.096	0.027	0.12	0.041	0.28	100	15
2. " + pyruvate	0*	-	-	-	-	0	0
3. " + pyruvate + dialysis (1)	0.055	0.061	0.12	-	1.1	83	22
4. " + pyruvate + dialysis (2)	0.083	0.024	0.11	0.062	0.29	94	16
5. NADH	-	-	-	-	-	-	1

* No NADH is detectable; however, in the presence of excess pyruvate, the assay procedure for NADH causes a conversion of enzyme-bound pyridine nucleotide to an unidentified fluorescent derivative.

Transhydrogenase was treated with a 500-fold excess of potassium pyruvate over enzyme-bound NADH (line 2). The nonfluorescent enzyme was then dialyzed against 200 volumes of Tris-Cl buffer, 0.02 M, pH 7.9. Line 3 shows the results of an experiment in which dialysis was continued for 27 hr, with three changes of buffer (200 volumes each time). Line 4 shows the results of an experiment in which dialysis was continued for 48 hr, with four changes of buffer. Fluorescence was determined on suitably diluted aliquots of enzyme, with a Farrand Fluorometer (thiamin filters).

stage of dialysis contains dissociable pyruvate, it may be difficult to obtain an accurate correlation between NADH content and fluorescence intensity. Dialysis of pyruvate-treated enzyme for 48 hr (line 4) causes the enzyme to return approximately to its initial condition. The dialyzed enzyme has 100% of its initial activity, and the spectrum is that of the original, untreated enzyme.

Fluorescence properties — Tryptophan and NADH emission peaks occur at 330 and 440 mμ respectively. Thus both these peaks are shifted to the ultraviolet when compared with the emission characteristics of the free compounds in aqueous solution. The 330 mμ fluorescence is excited at 280 mμ and the 440 mμ fluorescence at 280 and 350 mμ. Pyruvate quenches the 440 mμ emission peak of NADH, but not the tryptophan emission. When fluorescence is quenched with the minimum amount of pyruvate, a 2.5-fold excess of malate causes the reappearance of 70% of the initial fluorescence.

At enzyme concentrations of 2×10^{-7} M (as NADH), 50% quenching of NADH fluorescence occurs at the following molar concentrations: pyruvate, 3×10^{-6}; oxalacetate, 1.6×10^{-6}; α-ketoglutarate, 1.5×10^{-4} (immediate), 3.5×10^{-6} (after 50 min); oxamate, 10^{-2}. Fluorescence is also quenched by α-ketobutyrate, but not by acetaldehyde, acetone or acetoin, the latter three at a concentration of 2×10^{-3} M. Neither NAD^+ nor p-chloromercuribenzoate causes fluorescence quenching, i.e. NADH is not displaced from the enzyme by NAD^+ or the mercurial.

More exact data on keto acid dissociation constants are not available. Analysis of fluorescence titration data by the method of Klotz (1946) or Winer and Schwerdt (1959) suggests that the pyruvate binding sites are not equivalent and independent under the conditions used.

Substrate specificity — The inactivity of artificial electron acceptors has been mentioned. Apparently the bound NADH is not accessible to compounds such as phenazine methosulfate which can oxidize NADH spontaneously. With malate as donor, the keto acid may be replaced by α-ketobutyrate, but not by acetaldehyde, acetone,

acetoin, oxamate or α-ketoglutarate. The latter compound is a weak inhibitor. With pyruvate as the keto acid, DL-isocitrate does not replace L-malate.

DISCUSSION

The behavior of the transhydrogenase seems to be unique for a pyridinoprotein. As isolated, the enzyme contains a 3.5-fold excess of NADH over NAD^+, both nucleotides being firmly bound. The fluorescence of the bound NADH is quenched on addition of the oxidant (pyruvate). However, the reducing equivalents originally present in NADH remain enzyme-bound, since on dialysis (Table I) the enzyme returns to its original condition. It is not clear whether the complex between transhydrogenase and pyruvate is more accurately represented as E·NADH·pyruvate or E·NAD^+·lactate. Since the fluorescence of the enzyme is also quenched by α-ketoglutarate (a weak inhibitor) at low concentration and by oxamate at high concentration, it may be that quenching does not require complete transfer of hydrogen from NADH to keto acid. Schwerdt and Winer (1963) have suggested that the quenching of NADH fluorescence of lactic dehydrogenase by oxamate "arises from attraction of a hydride ion by the polarized carbonyl of oxamate in the ternary enzyme-NADH-oxamate complex, with resultant resonance of the pyridine ring into the nonfluorescent benzenoid form." In the case of the transhydrogenase, such a mechanism of quenching may occur even with enzymically active keto acids. An alternate structure involving reduced enzyme (EH·NAD^+·pyruvate) may be proposed for the nonfluorescent complex of the transhydrogenase. This is suggested by the work of Schellenberg (1965) on yeast alcohol dehydrogenase. Thus, one may visualize at least three kinds of complex, only one of which contains lactate. Whatever the structure of the complex, it is clear that the half reaction (oxidation of enzyme-bound NADH by pyruvate and dissociation of lactate) does not fit with the present data. If the nonfluorescent complex is an intermediate in transhydrogenation, further reaction of the complex with the reducing substrate (the hydroxy acid) is necessary in order to remove reducing equivalents from the enzyme.

ACKNOWLEDGMENT

We are indebted to Mr. D. R. Holladay and Dr. A. P. Pfuderer for the analytical ultracentrifuge studies.

REFERENCES

Allen, S. H. G. and Galivan, J. H., Federation Proc. 24, 531 (1965).
Delwiche, E. A., Phares, E. F. and Carson, S. F., J. Bacteriol. 71, 598 (1956).
Dolin, M. I., Phares, E. F. and Long, M. V., Federation Proc. 23, 427 (1964).
Klotz, I. M., Arch. Biochem. 9, 109 (1946).
Lowry, O. H., Roberts, N. R. and Kapphahn, J. I., J. Biol. Chem. 224, 1047 (1957).
Phares, E. F. and Long, M. V., Abstr. 130th Meeting Am. Chem. Soc., p. 62 c (1956).
Schwerdt, G. W. and Winer, A. D., in P. D. Boyer, H. Lardy and K. Myrbäck (editors), The Enzymes, Vol. 7, Academic Press, New York, 1963, p. 127.
Schellenberg, K. A., J. Biol. Chem. 240, 1165 (1965).
Winer, A. D., Biochemistry 4, 606 (1965).
Winer, A. D. and Schwerdt, G. W., J. Biol. Chem. 234, 1155 (1959).
Yphantis, D. A., Biochemistry 3, 297 (1964).