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# Catalytic and regulatory strategies of thermophilic lactate dehydrogenase: microscopic rate constants from kinetic isotope effects

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The lactate dehydrogenase of Bacillus stearothermophilus exists in dimeric and tetrameric forms, the latter favored by the regulatory effector, fructose-1,6-bisphosphate. The kinetic behavior of the two forms is different, the tetramer showing activation relative to the dimer at low pyruvate concentrations, but pyruvate inhibition at high concentrations. Deuterium isotope effects at the transferring hydride site of NADH (primary isotope effect) and the methyl group of pyruvate (secondary isotope effects) allow estimation of the microscopic rate constants for individual processes for both enzyme forms. In the dimer, entrance and exit to and from the active site is slow for pyruvate nd lactate, rapid for NAD. In the tetramer, entrance and exit for pyruvate and lactate are fast while NAD exit is slow. Hydride transfer occurs at similar rates in both forms. This model explains the main features of the kinetics of both forms of the enzyme.

## INTRODUCTION

### The lactate dehydrogenase of Bacillus stearothermophilus

The lactate dehydrogenase (BsLDH) of the thermophilic bacterium Bacillus stearothermophilus has been the object of intensive investigation, in particular by the groups led by Holbrook (ref. 1) and by Zuber (ref. 2). The thermophilic character of the organism has given rise to a thermostable enzyme which operates with high efficiency at 55°C, the temperature at which the results to be reported herein were obtained. The enzyme catalyzes the oxidation-reduction reaction of reduced nicotinamide adenine dinucleotide (NADH) and a proton with pyruvate or nicotinamide adenine dinucleotide (NAD) with L-lactate.

BsLDH is formed of monomeric single polypeptides. The structures of both eukaryotic LDHs (ref. 3) and BsLDH (ref. 4) have been determined crystallographically. The structures of the active sites and the general three-dimensional structures of the bacterial and eukaryotic enzymes are exceedingly similar, except for an N-terminal helix-

containing stretch of 12-14 residues present in the eukaryotic structure and missing in bacterial structures. This substructure appears to stabilize the aggregation of the eukaryotic enzyme into tetrameric form. The eukaryotic enzyme is not subject to regulation by fructose-1,6-bisphosphate (FBP).

In contrast, BsLDH is regulated by FBP. The regulatory effect has been shown (ref. 5) to correspond to the conversion of dimers of the monomeric subunit (the dimer being stabilized apparently mainly by helix-helix interactions) into tetramers, crosslinked by two FBP molecules in each tetramer. In the direction of pyruvate reduction by NADH, the tetrameric aggregate shows strong activation compared to the dimer at low and moderate concentrations of pyruvate; at sufficently high concentrations of pyruvate, however, the tetrameric enzyme suffers strong inhibition by pyruvate while the dimeric enzyme does not (ref. 2).

Determination of microscopic rate constants

The BsLDH system is so well-defined structurally, biophysically and in terms of the kinetics and mechanism of subunit aggregation and disaggregation, as a result of the studies of previous workers, that it ought to be possible to understand more fully its catalytic and regulatory properties at the most basic level. We report here in a preliminary manner the results of experiments intended to yield the so-called "microscopic rate constants" for catalysis by both dimeric and tetrameric forms of BsLDH at the biologically relevant temperature of 55°C. The term "microscopic rate constants" refers to constants for processes occurring along the reaction pathway, at a finer level of resolution than is given by the rate constants obtained by classical enzyme-kinetics studies. Ideally, one might like rate constants for the elementary chemical reactions but many features of the complexity of enzyme-catalytic systems and, in particular, the probable coupling between substrate reactions and structural alterations of the enzyme suggest that the apparent microscopic constants actually obtained will be themselves aggregates of rate constants for serial and parallel reactions at an unresolved level.

Kinetic isotope effects have proved an excellent means for estimating microscopic rate constants (ref. 6). Chart 1 outlines the relationship between kinetic isotope effects and the component rate constants in a serial steady-state kinetic system. The extension to parallel reactions is straightforward but we shall make use here only of the moethod for serial reactions. We shall apply the formulation of Stein (ref. 7), which is algebraically equivalent to the more widely-used formulation of Northrop (ref. 8); Stein's approach appears to us more straightforward and general. For example, Stein's method makes no assumptions about the presence or absence of isotope effects on any step.

