Isotope Effects and Temperature Dependences in the Action of the Glucose Dehydrogenase of the Mesophilic Bacterium Bacillus megaterium

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Abstract

The glucose dehydrogenase of the mesophilic bacterium Bacillus megaterium (optimal growth around 35 °C) exhibits non-linear Eyring temperature dependences from 25 to 55 °C in its catalysis of the oxidation by hydride-transfer to NAD$^+$ of the $\beta$-anomers of 1-\textit{\textit{\textdollar}}D-glucose and 1-\textit{\textdollar}D-glucose (rate constant $k_{\text{cat}}/K_M^{\beta}$). A break around 300K separates a high-T region from a low-T region. In the high-T region, isotopic enthalpies of activation within a considerable experimental error are equal to zero. In the low-T region, the enthalpies of activation are roughly equal for the isotopic substrates but are different from zero. An alternative treatment with Eyring plots taken as effectively linear produces enthalpies of activation having the unusual feature of being larger for the H-substrate (26 kJ/mol) than for the D-substrate (21 kJ/mol). Compensation of the enthalpic effect by a more positive entropy for the H-substrate then reproduces the isotope effects. For oxidation by NADP$^+$ of the same pair of isotopic glucose substrates, catalysis by the glucose dehydrogenase of Thermoplasma acidophilum, a thermophilic archaeon, leads to temperature dependences characterized by a high-T region and a low-T region separated by a gentle thermal transition (K. Anandarajah, K.B. Schowen, and R.L. Schowen, Z. phys. Chem. 2008, 222, 1333–1347). Tentative approaches to a mechanistic interpretation of both cases rely on models featuring configurational searches of the enzyme for tunneling states, followed by hydrogen-transfer tunneling, although explanations can be constructed also on the basis of simple transition-state stabilization without tunneling.

Keywords

enzyme-catalyzed hydride transfer; isotope effects; temperature dependence; glucose dehydrogenase

Introduction

With our colleagues around the world we mourn the loss our friend Professor Rory Anthony More O’Ferrall, and feel it a privilege to join in the publication of this Memorial Issue for a major figure of 20\textsuperscript{th} – Century physical organic chemistry. Some of Rory’s many and varied contributions to our field addressed isotope effects and their mechanistic interpretation\cite{1–14}. This is an aspect of science which over the past half-century has penetrated, mainly through the medium of physical organic chemistry, deeply into biochemical mechanisms\cite{15}. Our report emphasizes such a biochemical application.

In 2008 we reported\cite{16} temperature-dependence determinations of the protium/deuterium isotope effects for the hydrogen-transfer reaction of 1-$\text{\textdollar}$D-glucose and 1-$\text{\textdollar}$D-glucose with

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the redox cofactor nicotinamide adenine dinucleotide phosphate (NADP), as catalyzed by the glucose dehydrogenase of the archaean *Thermoplasma acidophilum* (the enzyme name abbreviated TaGDH), an organism that has evolved to an optimal growth temperature around 60 °C. Eyring plots were non-linear for the rate constant $k_{\text{cat}}/K_M \beta$, which describes the reaction in which the $\beta$-anomer of the substrate D-glucose binds to the E:NADP complex and is oxidized by hydride transfer from the anomic carbon to the NADP cofactor. A gentle transition suggesting a protein structural change around 37 °C divided a high-temperature region from a low-temperature region.

At high temperatures, the enthalpies of activation for both isotopic substrates were essentially the same and appeared to be smaller than the value expected from the isotope effects of around 1.6 (H/D). The high-temperature isotope effects thus appeared to be underdependent on temperature\(^\text{17–19}\). The entropies of activation differed in such a way that the Arrhenius A-factor isotopic ratio $A_H/A_D$ was 2.0 ± 1.6, consistent with a largely entropic origin for the isotope effect. Such findings have been previously observed in a number of enzyme systems, where the common interpretation has been that the enzyme possesses a binding site that places the substrates in a position that accommodates with little or no energy requirement a hydrogen-transfer event with quantum-tunneling such that the isotopic probability ratio for tunneling generates the observed isotope effect (thus an entropic isotope effect)\(^\text{17–19}\).

