UNUSUAL KINETIC PROPERTIES OF A DPN-LINKED LACTATE DEHYDROGENASE FROM BUTYRIBACTERIUM RETTGERI

Charles L. Wittenbe r

U. S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Dental Research, and the American Dental Association, Bethesda, Maryland

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A DPN-linked lactate dehydrogenase (LDH) has been partially purified from extracts of Butyribacterium rettgeri. While studying the substrate stereospecificity and coenzyme requirement for this enzyme, some unusual kinetic properties were observed. It will be shown in this report that the B. rettgeri LDH is unable to catalyze the reduction of DPN with lactate under conditions where this reaction can be demonstrated readily with LDH's from other sources. In addition, it will be shown that the rate of pyruvate reduction by DPNH, as catalyzed by the B. rettgeri LDH, increases in a sigmoidal fashion with increasing concentrations of pyruvate.

Materials and Methods - All cofactors employed in this study were purchased from the Sigma Chemical Company. Crystalline rabbit muscle LDH was a Boehringer product obtained from Calbiochem.

Cultivation of B. rettgeri in a semisynthetic medium (Kline and Barker, 1950) with glucose as the energy source and the preparation of cell-free extracts were as described previously (Wittenberger and Flavin, 1962). Isolation of the DPN-LDH from crude extracts and its separation from DPNH oxidase was accomplished by a combination of ammonium sulfate fractionations and DEAE-cellulose chromatography. The details of this purification, which yields approximately a 100-fold purified enzyme,will be published elsewhere.

A D(-)-specific LDH was obtained from Lactobacillus plantarum (Dennis and Kaplan, 1960). L. plantarum cells were kindly supplied by Dr. T. Shiota. Cells were suspended in cold 10 mM potassium phosphate buffer, pH 6.5, and passed through an Aminco French pressure cell two times. The resulting extract was centrifuged at 30,000 X g for 20 min and the supernatant fluid was then fractionated with solid ammonium sulfate. The fraction which precipitated at 40-80% ammonium sulfate saturation was employed as the source of the enzyme. These preparations also contained an L(+)-specific LDH (Dennis and Kaplan, 1960).

Lactate dehydrogenase activity was assayed either by following the pyruvate-dependent oxidation of DPNH at 340 mμ as previously described (Wittenberger and Haaf, 1964), or by measuring the lactate-dependent reduction of the 3-acetyl-pyridine analogue of DPN (APDPN) at 365 mμ (Kaplan and Ciotti, 1956). For the latter assay, each reaction cuvette contained 100 mM Tris-HCl, pH 8.0, 87.3 mM Na-D(-)- or Na-L(+)-lactate, 2 mM APDPN, and an amount of purified B. rettgeri LDH which gave a ΔO.D. at 365 mμ of .020-.100 per min. The final volume of each reaction mixture was one ml and reactions were initiated by the addition of enzyme. All reactions were followed in a Beckman Model DB spectrophotometer equipped with a Sargent Model SRL recorder.

Results and Discussion - The purified DPN-linked LDH from B. rettgeri is specific for the D(-)-stereoisomer of lactate and its activity with D(-)-lactate is not significantly affected by the presence of an equivalent amount of the L(+)-isomer (Table I). For comparative purposes, the activity of the enzyme with pyruvate as substrate and DPNH as the reductant is also shown.

The enzyme has a specific requirement for DPNH as the coenzyme. TPNH is completely inactive as a reductant in the conversion of pyruvate to lactate. The enzyme also exhibits specificity for pyruvate as the substrate. No oxidation of DPNH occurred with either α-ketoglutarate or oxaloacetate. DPNH was oxidized with α-ketobutyrate, but the reaction rate was only about 1/10 of that observed when pyruvate served as substrate.

Although the B. rettgeri LDH catalyzed the oxidation of lactate with

Table I

Substrate Stereospecificity of B. rettgeri LDH

Substrate	Cofactor	Specific activity‡
D(-)-lactate	APDPN	354
L(+)-lactate	APDPN	none detected
D(-)-lactate + L(+)-lactate	APDPN	343
Pyruvate	DPNH	2,712

Assay conditions were as described in Materials and Methods except when L(+)-lactate served as substrate, in which case twice as much enzyme was used.

‡ mμmoles APDPN reduced or DPNH oxidized per min per mg protein.