Stein's method represents the observed isotope effect on a rate constant, as obtained in kinetic experiments (e.g., the familiar $k_{cat}$ and $k_{cat}/K_m$ of one-substrate enzyme kinetics) as a weighted average of the isotope effects on the component "microscopic" rate constants that make up the observed rate constant. The isotope effects on the component rate constants are known as "intrinsic isotope effects" so Stein portrays the observed isotope effect as a weighted average of the intrinsic isotope effects. Most importantly, the weighting factor for each intrinsic effect is *the ratio of the observed rate constant to the microscopic rate constant*. Thus, the determination of a sufficient number of observed isotope effects, if the intrinsic isotope effects are known or can be estimated, can lead to a complete specification of the microscopic rate constants.

Chart 1

Derivation of Microscopic Rate Constants from Steady-State Isotope Effects

$(1/k) = (1/k_1) + (1/k_2)$: two-step steady-state rate expression

Stein, J. Org. Chem. 1981, 46, 3328-3330:

$$k_H(1/k_D) = k_H(1/k_{1D}) + k_H(1/k_{2D})$$

$$k_H/k_D = (k_H/k_{1H})(k_{1H}/k_{1D}) + (k_H/k_{2H})(k_{2H}/k_{2D})$$

$(k_H/k_{iH}) = w_i$: weighting factors; $(k_{iH}/k_{iD})$: intrinsic isotope effects

$$k_{iH} = k_H/w_i$$

After Northrop, Biochemistry 1975, 14, 2644-2651:

$$k_H/k_D = [k_{1H}k_{2H}(k_{1D} + k_{2D})]/[k_{1D}k_{2D}(k_{1H} + k_{2H})]$$

$$k_H/k_D = [(k_{2H}/k_{2D}) + C(k_{1H}/k_{1D})]/[1 + C]$$

$C = k_{2H}/k_{1H}$: commitment to catalysis; $k_{1H} = k_H(1 + C)/C$, $k_{2H} = k_H(1 + C)$

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## RESULTS

### Kinetics for dimeric and tetrameric enzyme forms

BsLDH follows the typical sequential ordered mechanism portrayed in Fig. 1, in which NADH (A) is first on, NAD (Q) last off and pyruvate (B) last on, lactate (P) first off. As the figure shows, there are four kinetic constants accessible from the kinetics in the direction of pyruvate reduction:

$k_{cat}/K_{iA}K_{mB}$: first order in NADH, first order in pyruvate;
$k_{cat}/K_{mA}$: first order in NADH, zero order in pyruvate;
$k_{cat}/K_{mB}$: zero order in NADH, first order in pyruvate;
$k_{cat}$: zero order in both NADH and pyruvate.

In addition, since pyruvate inhibition is observed for the tetrameric enzyme, a constant $K_{iB}$, characterizing this inhibition, can be obtained. The rate constants are presented in Table 1.

### Kinetic isotope effects

Two types of kinetic isotope effects were measured for each of two kinetic parameters, $k_{cat}/K_{mB}$ and $k_{cat}$. The primary isotope effect, denoted

TABLE 1. Effect of FBP-induced dimer-tetramer conversion on the kinetic parameters for a sequential ordered mechanism for BsLDH, pH 6, 55°C.

| Enzyme form (FBP conc) | $10^{-9}\ k_{cat}/K_{iA}K_{mB}$, $M^{-2}s^{-1}$ | $10^{-7}\ k_{cat}/K_{mA}$, $M^{-1}s^{-1}$ | $10^{-5}k_{cat}/K_{mB}$, $M^{-1}s^{-1}$ | $10^{-2}k_{cat}$, $s^{-1}$ |
|---|---|---|---|---|
| Dimer (0 mM) | 1.3 ± 0.2 | 2.3 ± 1.9 | 4.8 ± 0.5 | 103 ± 5 |
| Tetramer (2 mM) | 260 ± 130 | 4.3 ± 0.4 | 160 ± 120 | 85 ± 4 (a) |

(a) As saturation is approached, the rate begins to decrease for the tetramer as a result of pyruvate inhibition with $K_{iB}$ = 50 ± 20 mM. At very high pyruvate concentrations the rate is again constant with $k_{cat}$ = 21 ± 7 $s^{-1}$.

