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Solvent Isotope Effects and the Nature of Electrophilic Catalysis in the Action of the Lactate Dehydrogenase of *Bacillus stearothermophilus*

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Abstract—Deuterium oxide at atom fractions of deuterium from 0.0 to 0.97 has an effect of less than 20 % on the kinetic term k_{cat}/K_{mB} (believed to reflect the transition state for the hydride-transfer step) for the reduction of pyruvic acid by NADH at 55 °C, with catalysis by the tetrameric form of the lactate dehydrogenase of *Bacillus stearothermophilus*. This observation suggests that the hydride-transfer event is not assisted by protonic bridging to the carbonyl group being reduced. The results are consistent with protonic bridging only if an opposing isotope effect is present, for example from a generalized conformation or solvation change. The results are consistent with other forms of electrophilic catalysis.

Introduction

Professor J. Bryan Jones has been a leader in deploying the most basic concepts of chemical science together with the most powerful methods of molecular biology in a variety of basic and applied investigations of enzymes.[1] One of the systems of which our knowledge has had the benefit of these efforts has been the enzyme lactate dehydrogenase (LDH) from the thermophilic bacterium *Bacillus stearothermophilus*.

All investigators of this enzyme are much in the debt not only of Bryan Jones but also in particular to the laboratories of Holbrook[2] in Bristol and Zuber[3] in Zurich, from which a rich literature on the biophysical and biochemical properties of LDH has been published.

LDH is a good catalyst of the hydride-transfer reaction of NADH with pyruvate. Kirby and Walwyn[4] have constructed an intramolecular model compound to simulate the NADH/pyruvate pair in the LDH active site. Their compound undergoes the intramolecular redox reaction with a first order rate constant around 10^{-3} s^{-1} in 20 %-dioxan–water at 39 °C. We believe that at 55 °C the corresponding redox reaction in the ternary complex of LDH occurs with a rate constant[5] of 10^{4-5} s^{-1}, yielding a catalytic acceleration factor of 10^{7-8}-fold. This factor corresponds to a net transition-state stabilization free energy of 40–46 kJ mol^{-1}.

Holbrook and his coworkers have identified a reasonable source for this transition-state stabilization.[2] Structural data are consistent with an approximation between the imidazolium side-chain of His 195 and the oxygen of the substrate keto group at the transition-state. Such an approximation could lead to three plausible models for transition-state stabilization, described here for the direction of pyruvate reduction:

1) Proton-transfer could occur from imidazolium before the hydride-transfer transition-state is reached. Increased affinity of the partially positive keto group for the transferring hydride ion would result in stabilization of the transition state. The transition-state on this model would have no protonic bridging between His-imidazole nitrogen and substrate keto-oxygen.
2) General acid catalysis of hydride-transfer, with a stabilizing transition-state protonic bridge, could occur.
3) Proton-transfer could occur after the hydride-transfer transition-state. Electrostatic stabilization by the cationic His-imidazolium of the oxyanion developing in the hydride-transfer transition-state could occur. Here too there would be no transition-state protonic bridge.

Protonic bridging in transition states for general acid–base catalysis commmonly[6] generates kinetic solvent isotope effects k_{HOH}/k_{DOD} of *ca* 2-4 (where HOH denotes protium oxide and DOD denotes deuterium oxide). The solvent isotope effect for the hydride-transfer event should therefore distinguish model 2, which should exhibit an effect of 2–4, from models 1 and 3, which should exhibit no solvent isotope effect or at most small equilibrium or secondary solvent isotope effects.

We have previously[5] observed a primary isotope effect of 5.5 (NAD[H] versus NAD[D]) and a secondary isotope effect ($CH_3COCOOH$ versus $CD_3COCOOH$) of 0.91 for the kinetic term k_{cat}/K_{mB} in the action of the dimeric form of LDH (absence of fructose-1,6-bisphosphate) at 55 °C. This indicates that the hydride-transfer transition-state is largely rate-limiting for this kinetic term, which must therefore correspond to the conversion of EA (enzyme:NADH complex) and pyruvate to the hydride-transfer transition state. We can thus carry out the needed solvent isotope-effect measurements by determination of k_{cat}/K_{mB} in HOH and DOD.