At temperatures below the transition temperature, the temperature dependences were consistent with an isotopic difference in enthalpies or energies of activation as large as 6–7 kJ/mol, corresponding to much larger isotope effects (ca. 10 – 12 (H/D)) than the values observed (ca. 2–3 (H/D)). Correspondingly the isotopic differences in entropy of activation were such as to make the Arrhenius $A_H/A_D$ ratio apparently quite small (0.12 ± 0.15), thus correcting the values calculated from the enthalpies back to near the observed values. This behavior represents an overdependence on temperature\(^\text{17–19}\) of the sort seen long ago for non-enzymic reactions and at that time typically treated by the tunneling-correction formalism of Bell\(^\text{20–21}\). This suggested to us that the structural transition around 37 °C had removed the prepared site that facilitated tunneling in the higher temperature regime to which *Thermoplasma* is evolutionarily adapted, with tunneling still being the mode of hydrogen transfer at lower temperatures but in a manner more characteristic of non-enzymic reactions.

In the present work, we present a preliminary study of the situation for a glucose dehydrogenase (BmGDH) from a mesophilic organism, *Bacterium megaterium*, with an evolutionary history mainly at lower temperatures and optimal growth in the neighborhood of 30 °C. Figure 1 gives an idea of the phylogenetic relationship between *Bacterium megaterium* and *Thermoplasma acidophilum*.

**Results**

Figure 2 shows Eyring plots for the rate constants $k_{\text{cat}}/K_M \beta$ of oxidation by the isotopic $\beta$-anomers of D-glucose with catalysis by BmGDH at temperatures from 15 to 40 °C. One approach to the treatment of the data is to note an apparent biphasic nature in both data sets, with a steeper slope at low temperatures going over to a nearly flat dependence at high temperatures. This approach yields the two essentially linear regions shown, which in turn produces the enthalpies and entropies of activation for the two isotopic substrates, as shown in the upper part of Table 1. The lower part of Table 1 gives the quasi-thermodynamic properties of activation for the *Thermoplasma* enzyme TaGDH, to be used in a later comparison.
A simpler approach might be to acknowledge the considerable errors that afflict these data, and treat both data sets as a single linear set. When the rate constants from steady-state experiments are included, with the data for each isotopic substrate taken to form a single monophasic Eyring relationship then the properties shown in the last row for BmGDH in Table 1 (“monophasic treatment”) are found.

**Discussion**

In this section, we first propose tentative ways in which the data for BmGDH, reported in the present paper, might be interpreted in molecular terms. Then we compare briefly these ideas with the mechanistic models for TaGDH, developed earlier and reviewed in the introduction to the present paper. A theme of these models, stemming from Klinman but now widely adopted\[18\], is a hydrogen-transfer event that is preceded by a series of mainly local conformation changes as the enzyme explores structures surrounding the substrate-cofactor assembly in the ternary enzyme complex (a “configurational search”), with the actual transfer occurring by quantum tunneling when the configurational search arrives at a structure that especially favors tunneling. The search itself might be conceived, to give two examples, as a sequence of random motions or a systematic generation of “tunneling states,” such as could arise through molecular evolution. Finally, we consider the possibility that the reaction occurs without tunnelling, with transition-state stabilization by the enzyme (see Liu & Warshel, pp 242 – 267 in ref. [18]).

**Tentative model for the BmGDH data, monophasic treatment (Table 1)**

Table 1 indicates that a simple monophasic Eyring behavior is capable of fitting the BmGDH data but with a feature that may seem difficult to explain. The difference between the enthalpies of activation (5 kJ/mol) is capable of generating an isotope effect of 7 to 8-fold near room temperature but it is the H-substrate that exhibits the larger enthalpy – thus the isotope effect in the absence of entropic contributions would be the reciprocal of 8–9, i.e., a very large inverse isotope effect. The entropic effect is of course large and corresponds to $\Delta H/\Delta S = ca. 20$, so that the observed isotope effects around 2 (H/D) are reproduced.