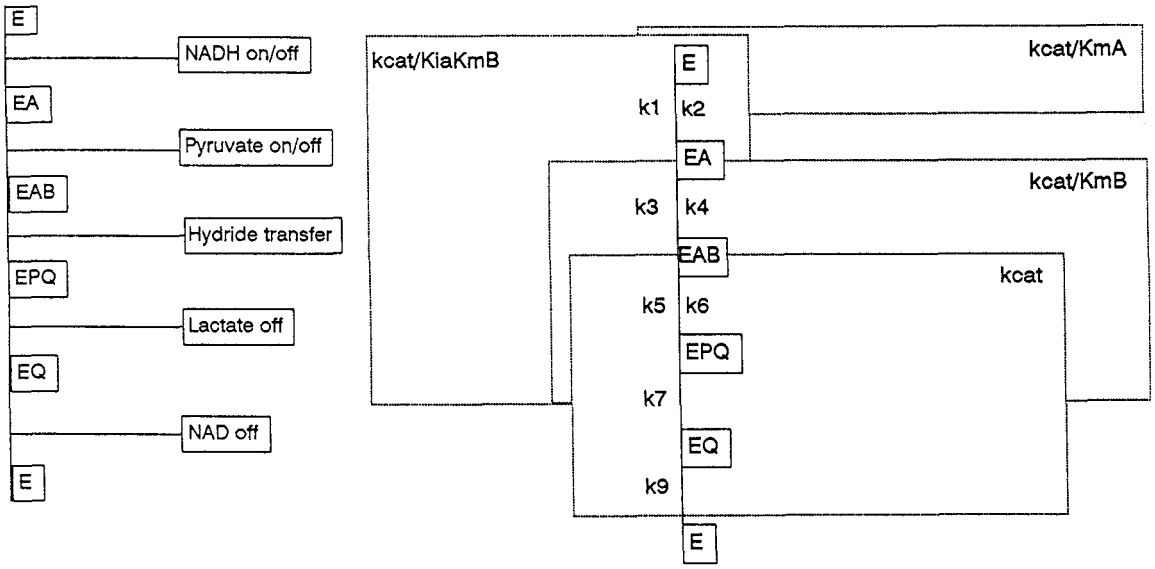


Fig. 1. *Left:* Sequential ordered mechanism for BsLDH with designations of the main mechanistic events occur in the interval between each potentially accumulating enzyme form. *Right:* Definitions of the four kinetically accessible parameters. Each box has a top border at the initial state for the parameter and a bottom border at the final transition state. Each parameter except $k_{cat}$ has only the single initial state indicated; $k_{cat}$ has all initial states within its box. The microscopic rate constants are also shown. Dead-end inhibition by pyruvate (B) is omitted.

P below, was determined for deuterium labeling at the "A" position of NADH, which is stereospecifically transferred in the BsLDH reaction. The second isotope effect arose from labeling of the three methyl hydrogens of pyruvate. This is a $\beta$-deuterium secondary isotope effect, denoted S below. Rates were determined spectrophotometrically from the NAD/NADH absorbance change.

The isotope effects expected for each of the microscopic processes can be estimated from available data with some precision, and by analogy with considerably lower precision. The expected ranges of the intrinsic isotope effects are shown in Fig. 2, left side. The main principles involved are these:

(a) Intrinsic isotope effects for processes before the hydride-transfer step are taken to be unity, thus neglecting small effects due to binding, solvation changes, etc.

(b) Intrinsic isotope effects for processes with inital states before but transition states after the hydride-transfer step should be the equilibrium effects, which have been measured by Cleland and his coworkers (ref. 6).

(c) Intrinsic isotope effects for processes with both initial and transition states following the hydride-transfer step were taken to be equal to unity, again neglecting small effects from differential interaction with the enzyme, etc.

(d) The intrinsic isotope effects for the hydride-transfer step were taken to be roughly 4-7 for the primary effect P (although this range turns out to play no role) and between unity and the equilibrium effect of 0.87 for the secondary effect S.

The right side of Fig. 2 shows the effects actually measured for the two kinetic constants with the dimeric form of the enzyme in the absence of FBP and the tetrameric form of the enzyme in the presence of 2 mM FBP.

## DISCUSSION

### Catalytic and regulatory features for BsLDH at 55°C

The data of Table 1 indicate several characteristics. First tetramerization from the dimer has little effect on the rate of binding of NADH to free enzyme ($k_{cat}/K_{mA}$). The rate constant $k_{cat}/K_{mB}$ is substantially increased, indicating that transition-states after NADH binding and up through lactate release are substantially stabilized in the tetramer. Finally the value of $k_{cat}$ is only very little affected by dimer-to-tetramer conversion.

In addition, as mentioned before, inhibition by pyruvate is detectable as concentrations approach 50 mM with the tetramer but is indetectable with the dimer, suggesting that the value of $K_{iB}$ for the dimer is greater than 500 mM. All of these differences should be seen in the light of the structure of the tetrameric form of BsLDH, which exhibits an electronic and steric environment for the active site which should be exceedingly similar to that for the dimer (ref. 4). The single difference is that the relatively nearby FBP binding site is occupied in the tetramer but not in the dimer.

