Effects of Macromolecular Crowding on Alcohol Dehydrogenase Activity Are Substrate-Dependent

A. E. Wilcox, Micaela A. LoConte, and Kristin M. Slade*

Department of Chemistry, Hobart and William Smith Colleges, Geneva, New York 14456, United States

ABSTRACT: Enzymes operate in a densely packed cellular environment that rarely matches the dilute conditions under which they are studied. To better understand the ramifications of this crowding, the Michaelis–Menten kinetics of yeast alcohol dehydrogenase (YADH) were monitored spectrophotometrically in the presence of high concentrations of dextran. Crowding decreased the maximal rate of the reaction by 40% for assays with ethanol, the primary substrate of YADH. This observation was attributed to slowed release of the reduced $\beta$-nicotinamide adenine dinucleotide product, which is rate-limiting. In contrast, when larger alcohols were used as the YADH substrate, the rate-limiting step becomes hydride transfer and crowding instead increased the maximal rate of the reaction by 20−40%. This work reveals the importance of considering enzyme mechanism when evaluating the ways in which crowding can alter kinetics.

Cellular concentrations of proteins, nucleic acids, and polysaccharides are tens to hundreds of times greater than those employed for the dilute conditions used in most biophysical studies.\(^1\)\(^-\)\(^3\) To better mimic and understand the ramifications of this densely packed environment, high concentrations of polymers such as dextran, ficoll, or polyethylene glycol are often added to in vitro conditions. These experiments, in combination with theoretical and computational work, have revealed that macromolecular crowding significantly alters protein dynamics, diffusion, and chemical equilibria, as well as other biophysical properties.\(^4\)\(^-\)\(^6\)

Recent experiments have applied these techniques in combination with Michaelis–Menten kinetics to better understand how crowding affects enzyme activity.\(^7\)\(^,\)\(^8\)

In general, crowding has little to no effect on the Michaelis constant, $K_m$, of enzymes.\(^9\) Several studies with diffusion-limited enzymes report small increases in $K_m$ due to diffusion resistance.\(^10\)\(^-\)\(^12\) In the overwhelming majority of cases, however, the presence of crowders results in slight decreases in $K_m$.\(^9\)\(^,\)\(^13\)\(^-\)\(^16\) This reduction is frequently attributed to changes in activity coefficients and increased effective concentrations that can enhance substrate binding.\(^17\)\(^-\)\(^20\)

In contrast to $K_m$, the effects of crowding on $V_{max}$, the maximal velocity, are more varied and appear to depend on numerous, sometimes competing factors. For example, crowding has been reported to decrease enzyme activity by enhancing product inhibition,\(^21\) changing solution polarity,\(^18\) or slowing diffusion\(^10\)\(^,\)\(^22\)\(^,\)\(^23\) and thus reducing the frequency of substrate–enzyme encounters.\(^12\)\(^,\)\(^24\)\(^,\)\(^25\) At the same time, enhancements in enzyme activity can occur due to conformational changes,\(^26\)\(^-\)\(^29\) restricted internal dynamics,\(^20\) or changes in oligomerization state.\(^30\) Because the relative influence of these factors is not well understood, crowding trends for enzyme kinetics are difficult to predict. Studies have begun to investigate how enzyme size and crowding agent properties determine the dominant factors in crowding effects,\(^31\)\(^-\)\(^33\) yet to the best of our knowledge, no one has directly examined the role of enzyme mechanism. Fortunately, yeast alcohol dehydrogenase (YADH) provides a unique opportunity to do so because the rate-limiting step of the mechanism depends on its substrate.

YADH is a 150 kDa tetramer that catalyzes the reversible oxidation of an alcohol (S) to an aldehyde or ketone (P) using the cofactor NAD$^+$ as illustrated in Scheme 1. The reaction consists of a stereospecific hydride transfer from the alcohol to the cofactor and a series of conformational changes that are likely to be influenced by crowding.\(^34\) For the natural substrate ethanol (EtOH), the slow step of the mechanism is the release...
of the NADH product.\textsuperscript{35} For larger alcohols, such as isopropanol and benzyl alcohol, the hydride transfer becomes rate-limiting.\textsuperscript{36–38} Thus, this system can provide further insight into whether the rate-limiting step (RLS) of a single enzyme plays a role in determining how crowding influences enzyme kinetics.