It might well be possible to rationalize this behavior, although we do so only briefly in the absence of better data (see Concluding Remarks). For example, the catalytic strategy of BmGDH throughout the temperature range studied could involve forcing the H-substrate (on which evolutionary selection has operated) and the NAD$^+$ cofactor into an efficient tunneling configuration. This event could require an enthalpic expenditure relative to the D-substrate of 5 kJ/mol but could result in a higher tunneling probability reflected in the more positive entropy of activation (~ 73 vs. ~ 98 J/K-mol). The lower enthalpy for the D-substrate could result then from a failure of the forcing event to produce efficient tunneling and reaction through a lower-energy channel with less efficient tunneling, consistent with the more negative entropy of activation. To support this idea adequately would involve considerable further experimentation or computation.

**Tentative Model for the BmGDH data, biphasic treatment (Figure 1, Table 1)**

The biphasic model was fit by simply segregating the data into a three point set above about 27 – 29 °C and a three-point set below this temperature range. Each set was then fit to an Eyring expression, producing the results shown in Figure 1 and Table 1, where the large error limits shown in the table reflect largely the sparseness of the two data sets. Here too, only the briefest interpretative scheme will be described.
In the high-temperature range, both H-substrate and D-substrate show very small enthalpies of activation, possibly zero. These are coupled in both cases to very negative values of the entropies. A simple mechanistic model could posit a very mobile enzyme structure at the higher temperatures, yet still containing relatively rare binding sites in which substrate and cofactor are so situated as to favor efficient tunneling. The system executes a search for such a site, which requires little or no energy but has a low probability of success: the barrier to reaction is thus purely entropic. The entropy seems more positive for the H-substrate, as expected for a higher tunneling probability for protium relative to deuterium, once the tunneling configuration has been located and filled by substrate and cofactor.

In the low-temperature range, both isotopic substrates again show roughly the same enthalpy of activation but now the value is considerable in magnitude, seemingly around 42–44 kJ/mol. This value is consistent with the stiffening of enzyme structure at lower temperatures, so that the search for tunneling configurations has a higher enthalpic cost. The search should be the same process for both isotopic substrates, so the enthalpic cost is the same for both. The entropic cost is now much less negative, perhaps suggesting some systematic character in the search, perhaps arising from evolutionary selection. Finally the mean value of the entropy of activation is slightly higher for the H-substrate again consistent with its expected higher tunneling probability once the substrate and cofactor have been installed in the prepared tunneling configuration.

Comparison of the results for the enzymes from *Thermoplasma acidophilum* and from *Bacillus megaterium*

This extremely preliminary study seems to suggest that these two enzymes, catalyzing two near-identical reactions, but bringing to the task two very different evolutionary histories of their host organisms over the past 4 billion years, both seem to employ tunneling mechanisms in catalysis but in ways that respond quite differently to variations in temperature. When truly adequate data become available, it may be possible to reach more exact conclusions about the matter.

Models other than a configurational search leading to tunnelling

The preliminary character of the data reasonably requires that non-tunneling models involving only transition-state stabilization by the enzyme be considered. For the monophasic treatment, the most difficult features to explain by any model are the facts that (a) the enthalpy of activation is substantially larger for the H-substrate than for the D-substrate so that the enthalpic isotope effect is large and inverse and (b) this inverse apparent effect is countervailed by a large entropy difference in the opposite direction. There is basically no strong reason to prefer the tunneling model on this data treatment, other than its capacity to accommodate these kinds of enthalpy/entropy relations more readily than the transition-state stabilization models. For the biphasic treatment at high temperatures, both isotopic values of the enthalpy of activation are roughly equal to zero and the entropies (less negative for the H-substrate) generate the entire isotope effect. This behavior is readily reconciled (see above) with the search/tunneling model. A transition-state stabilization model might also be accommodated with enzymic stabilization just overcoming the enthalpic cost of transition-state formation. The negative entropies might reflect in large part the entropic cost of the interaction, the difference between then reflecting the greater tunneling probability for the H-substrate. The biphasic data at lower temperatures are much more of a familiar shape and can also be rationalized (as above) by the search/tunneling model or on the transition-state stabilization model with both an enthalpic and an entropic component of stabilization. When far better data are in hand, the requisite theoretic work may lead to a distinction.
Experimental Section