APDPN as the acceptor (Table I), this reaction could not be demonstrated easily with DPN as the acceptor. Table II shows that under conditions where the L(+)-specific LDH from rabbit muscle and a D(-)-specific enzyme from L. plantarum readily catalyzed the reduction of DPN with lactate, no reduction of DPN could be observed with the B. rettgeri LDH. Even with a 4-fold excess of B. rettgeri LDH, compared to the L. plantarum or rabbit muscle enzymes, only a trace level of DPN-reducing activity could be demonstrated.

It was considered that the markedly decreased ability of the B. rettgeri LDH to catalyze the reverse of the physiological reaction might reflect a relatively low affinity of the enzyme for DPN. As an indirect test of the DPN-binding capacity of the enzyme, the effect of DPN on the reduction of APDPN by lactate was studied. A summary of some of these experiments is given in Fig. 1, where the results are expressed in the Lineweaver-Burk (1934) double reciprocal plots. It may be seen that both DPN and DPNH are effective

Table II

DPN Reduction by Lactate as Catalyzed by Lactate Dehydrogenases from Various Sources

Source of enzyme	Substrate	Units LDH‡ assayed	mμmoles DPN reduced/min
Rabbit muscle	L(+)-lactate	50	21.9
L. plantarum	D(-)-lactate	50	5.7
B. rettgeri	D(-)-lactate	50	0
B. rettgeri	D(-)-lactate	200	1.1

Assay conditions were as described in Materials and Methods for the APDPN assay except that 2 mM DPN was substituted for APDPN and reactions were followed at 340 mμ.

‡Units were determined by assay of the enzymes with DPNH and pyruvate as described in Materials and Methods. One unit is the amount of enzyme which oxidizes one mμmole of DPNH per minute. The L. plantarum preparation employed here contained both D(-)- and L(+)-specific LDH's. In order to estimate what percent of DPNH oxidation by pyruvate was due specifically to the D(-)-enzyme, the preparation was assayed in the presence of an amount of oxamate which inhibited the L(+)-enzyme activity by over 90%, but which was without effect on the activity of the D(-)-enzyme (Dennis and Kaplan, 1960).

inhibitors of APDPN reduction and that the inhibition, in both cases, is competitive with respect to APDPN. It has been found in other studies that neither TPN nor TPNH act as inhibitors of APDPN reduction when used at concentrations equivalent to those shown here for DPN and DPNH.

The kinetic constants calculated from the data in Fig. 1 are given in Table III. The Ki value for DPN has varied somewhat with different enzyme preparations and values ranging from 4.32×10^{-4} M to 7.84×10^{-4} M have been obtained. These values, however, fall well within the range of reported K_M values for DPN for LDH's from other sources. For example, the K_M for DPN

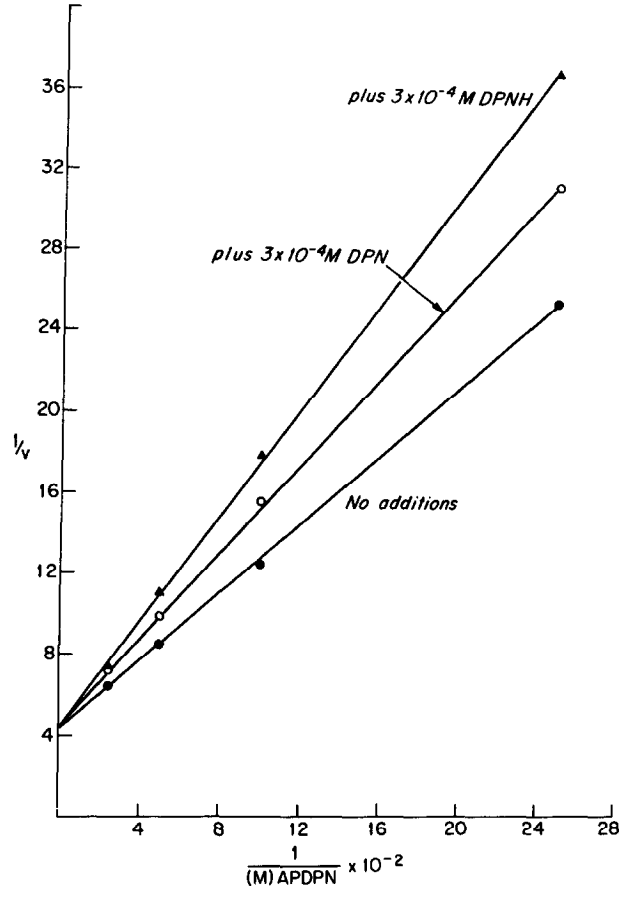

Fig. 1. Effect of DPN or DPNH on APDPN reduction by lactate. Assays were as described in Materials and Methods except APDPN concentrations were varied between the limits shown. Where indicated, 1 x 10^{-4} M DPN or DPNH was included in the reaction mixture.