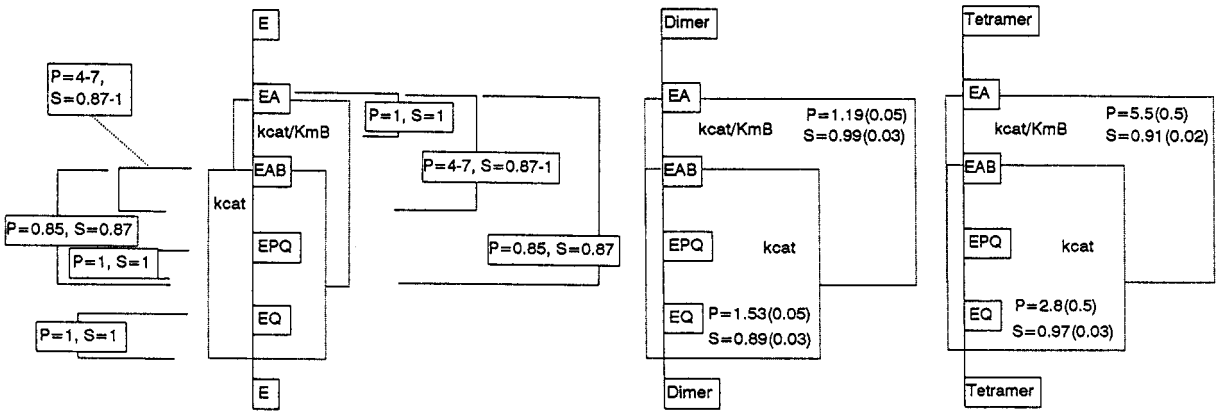


Fig. 2. *Left:* Expected intrinsic isotope effects for the primary (P) label in NADH and the secondary (S) label in pyruvate for processes contributing to the two indicated kinetic parameters. The values were estimated as described in the text. *Right:* Measured isotope effects for the two kinetic parameters for the dimeric enzyme (no FBP) and the tetrameric enzyme (2 mM FBP) at 55°C and pH 6.

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Calculation of the microscopic rate constants: general assumptions

The values of the constants are shown in Fig. 3. Some of the values are independent of the isotope-effect measurements. The on-rate and off-rate constants for NADH are available from $k_{cat}/K_{mA}$ (on = k1 in Fig. 1) and $K_{ia}$ (off/on = k2/k1). The main assumption entering the determination of the remainder is that the primary and secondary effects for $k_{cat}/K_{mB}$ for the tetramer (P = 5.5, S = 0.91) are in fact the intrinsic effects for hydride transfer. The large value of P and the value of S intermediate between S = 1 and S = 0.87, the equilibrium value, are both consistent with this assumption. This means both lactate release and pyruvate binding must have transition states substantially lower in free energy than that for hydride transfer. Next, the value of P for $k_{cat}$ for the tetramer is reduced from 5.5, signalling the incursion of another partially rate-limiting process. Changes in P and S suggest that this new process has both P and S =1. The process could be either lactate release from EPQ or NAD release from EQ. We argue it is the latter for reasons given below. Assuming that the intrinsic effects apply also to the dimer (because of the similar active-site environments), we note that for $k_{cat}$ for the dimer the value of P is reduced to 1.5 from the intrinsic value of 5.5 by a partially rate-limiting process that changes the intrinsic S of 0.91 either not at all or to a more inverse value. This cannot be release of P from EPQ or release of Q from EQ (S = 1 in