It is generally advisable, in measuring solvent isotope effects on enzymic reactions, to carry out a so-called 'proton inventory', or determination of the desired kinetic parameter in mixtures of HOH and DOD as well as in the pure isotopic solvents.[7] Such determinations address the concern that the observed solvent isotope effect may be a composite effect coming from many sites in the enzyme structure, such as backbone NH bonds, and thus might not have mechanistic significance. In a proton inventory, a linear dependence of the rate constant on n, the atom fraction of deuterium in the isotopic solvent mixtures, indicates that one exchangeable protonic site generates the entire solvent isotope effect; this is the expected result for general acid–base catalysis involving a single acid–base functional group. If instead a generalized conformation or solvation change produces the effect, the rate constant will be an exponential function of n.

The strategy of measuring the entire kinetics in a number of isotopic solvent mixtures in order to obtain the kinetic parameter k_{cat}/K_{mB} as a function of n would be tedious. Furthermore, the correlation among the steady-state kinetic parameters would lend a relatively large estimated error to the values thus obtained.

Instead, we have made use of the approach outlined in Chart 1. There it is shown that with an appropriate choice of concentrations of the first substrate in the ordered mechanism, NADH or A, and the second substrate, pyruvate or B, the observed kinetics will yield the desired rate constant directly. We report here the results of such experiments.

Chart 1. Determination of $E_t k_{cat}/K_{mB}$ as a first-order relaxation constant

At 55 °C, pH 6.6, the kinetic law for the rate of change in NADH concentration, as measured by the change in absorbance at 380 nm is:

$$(v - v_b)^{-1} = (E_t\ k_{cat}/K_{mA})^{-1}A^{-1} + (E_t\ k_{cat}/K_{mB})^{-1}B^{-1} + (E_t\ k_{cat}/K_{mB}K_{ia})^{-1}(AB)^{-1} + (E_t\ k_{cat})^{-1}$$

where E_t is the enzyme concentration, A is the concentration of NADH, B the concentration of pyruvate, $v = -dA/dt$ is the rate of conversion of NADH to NAD$^+$, and v_b is the background rate of loss of NADH in the absence of enzyme, which can be rapid at 55 °C.

When A is large compared to K_{mA} and B is small compared to K_{mB}, then

$$(v - v_b)^{-1} = (E_t\ k_{cat}/K_{mB})^{-1}B^{-1}(1 + [K_{ia}/A])^{-1}$$

and if A is further large compared to K_{ia}, then

$$v = -dA/dt = (E_t\ k_{cat}/K_{mB})B = v_b$$

where v_b will be a constant if A is so large that the loss of A during the experiment is negligible. Then the integrated expression becomes:

$$A = A_0 - B_0(1 - e\text{-}[E_t k_{cat}/K_{mB}]\ t) - v_b t$$

Thus the absorbance at 380 nm will relax from the initial absorbance in a first-order manner to a zero-order loss of absorbance as a result of the background reaction. The time constant for the relaxation process is $(E_t k_{cat}/K_{mB})$.

Results

Kinetics

Figure 1 shows the time course at 55 °C of absorbance at 380 nm for A = [NADH] = 3 mM (*ca* 15-fold the value of K_{mA} and 50-fold the value of K_{ia} determined previously[5]), B = [pyruvate] = 52 µM (one-tenth the value[5] of K_{mB}) in the presence of lactate dehydrogenase from *Bacillus stearothermophilus* at pH 6.0. As expected from the treatment in Chart 1, the system undergoes first-order relaxation from the initial absorbance to the zero-order rate of degradation of NADH. The time constant for exponential relaxation should be $E_t k_{cat}/K_{mB}$. The value thus obtained in this work with commercial enzyme for k_{cat}/K_{mB} in protium oxide is $5.5 \pm 0.5 \times 10^6$ M^{-1}s^{-1}, in adequate if unimpressive agreement with the value of $16 \pm 12 \times 10^6$ M^{-1}s^{-1} obtained from steady-state kinetic studies[5] with highly purified enzyme from Prof. H. Zuber.

Proton inventory

Figure 2 shows the values of the first-order relaxation rate constants obtained at five mole fractions of DOD. The overall solvent isotope effect is 0.87 ± 0.09.

Discussion

Absence of a large solvent isotope effect

The absence of a normal solvent isotope effect of 2–4 and indeed the observation of a slightly faster rate in DOD suggests that protonic bridging is not occurring in the transition-state for hydride-transfer, and thus that hydride-transfer is not assisted by general acid catalysis. Although this conclusion must be attenuated in force as described below, it is the most plausible deduction from the observations, and its implications may be considered briefly.