\section*{EXPERIMENTAL PROCEDURES}

\textbf{Chemicals.} Isopropanol, isopropanol-\textsubscript{d\textsubscript{6}}, \(\beta\)-nicotinamide adenine dinucleotide hydrate (NAD\textsuperscript{+}), bovine serum albumin (BSA) as purified lyophilized powder, alcohol dehydrogenase (EC 1.1.1.1) from \textit{Saccharomyces cerevisiae} (YADH, 361 units/\text{mg}) as a lyophilized powder, and dextran polymers from \textit{Leuconostoc mesenteroides} (\textit{~}9–11, 150, and 200 kDa) and \textit{Leuconostoc} spp. (\textit{~}40 and 450–650 kDa) were obtained from Sigma-Aldrich. Dextran polymers from \textit{L. mesenteroides} were also purchased from Molecular Probes (\textit{~}86 kDa). \(\beta\)-Nicotinamide adenine dinucleotide disodium salt trihydrate, in its reduced form (NADH), was purchased from Amresco. Absolute anhydrous ethanol was purchased from Pharmco-Aaper, and anhydrous ethanol-\textsubscript{d\textsubscript{6}} was from Cambridge Isotope Laboratories. Glucose and sodium pyrophosphate were from Acros Organics. Solutions were prepared with 100 mM pyrophosphate buffer (pH 8.9), and the pH of crowding agent solutions was corrected to 8.9 before their use.

\textbf{Michaelis–Menten Kinetic Assays.} YADH activity at 25 \(^\circ\text{C}\) was determined by monitoring the change in NADH absorbance at 340 nm every 6 s for 6 min in a 96-well plate with a Molecular Devices SpectraMax 190 spectrophotometer while the plate was being shaken. Each well contained 110 mM ethanol or ethanol-\textsubscript{d\textsubscript{6}}, 0.125–4.00 mM NAD\textsuperscript{+}, 0.024 unit/\text{mL} (0.45 nM) YADH, and 300 g/L crowding agent. The YADH stock solution was prepared by adding 8 \(\mu\text{L}\) of a 2 g/L enzyme solution to 5992 \(\mu\text{L}\) of 1 g/L BSA, which served to stabilize and maintain enzyme activity.\textsuperscript{39} Addition of ethanol initiated the enzymatic reaction, but adding the reagents in a different order did not have an effect on the resulting rate. The assay was repeated using 550 mM isopropanol or isopropanol-\textsubscript{d\textsubscript{6}} in place of ethanol and 0.25–12.5 mM NAD\textsuperscript{+}. The isopropanol stock solution was kept warm throughout the experiment to prevent precipitation. Addition of YADH initiated the reaction. In the absence of YADH or one of the substrates, the absorbance in the well did not change with time, indicating no detectable enzyme activity. For a given set of conditions, an assay was repeated eight times.

\textbf{Michaelis–Menten Data Analysis.} Because saturating alcohol concentrations were employed, the YADH-catalyzed oxidation of ethanol or isopropanol by NAD\textsuperscript{+} can be treated as single-substrate reactions with the following scheme:

\[
E + S \xrightleftharpoons[k_{-1}]{k_1} ES \xrightarrow{k_{\text{cat}}} E + P
\]

where E is the enzyme, S is the substrate, and \(k_{1}, k_{-1}, \text{ and } k_{\text{cat}}\) are rate constants as first described by Brown.\textsuperscript{40} The maximal rate, \(V_{\text{max}}\), and the Michaelis constant, \(K_m\), can then be determined from the Michaelis–Menten equation:\textsuperscript{41}

\[
v_0 = \frac{V_{\text{max}} [S]}{K_m + [S]} \quad (2)
\]

Initial enzymatic rates, \(v_0\), were obtained by taking the maximal slopes from absorbance versus time plots using SoftMax Pro version 6.3. A single Michaelis–Menten curve was then constructed by averaging eight initial rates per substrate concentration [S], for eight concentrations, using SigmaPlot to determine \(K_m\) and \(V_{\text{max}}\). \(K_m\) and \(V_{\text{max}}\) values obtained under crowded conditions were normalized to the values obtained in buffer only, yielding relative kinetic values.