Materials

D-(-)-Glucose (product no. G7528), D-glucose-1-d (97% D, 31,081-6), β-NAD+ sodium salt hydrate (N0505), Trizma hydrochloride (T6666), and bovine albumin (A7906) were purchased from SigmaAldridge Co. LLC. Polypropylene sample vials (03-338-1C and 1E) and siliconized polypropylene tubes (05-541) were purchased from Fisher Scientific Co. Nanopure water was buy use of a LABCONCO water purification system.

Glucose dehydrogenase from Bacillus megaterium (EC 1.1.1.47) was purchased from SigmaAldrich Co. LLC. (product no. G7653, chromatically purified, lyophilized powder) and used as supplied. The molar concentration of the enzyme in stock solutions was determined from the measured activity with the activity of pure enzyme taken as 550 units/mg. Activity assay conditions were 100 mM D-glucose, 10 mM NAD+, pH 8.0, 25.0 °C. Enzyme stock solutions contained 1 mg/mL bovine serum albumin and were stored in siliconized polypropylene tubes.

Kinetic measurements

Most of the kinetic determinations were conducted at enzyme concentrations of 50–60 nM, D-glucose concentrations of 10 µM (K_M β = 15 mM, β-β-L-D-glucose, L = h, d), and NAD+ (substrate A) concentrations of 10 mM (K_MA = 0.24 mM). Thus the reaction under observation was EA + β = EP +Q, P = NADH, Q = D-gluconolactone. As was earlier found for the TaGDH, the spectroscopic time-course of NAD+ reduction at 366 nm (Δε = 2808 M⁻¹) exhibits a characteristic form. There is an initial generation of NADH in an exponential wave, corresponding to the pseudo-first-order reaction of EA with the β-anomer of D-glucose in large excess, going over to a much slower linear increase in absorbance as the α-anomer is exhausted and is thereafter generated by a slow, non-enzymic mutarotation of the remaining β-anomer. This process is described by eq. (1):

\[ \text{Absorbance at 366nm/Δε=initial value+β}_0 (1 - \exp[-kt]) + vt \]  

where t is the time from initiation of the experiment, β_0 is the initial concentration of β-anomer in the proio- or deuterio-D-glucose substrate at anomeric equilibrium, k = (k_cat/ K_M β)e_0 with e_0 the total enzyme concentration which will be equal to [EA] under the reaction conditions, and v = (d [Q]/dk) where is the concentration of the enzymically inactive β-anomer of the labeled D-glucose and k is the rate constant for first-order, non-enzymic conversion of the β-anomer to the β-anomer. The β-anomer is then rapidly consumed in the enzymic reaction so that the anomeric equilibrium is never achieved. Since Q_0 is less than K_M β 15,000 and [NAD+] is around 40K_MA, the use of eq. (1) leads to a good value of k_cat/K_M β for each isotope substrate. Complete steady-state analyses were conducted at three temperatures and roughly confirmed the values of k_cat/K_M β and the isotope effects from the transient-phase analyses, but the experimental errors were relatively large.

Concluding Remarks

Readers of the Journal of Physical Organic Chemistry will have noted deficiencies in the data shown in Figure 1 and Table 1. The experimental measurements are too sparse and will require supplementation with more measurements before confident conclusions can be reached. The error estimates both for measurements and for calculated values are larger than would be necessary for reaching confident conclusions. Beyond these problems, we do not know whether the small isotope effects observed are purely effects for the hydrogen-transfer
step (intrinsic isotope effects) and to ascertain the true situation would be a very considerable undertaking.

We, however, are in no position to address these deficiencies: Dr. Anandarajah died tragically several years ago. The laboratories of KBS and RLS have long been closed. Our reason for publishing the data at present is that we believe they suggest, even in their imperfect state, some fascinating properties of these two systems so that pursuit of a complete characterization could be rewarding. It is our hope that interested persons will give the problem whatever effort they feel it merits.