Table III

Kinetic Constants for B. rettgeri LDH

K_M APDPN	Ki DPN	Ki DPNH
(M)	(M)	(M)
1.85 x 10^{-3}	7.84 x 10^{-4}	4.55 x 10^{-4}

for Bacillus subtilis LDH is 9 x 10^{-4} M at pH 7.2 (Yoshida, 1965); the value for rat liver LDH is 1.6 x 10^{-4} M at pH 8.6 (Anderson, et al., 1964); and the K_M's for DPN for various heart and brain LDH isozymes range from 0.88 x 10^{-4} M to 1.6 x 10^{-4} M at pH 8.7 (Nisselbaum, et al., 1964). The data from Fig. 1 show that DPN is effectively bound by the B. rettgeri LDH and it appears unlikely, therefore, that the greatly reduced ability of this enzyme to catalyze the reduction of DPN with lactate is due to a markedly lowered affinity of the enzyme for the pyridine nucleotide coenzyme.

It was also considered that the difficulty in demonstrating the conversion of lactate to pyruvate with the B. rettgeri LDH might be due to a low affinity of the enzyme for lactate. Employing APDPN as the acceptor, the K_M for lactate was found to be 0.11 M at pH 8.0. This value is about 3-4 times higher than the K_M for lactate (with APDPN as acceptor) reported for the D(-)-specific LDH from L. plantarum (Dennis and Kaplan, 1960). It is uncertain, however, whether this modestly decreased affinity of the B. rettgeri LDH for

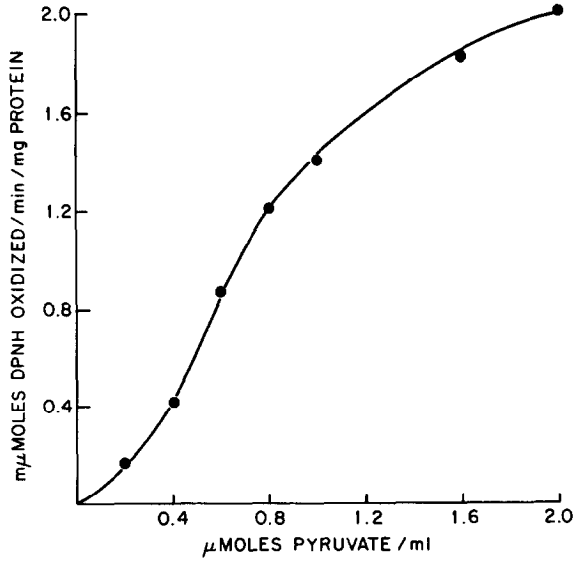

Fig. 2. Effect of pyruvate concentration on the rate of DPNH oxidation. Assay conditions were as described in Materials and Methods, except pyruvate concentrations were varied as shown.

lactate provides a complete explanation for the markedly reduced capacity of this enzyme to catalyze the conversion of lactate to pyruvate with DPN as the cofactor. The possibility remains that the K_M for lactate with DPN as the cofactor is much higher than that observed when APDPN serves as the acceptor. This possibility has not been tested, as yet, because of the difficulty in demonstrating the reverse of the physiological reaction under these conditions.

Tarmy and Kaplan (1965) have recently purified an L(+)-specific LDH from Escherichia coli, which also has a greatly reduced ability to catalyze the reverse of the physiological reaction. When this enzyme was assayed in the direction of pyruvate reduction by DPNH, the reaction rate, at low pyruvate concentrations, was shown to be a sigmoidal function of the pyruvate concentration (Tarmy and Kaplan, 1965). The B. rettgeri D(-)-specific LDH exhibits a similar deviation from Michaelis-Menten kinetics as shown in Fig. 2. This type of kinetic response is analogous to that observed with a number of enzymes, which have been shown to be allosteric with respect to a variety of effector ligands. Whether this behavior is indicative of an allosteric protein in the case of the B. rettgeri LDH is, as yet, unresolved. It is interesting to speculate, however, that the inability of this enzyme to catalyze effectively the conversion of lactate to pyruvate might reflect a requirement for some physiological activator ligand. In this regard, it has been shown that rabbit muscle LDH isozyme 5 is an allosteric protein and is activated by a number of physiological metabolites (Fritz, 1965). In addition, LDH's from several species of Streptococcus have been shown to be activated by fructose-1-6-diphosphate (Wolin, 1964). Studies designed to characterize the nature of the unusual kinetic properties exhibited by the B. rettgeri LDH are now in progress.

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