\textbf{Benzyl Alcohol YADH Kinetic Assays.} The reaction progress of YADH was followed using a 96-well plate Biotek Eon spectrophotometer. Each well contained 75 mM benzyl alcohol or ethanol, 1.5 mM NAD\textsuperscript{+}, and 12 units/\text{mL} (0.225 \(\mu\text{M}\)) YADH in the presence of 300 g/L glucose or dextran. Concentrations of all substrates were chosen to be saturating, and 100 mM pyrophosphate buffer (pH 8.9) was used to bring the final volume to 200 \(\mu\text{L}\). All solutions were kept on ice to preserve their integrity. The YADH stock was prepared by adding 1.2 \(\mu\text{L}\) of a 1 g/L solution of YADH to 1.8 g/L of 1 g/L BSA, which served to stabilize and maintain enzyme activity. Immediately following the addition of 10 \(\mu\text{L}\) of the YADH stock to the assay, the rate of NADH formation was measured at 340 nm every 40 s for 6 min. All assays were repeated 18 times. Initial enzymatic rates were obtained by taking the maximal five-point slopes from absorbance versus time plots using Gen5 software. These initial rates recorded in the presence of glucose or dextran were then normalized to the initial rates obtained in buffer only, yielding relative rates.

\textbf{Fluorescence Studies.} All fluorescence experiments were performed at 25 \(^\circ\text{C}\) using a PerkinElmer LS 45 fluorimeter with excitation and emission slits set to 10 nm. A 0.09 \(\mu\text{M}\) YADH solution was titrated with glucose, 150 kDa dextran, or buffer. To monitor the resulting tryptophan fluorescence, an excitation wavelength of 295 nm was used to record emission spectra from 300 to 400 nm. Fluorescence intensities from both titrations were corrected for dilution.

\textbf{Coenzyme Binding.} Because coenzyme binding quenches YADH fluorescence, an excitation wavelength of 295 nm and an emission wavelength of 340 nm were used to monitor tryptophan fluorescence. Titration experiments were performed by adding small aliquots of 75.4 mM NAD\textsuperscript{+} or 2.11 mM NADH to 3.0 \(\mu\text{L}\) of 0.90 \(\mu\text{M}\) YADH in 100 mM pyrophosphate buffer (pH 8.9) in the presence or absence of 300 g/L glucose. Data were analyzed as previously described.\textsuperscript{42} In short, background readings were subtracted and corrections made to compensate for enzyme dilution. The fractional saturation of coenzyme, \(\theta\), was determined from

\[
\theta = \frac{F^0 - F}{F^0 - F'} \quad (3)
\]

where \(F^0\) is the fluorescence intensity in the absence of coenzyme, \(F\) is the fluorescence intensity at saturating concentrations, and \(F\) is the intensity after addition of a given concentration of coenzyme, [C]. SigmaPlot software was used to determine the dissociation constant, \(K_{\text{D}}\), from...
RESULTS

The initial rates for the YADH-catalyzed oxidation of alcohol were monitored spectroscopically for varying concentrations of NAD⁺. The resulting Michaelis–Menten curves (Figure 1) provided $K_m$ and $V_{max}$ parameters. To account for day-to-day variability in the assays, relative values were determined by dividing the $K_m$ and $V_{max}$ parameters obtained in glucose or dextran by values obtained concurrently in the absence of these sugars.

The results indicate that the effects of crowding depend on the alcohol used as the YADH substrate. While the presence of glucose or dextran decreases the rate of YADH activity for ethanol oxidation, these sugars tend to enhance YADH activity for isopropanol oxidation (Figure 2A). Similar rate enhancements were also observed when benzyl alcohol was used as the YADH substrate (Figure 3). The relative $K_m$ values for NAD⁺ are consistently larger when isopropanol is used as the substrate in place of ethanol (Figure 2B). While Figure 2B is suggestive of a potential dextran size dependence for ethanol oxidation,

$$\theta = \frac{[C]}{K_D + [C]}$$

(4)

Figure 1. Concentration-dependent effects of dextran on YADH Michaelis–Menten plots. Assays containing YADH, (A) ethanol or (B) isopropanol, and varying concentrations of NAD⁺ were performed in the presence of 0 (black), 25 (green), 100 (purple), 200 (blue), or 300 g/L (red) 150 kDa dextran in 100 mM pyrophosphate buffer (pH 8.9). Error bars represent standard deviations ($n = 8$).