In the absence of general acid protonic bridging (which itself may possibly but not necessarily[8] involve proton-transfer concerted with hydride-transfer), proton-transfer to the keto/hydroxyl oxygen of pyruvate/lactate must occur either before or after hydride-transfer. If the proton-transfer precedes hydride-transfer in the direction of pyruvate reduction, then an oxonium intermediate is generated. If the LDH active site was fully exposed to the aqueous environment, the attractive electrostatic interaction between the oxonium center and the transferring hydride ion would be reduced by the higher dielectric constant of an aqueous environment in comparison to a protein environment from which water has been excluded. In addition, such a reactive species as an oxonium ion might well undergo abortive nucleophilic attack by water or buffer species. If the proton-transfer succeeds hydride-transfer, then negative charge will accumulate on the substrate keto-oxygen in the transition-state, with stabilization of the transition-state

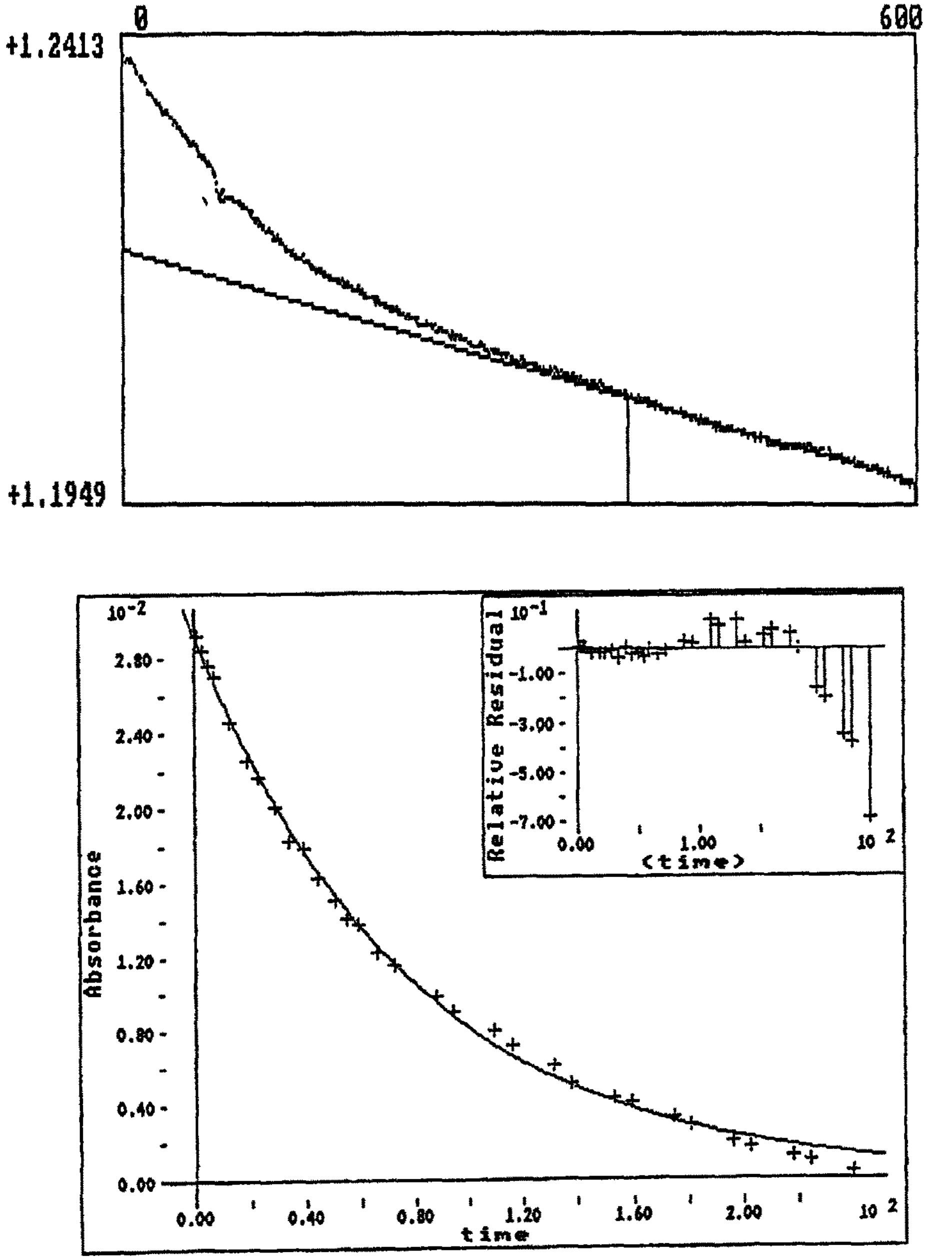

Figure 1. Upper panel: absorbance at 380 nm as a function of time (in hundreds of seconds) in a typical experiment. Lower panel: Fit of an exponential function to $[abs - abs(t = 0) - v_b t]$ versus t, where v_b is the slope of the linear part of the data at long times.

resulting from electrostatic interaction with the cationic His-imidazolium. If the LDH active site was fully exposed to the aqueous environment, then the stabilizing electrostatic interaction would again be reduced as described for an oxonium-like transition-state.