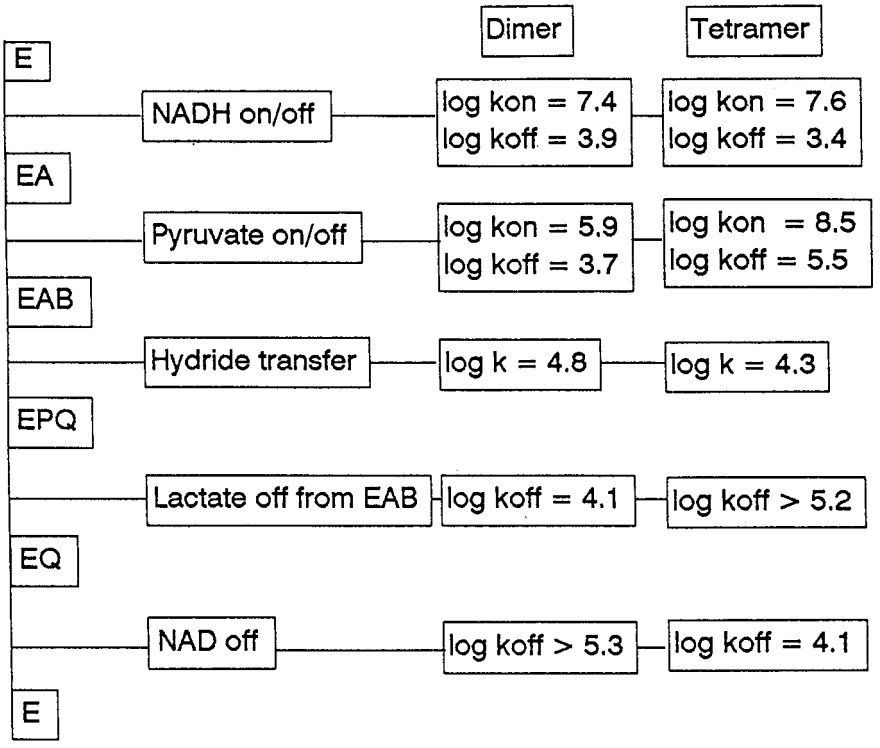


Fig. 3. Values of rate constants estimated as described in the text for the microscopic events during catalysis by the dimeric and tetrameric forms of BSLDH.

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both cases) but could be release of lactate P with an initial state EAB (partially reversible hydride transfer). Finally the almost negligible value of P for the dimeric $k_{cat}/K_{mB}$ suggests pyruvate binding to be essentially rate-limiting for the dimer.

Overall qualitative model. The qualitative picture indicates slow addition of pyruvate and slow release of lactate in the dimer, rapid addition of pyruvate and rapid release of lactate in the tetramer. Indeed, if we take lactate release to be rapid in the tetramer even at saturation, so that the process that reduces P from 5.5 to 2.8 is Q release from EQ, which accumulates, then we have a simple explanation of the strong pyruvate inhibition observed for the tetramer but not the dimer. EQ is the target for dead-end inhibition by pyruvate, which can occupy the lactate site. If EQ accumulates with the tetramer but not the dimer, dead-end inhibition will be more important with the tetramer than the dimer. We have therefore adopted this view, and the resulting set of assumptions leads to the values of the microscopic rate constants shown in Fig. 3.

Quantitative conclusions

The estimates in Fig. 3 suggest the following conclusions, which apply to the cycling steady-state species at 55°C, pH 6:

(a) The on and off rate constants for NADH are unaffected by dimer-to-tetramer conversion, in agreement with the similar situations of the active sites.

(b) Dimer-to-tetramer conversion results in an effective catalysis of the on/off reaction of pyruvate, the on-rate constant increasing by about 400-fold, the off-rate constant nearly 100-fold.

(c) Hydride transfer is unaffected in rate by the conversion, again consistent with the similar active-site situations.

(d) Lactate release (initial state EAB) is speeded by dimer-to-tetramer conversion, while NAD release (initial state EQ) is slowed by about the same factor. This compensatory change in rates, coupled with the constant rate of hydride transfer, accounts for the fact that $k_{cat}$ is the same for dimer and tetramer.

(e) The values lead to an estimate, from the relative populations of EQ in the two cycling steady states, of $K_{iB}$ about 500-600 mM for the dimer. This would indeed be indetectable under our conditions. This provides a model for substrate inhibition in the tetramer but not the dimer.

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REFERENCES

1) C.R. Dunn, H.M. Wilks, D.J. Halsall, T. Atkinson, A.R. Clarke, H. Muirhead, and J.J. Holbrook, Phil. Trans. Roy. Soc. London B 332, 177-184 (1991) and references given therein.

2) M. Kotik and H. Zuber, Biochemistry 31, 7787-7795 (1992) and references given therein.

3) H.H. Hogrefe, J.P. Griffith, M.G. Rossman, and E. Goldberg, J. Biol. Chem. 262, 13155-13162 (1987) and references given therein.

4) K. Piontek, P. Chakrabarti, H.-P. Schär, M.G. Rossman, and H. Zuber, Proteins: Strcuture, Function and Genetics 7, 74-92 (1990); D.B. Wigley, S.J. Gamblin, J.P. Turkenburg, E.J. Dodson, K. Piontek, H. Muirhead, and J.J. Holbrook, J. Mol. Biol. 223, 317-335 (1992).

5) A.R. Clarke, T. Atkinson and J.J. Holbrook, Trends Biochem. Sci. 14, 101-105 (1989).

6) P.F. Cook, Enzyme Mechanisms from Isotope Effects, pp. 181-202 (D.B. Northrop); pp. 203-230 (P.F. Cook), CRC Press, Boca Raton, Florida, 1991.

7) R.L. Stein, J. Org. Chem. 46, 3328-3330 (1981).

8) D.B. Northrop, Biochemistry 14, 2644-2651 (1975).