Figure 2. Effects of crowding on YADH kinetic parameters depend on the substrate and are independent of dextran size. Assays containing YADH, ethanol (black) or isopropanol (red), and varying concentrations of NAD⁺ were performed in the presence of 300 g/L glucose or various sizes of dextran (kilodaltons). (A and C) $V_{max}$ and (B and D) $K_m$ values from the resulting eight-point Michaelis–Menten curves were normalized to values acquired in the absence of dextran or glucose to yield the relative kinetic constants shown above ($y$-axis). Error bars represent ± one standard error ($n = 8$). Asterisks (*$p < 0.0003; **p < 0.06; ***p < 0.1$) indicate a significant difference in the relative $V_{max}$ of (A) ethanol vs isopropanol or (C) dextran vs glucose (Student’s two-tailed t test). For ethanol, Student’s two-tailed t tests failed to show any statistical difference between the relative (C) $V_{max}$ (all $p$ values of >0.1) or (D) $K_m$ (all $p$ values of >0.5) for any two sizes of dextran.

Figure 3. Effects of crowding on YADH activity are altered when benzyl alcohol is substituted for ethanol substrate. YADH assays containing saturating concentrations of either ethanol (black) or benzyl alcohol (red) and NAD⁺ were monitored in the presence of 300 g/L glucose or 150 kDa dextran. These initial rates were divided by initial rates obtained in buffer only to yield relative rates ($y$-axis). Error bars represent ± one standard error ($n = 18$). To compare the ethanol and benzyl alcohol rates, $p$ values (two-tailed) were calculated for t tests assuming equal variance.
testing a wider range of dextran dimensions reveals that crowding effects on both $V_{\text{max}}$ and $K_m$ are independent of polymer size (Figure 2 C,D).

To confirm that the opposing crowding effects with ethanol and isopropanol are due to different rate-limiting steps of the enzyme, the YADH assays were repeated with deuterated substrates (Figure 4). No difference in relative $V_{\text{max}}$ values should be observed with different alcohol isotopes if crowding primarily impedes NADH release. In contrast, if crowding instead influences hydride transfer, different relative $V_{\text{max}}$ values are likely, in part, because deuterium requires a donor–acceptor distance (DAD) for the hydrogen tunneling process shorter than that of hydrogen. For isopropanol, the data are consistent with the latter, showing that the relative $V_{\text{max}}$ value with hydrogen was only two-thirds the value of the perdeuterated substrate. In contrast, the presence of dextran decreased the YADH activity with ethanol by 40% regardless of whether the alcohol was deuterated. The drastic increase in the relative $V_{\text{max}}$ when deuterating isopropanol contrasted with the lack of an effect for ethanol suggests that the implications of crowding on YADH kinetics are related to its rate-limiting step.

The decrease in $K_m$ values observed with crowding is frequently attributed to enhanced substrate–enzyme binding. To investigate this possibility, ligand binding curves generated from fluorescence measurements were used to determine the dissociation constant, $K_D$, of NAD$^+$ for YADH (Figure 5). Because tryptophan fluorescence of YADH is quenched by coenzyme binding, titrating this enzyme with increasing concentrations of NAD$^+$ resulted in a decrease in fluorescence intensity (Figure 6). Under dilute conditions (absence of glucose or dextran) with isopropanol as the substrate, the resulting $K_D$ value was within error of the $K_m$ for NAD$^+$ ($p$ value = 0.9, from a Student’s two-tailed $t$ test), yet when ethanol was the substrate, the $K_m$ $K_m$ value was statistically different ($p$ value = 0.002, from a Student’s two-tailed $t$ test) from the corresponding $K_D$ value (Table 1). Furthermore, the presence of glucose had little to no influence on YADH–coenzyme binding with a relative $K_D$ for NAD$^+$ of $0.91 \pm 0.03$. Corresponding values could not be obtained with dextran solutions because the first addition of NAD$^+$ to a YADH solution containing dextran resulted in an increase in fluorescence intensity, rather than the expected decrease observed in buffer (Figure 6A). Similar effects were observed for NADH titrations with YADH in the presence of dextran (Figure 6B).