Both of these arguments suggest that, particularly if general acid catalysis is not the source of transition-state stabilization in the hydride-transfer transition state for LDH action, then the isolation of the LDH active site from the aqueous environment should lead to more effective transition-state stabilization and/or to the avoidance of abortive diversion of reaction intermediates. This is entirely consistent with a great variety of structural and dynamical information[2,3] that connects catalysis by LDH with the opening and closing of an active-site peptide loop (approximately residues 98–110). This loop is generally presumed to close across the active site during the hydride-transfer event, sequestering the reactants from the external environment, much as in the paradigmatic case of the similar device in triosephosphate isomerase.[9]

Thus the simplest interpretation of these results, that general acid catalysis is not the source of catalytic power in the LDH acceleration of hydride-transfer from NADH to pyruvate in the ternary enzyme–substrate complex, leads to a model in accord with existing structural and dynamic information. However, as we shall now see, general acid catalytic bridging in the hydride-transfer transition state still cannot be excluded with strong reliability.

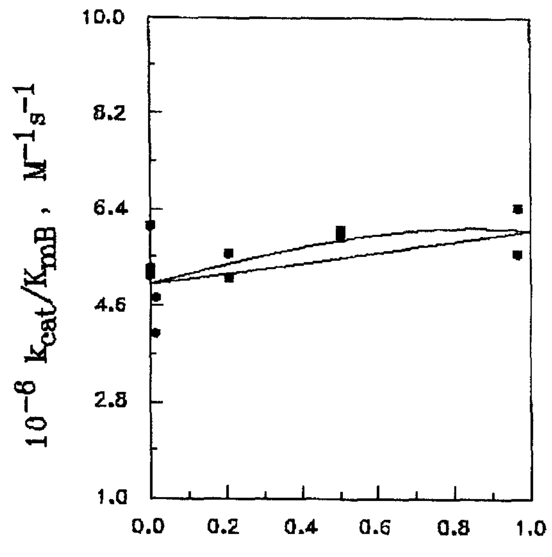

Figure 2. Proton inventory for the rate constant k_{cat}/K_{mB}. The upper curve is the function $k_n = k_0(1 - n + n/2)(2.4)^n$. The lower curve is the function $k_n = k_0(1.2)^n$.

Possibility of opposing isotope effects

Chart 2 outlines the expectations for proton inventory experiments on the basis of various models. If a single-site isotope effect is seen, as in donation of histidinium proton to pyruvate carbonyl concerted with hydride-transfer, then a linear dependence of rate constant on n is expected. If multi-site isotope-effects arise, an exponential dependence is expected.

Most importantly, both single-site and multi-site effects may arise simultaneously. When a single-site effect and a multi-site effect of roughly equal magnitude but opposite direction (one normal, the other inverse) occur, a curious phenomenon ensues. The overall solvent isotope effect — the ratio of rates in pure HOH and pure DOD — will be close to unity, or exactly unity if the opposing effects are of equal magnitude. But in mixtures of HOH and DOD, the cancellation does not hold throughout. For example, the last equation in Chart 2 shows that opposing multi-site and single-site effects of 2 will produce a partial isotope effect at $n = 0.5$ (a midpoint isotope effect) of $k_{0.5}/k_0$ of 1.06. Opposing effects of 3 will produce a midpoint isotope effect of 1.15, and opposing effects of 4 will produce a midpoint isotope effect of 1.25. Thus for a case in which a general acid proton-bridging normal isotope effect of 2–4 is opposed by an multi-site isotope effect (e.g. from a conformational change of the protein), the rate constants in HOH and DOD will be identical but the rate constants will be larger by up to 25 % in mixtures of HOH and DOD.

Conclusion

Figure 2 shows the proton inventory data compared to two models. In one, the small inverse isotope effect is assumed to arise from a multi-site model (generalized conformation or solvation change). In the other, a transition-state one-site normal isotope effect of 2 is assumed to be opposed by a multi-site effect of 2.4. Neither model can be excluded by the data. Thus while the simplest explanation of our findings may be an absence of general acid catalysis, no definite conclusion to this effect may be reached.