Acknowledgments

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References

19. See particularly Ch. 6 and Ch. 13 in Ref. 18.
Figure 1.
Schematic representation of the phylogenetic “tree of life” as determined from comparison of small-subunit ribosomal RNA sequences[22], illustrating the separation in phylogenetic terms between the two organisms that host the two glucose dehydrogenases we compare in this paper. Note that (a) Thermoplasma acidophilum is included in the group of extreme halophiles at upper right (first entry, with single asterisk, under the Euryarchaeota; (b) Bacillus megaterium (this work) is among the Bacteria at lower left, specifically the Gram-positive bacteria, fourth entry, bearing a double asterisk. According to the Time Tree[23] website (www.timetree.org) these two organisms last shared an evolutionary ancestor about 4 billion years ago.
Figure 2.
Eyring plots of the rate constants for reaction of 1-<i>h</i>-D-glucose (filled squares) and 1-<i>d</i>-D-glucose (filled circles) with the BmGDH:NAD<sup>+</sup> complex at temperatures from 15 to 40 °C. Both isotopic data sets appear to fall into a high-T range with very small or zero slopes and a low-T range with larger (and roughly equal slopes). Table 1 shows the enthalpies and entropies of activation calculated from this treatment of the data. Error limits are omitted for clarity but are large; errors in the rate constants ranged from 7% to 18% and averaged 11%. See the Discussion for alternative methods of treating the data.
Table 1
Comparison of the Temperature Dependences of the Primary Isotope Effects for Oxidation of \(1-L-\beta-D\)-glucose \((L = h, d)\) with Catalysis by the Glucose Dehydrogenases of the Mesophile *Bacillus megaterium* (this work) and the Thermophile *Thermoplasma acidophilum*[^16]

<table>
<thead>
<tr>
<th>Temperature regime</th>
<th>Quasi-thermodynamic properties of activation ((k_{cat}/K_M)^\circ) for oxidation of (1-L-\beta-D)-glucose ((L = h, d)) by NAD(^+) with catalysis by the glucose dehydrogenase of <em>Bacillus megaterium</em>[^a]</th>
<th>(\Delta^\circ H) kJ/mol</th>
<th>(\Delta^\circ S) J/K-mol</th>
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<tbody>
<tr>
<td>High-T: &gt; 300 – 302 K ((27 – 29 , ^\circ C))</td>
<td>2.1 ± 5.4 0.42 ± 8.0 –152 ± 18 –164 ± 26</td>
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<tr>
<td>Low-T: &lt; 300 – 302 K ((27 – 29 , ^\circ C))</td>
<td>42 ± 10 44 ± 12 – 18 ± 34 – 21 ± 41</td>
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<tr>
<td>Monophasic treatment with inclusion of steady-state data</td>
<td>26 ± 3 21 ± 4 – 73 ± 10 – 98 ± 11</td>
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<th>Temperature Regime</th>
<th>Quasi-thermodynamic properties of activation ((k_{cat}/K_M)^\circ) for oxidation of (1-L-\beta-D)-glucose ((L = h, d)) by NADP(^+) with catalysis by the glucose dehydrogenase of <em>Thermoplasma acidophilum</em>[^b]</th>
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</tr>
</thead>
<tbody>
<tr>
<td>High-T: &gt; 310 K ((37 , ^\circ C))</td>
<td>30 ± 5 28 ± 8 – 58 ± 13 – 69 ± 23</td>
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<tr>
<td>Low-T: &lt; 310 K ((37 , ^\circ C))</td>
<td>54 ± 5 55 ± 2 + 14 ± 5 + 137 ± 8</td>
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</table>

[^a]: Conditions: pH 8.0 (0.1 M Tris); 10 mM NAD\(^+\); 10 µM D-glucose; [BmGDH] 1.32–3.68 µM.

[^b]: Conditions: pH 7.0 (0.1 M BisTris); 0.8 mM NADP\(^+\); 10 µM D-glucose; [TaGDH] 0.87–2.96 µM.