On the basis of the drastic difference in YADH tryptophan fluorescence in the presence and absence of dextran (Figure 6), additional fluorescence experiments were conducted to investigate the effects of this polymer on YADH. The combined intensity decrease and red-shift in fluorescence with increasing 150 kDa dextran concentration indicate a change in the microenvironment of the YADH tryptophan residues (Figure 7). In contrast, the YADH fluorescence is essentially unaltered by the addition of glucose.

**DISCUSSION**

The effects of dextran and glucose on the kinetics of alcohol dehydrogenase depend on the alcohol employed in the assay. This result is most likely due to the substrate-dependent mechanism of YADH. Specifically, crowding decreases the rate of ethanol oxidation, which is limited by product release. In contrast, when YADH exhibits slow catalysis through the use of isopropanol or benzyl alcohol as its substrate, crowding enhances enzymatic activity (Figures 2A and 3). Moreover, the concentration-dependent effects of dextran differ for ethanol (Figure 1A) and isopropanol (Figure 1B). This observation further supports our conclusion that the mechanism by which crowding alters YADH activity is different for ethanol and isopropanol oxidation.

A single study previously called attention to the substrate-specific effects of crowding, attributing the observation instead to differences in the chemical interactions of each substrate with the surrounding crowders. To verify that the substrate-specific crowding effects observed in our study are due to different rate-limiting steps of the enzyme and not differences in the chemical properties of these substrates, the YADH assays were repeated with deuterated alcohols. The rate of hydride transfer during YADH catalysis is affected by the specific isotope used in the alcohol substrate, but the release of NADH from the active site is not. The fact that isotopic substitution of the substrate altered the relative $V_{\text{max}}$ for isopropanol oxidation but not ethanol oxidation (Figure 4) suggests that crowding does not influence the same step of these two reactions even though they are catalyzed by the same enzyme. Taken together with Figures 2A and 3, this work provides the first evidence that effects of crowding on enzyme kinetics depend on the rate-limiting step of the mechanism.
V_max Analysis. Our previous work with YADH used electrochemical experiments to correlate the decrease in V_max for ethanol oxidation in glucose with slowed small-molecule diffusion.23 This observation is consistent with numerous crowding reports that attribute decreased enzymatic activity to the reduced frequency of substrate encounters.12,24,25 For example, lactate dehydrogenase (LDH) operates very near or at diffusion-limited rates,44 such that crowding slows its activity by impeding substrate–enzyme encounters.15 However, this explanation is inappropriate for YADH because ethanol

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<th>K_m (μM) with ethanol</th>
<th>K_m (μM) with isopropanol</th>
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<td>270 ± 90</td>
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Figure 6. Coenzyme binding of YADH. Tryptophan fluorescence (λ_ex = 295 nm; λ_em = 340 nm) of 0.90 μM YADH was monitored with sequential the additions of (A) NAD⁺ or (B) NADH in the presence (red) or absence (black) of 300 g/L 150 kDa dextran.

Figure 7. Effect of glucose and dextran on the tryptophan fluorescence of YADH. A 0.09 μM YADH solution was titrated with (A) glucose or (B) 150 kDa dextran. Using an excitation wavelength of 295 nm, the resulting emission spectra were corrected for dilution. (C) The 150 kDa dextran elicited a detectable 0–14 nm red-shift in the emission wavelength of maximal fluorescence.
oxidation is limited by NADH release. Thus, the decrease in YADH activity is more likely to arise from glucose and dextran impeding diffusion of the product from the active site. This justification is indeed supported by the isopropanol and benzyl alcohol data. When NADH release is not the slow step, both glucose and dextran increase YADH activity instead.