Chart 2. Proton-inventory expectations[7]

The Gross–Butler equation describes the expected general dependence of a rate constant on the mole fraction of deuterium n in the aqueous medium:

$$\frac{k_n}{k_o} = \frac{\prod(1-n+n\varphi_i)}{\prod(1-n+n\varphi_j)} = \frac{TSC}{RSC}$$

where the ϕ_i and ϕ_j are isotopic fractionation factors (reciprocal equilibrium isotope effects for exchange of deuterium out of bulk water into the *i*th and *j*th sites), *TSC* means 'transition-state contribution' and RSC means 'reactant-state contribution.'

General-acid catalysis (single-site isotope effect)

If there is only one transition-state site that contributes an isotope effect and all other sites have either $\phi = 1$ or $\phi_i = \phi_j$, then the Gross–Butler equation reduces to:

$$\frac{k_n}{k_o} = (1-n+n/[k_H/k_D]).$$

Here ϕ for the single contributing site has been replaced by the reciprocal isotope effect generated at that site.

This is the result expected for simple protonic bridging at one exchangeable site (general acid catalysis).

Generalized conformation or solvation change (multi-site isotope effect)

If many sites each contribute a small isotope effect, so that all the ϕ are close to unity then:

$$\prod(1+n[\varphi-1]) \approx \prod e^{n[\varphi-1]} = e^{n\Sigma[\varphi-1]} = Z^n$$

In such a case, either *TSC* or *RSC* or both become exponential functions of n.

Combined single-site and multi-site effects

Both single-site and multi-site isotope effects may occur together:

$$\frac{k_n}{k_o} = (1-n+n/[k_H/k_D])z^n.$$

Experimental Section

Materials

Lactate dehydrogenase (EC 1.1.1.27) from *Bacillus stearothermophilus* was obtained from Sigma (catalog no. L-3514), as was NADH (β-nicotinamide adenine dinucleotide disodium salt), fructose-1,6-bisphosphate, dithiothreitol (DTT), bovine serum albumin (BSA) and

ADA (*N*-[2-acetamido]-2-iminodiacetic acid monosodium salt). Deuterium oxide was obtained from Aldrich.

Kinetic measurements

Rates of conversion of NADH to NAD^+ were measured at 380 nm (0.5 cm, 1 mL cuvet) with use of a Cary 118 or Shimadzu UV160 spectrophotometer interfaced to a Zenith Z-158 microcomputer for storage of digital absorbance/time data. The cell-compartment temperature was maintained at 55.0 ± 0.5 °C with a Lauda circulating thermostat. Solutions contained 3 mM NADH, 2 mM FBP, 1.5 nM LDH (formal dimer concentration), 52 μM pyruvate initially and were buffered at pH 6.00 (pD 6.50) with ADA. Also present from the enzyme stock solution were 18 $\mu g\ mL^{-1}$ DTT and 9 $\mu g\ mL^{-1}$ BSA. In isotopic solvent mixtures, the pL was maintained at the 'corresponding' value (equivalent points on pL/rate profile) by using a constant ratio of buffer components in all solvents.[7c] Enzyme stock solutions for all runs were prepared with n = 0.50; the 55-fold dilution of enzyme stock solution makes the effect on isotopic composition of the solvent negligible. Solutions containing enzyme, buffer and FBP were incubated for 3–5 min at 55 °C, then NADH was added and the solutions were incubated a further 10 min (a non-linear phase in the spontaneous decomposition of NADH at this temperature passes during this time). Reactions were then initiated by addition of pyruvate and mixing.

Data reduction

The absorbance at 380 nm was fitted by non-linear least squares to the equation

$$abs = abs(t{=}0) + \alpha\,(1 - e^{-\beta t}) + v_b t$$

where α measures the decrease in absorbance from the complete reduction of pyruvate and $\beta = E_t k_{cat}/K_{mB}$ (see Chart 1).

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8. General acid–base catalysis does not imply net proton transfer in the step that experiences general catalysis, only that protonic bridging occurs in the transition-state (most common models for general catalysis assume net proton-transfer but nothing requires it: see the discussion in Schowen, R.L. *Mechanistic Principles of Enzyme Activity*, pp 119–168, Liebman, J.F.; Greenberg, A., Eds; VCH Publishers: New York, 1988, especially p. 165). In LDH action, net proton transfer of course occurs at some point in the conversion of pyruvate to lactate, but there is no way at present to know when.

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