Consistent with our previous work, the relative $V_{\text{max}}$ for ethanol oxidation in the presence of glucose is lower than that for dextrans (Figure 2). We previously proposed that the presence of dextran adds an additional macromolecular crowding effect that partially offsets the decreased activity observed with glucose (from slowed product release). At the time, however, no direct evidence of the specific source of this effect was available. Figure 7 now provides more insight, revealing that dextran affects the YADH structure in ways that its small-molecule counterpart cannot. Specifically, the addition of dextran to YADH solutions results in a red-shift and a decrease in fluorescence intensity, indicating a change in the tryptophan microenvironment. In contrast, the YADH fluorescence is essentially unaltered by the addition of glucose, suggesting the effect with dextran is not due to polarity or other chemical effects. Similar trends in tryptophan fluorescence have been observed for at least four other enzymes, suggesting that crowding commonly induces structural changes in enzymes. This structural change most likely is the system’s way of reducing excluded volume effects and compensating for the additional entropy pressure imposed by crowding.

In contrast, both dextran and glucose have similar effects on isopropanol oxidation, suggesting that the observed rate enhancement is more likely a result of chemical effects than crowding (Figure 2A). Because the rate of the hydride transfer, the slowest step for this mechanism, depends on the distance between the donor and the acceptor within the active site, these sugars likely compact the YADH globule, which subsequently optimizes the transfer distance. This claim is supported by a study analyzing the effects of osmolytes on the rate constants and structure of lactate dehydrogenase (LDH). Specifically, TMAO stabilizes LDH by compacting it into a protein globule, which enhances the hydride transfer crucial for catalysis and decreases the activation energy by 15-fold. While TMAO is chemically distinct from glucose, it exhibits chemical properties comparable to those of the osmolyte betaine, which has been used to study YADH. In fact, betaine and glucose have similar effects on YADH stabilization. Thus, it is likely that the effects of glucose on YADH compaction parallel those observed with LDH in the presence of TMAO. It then follows that the rate enhancement observed with glucose and dextran results from stabilization and compaction of YADH, which streamlines the efficiency of the hydride transfer.

The one exception to the dextran rate enhancement of isopropanol oxidation occurred in the presence of the largest dextran size (Figure 1A, red). At the concentrations of dextran employed in our experiments, theory suggests that polymer entanglement creates a depletion layer that can cage YADH together with substrates and products. This caging could further impede NADH release. Because the depletion layer becomes thicker with increasing polymer size, the largest dextran is presumably the most effective at caging. Consequently, at the largest dextran size, the effect from caging and slowed product release may dominate over the competing effect of the crowding-enhanced hydride transfer. Alternatively, the observed reduction in YADH activity for 450–550 kDa dextran could be a result of enhanced substrate inhibition from caging. The presence of dextran has previously been shown to augment inhibition effects, potentially by increasing the effective concentration of the inhibitor. Furthermore, NAD$^+$ inhibition of YADH has been well documented at high coenzyme concentrations in the absence of crowding. Nonetheless, future experiments are necessary to determine if substrate inhibition, slowed product release, or an additional factor is responsible for the observed decrease in the extent of isopropanol oxidation with large dextrans.

### $K_m$ Analysis

The Michaelis constant is defined by the equation $K_m = (k_{-1} + k_{\text{cat}})/k_f$ using the rate constants in eq 1. For reactions in which the chemistry is slow, $k_{\text{cat}}$ is often small relative to $k_{-1}$, such that the $K_m$ can be approximated as the dissociation constant ($K_D = k_{-1}/k_{\text{cat}}$). As a result of this assumption, improved substrate binding has been widely used to rationalize the observed decreases in $K_m$ with crowding. For YADH, however, this is not an appropriate explanation.

Even in dilute solutions, the $K_m$ value associated with NAD$^+$ is 3-fold greater than the NAD$^+$ $K_m$ value for ethanol oxidation (Table 1), suggesting that $K_m$ reflects more than merely coenzyme binding and that catalysis is not slow enough for $k_{\text{cat}}$ to be negligible in this system. Furthermore, if $K_m$ was a reflection of binding ($K_m \approx K_D$), the $K_m$ values for NAD$^+$ should be similar regardless of the alcohol used in the assay. However, the $K_m$ for NAD$^+$ in dilute solution increases by 3-fold when ethanol is replaced with isopropanol (Table 1).

The observations mentioned above are consistent with our knowledge of the YADH mechanism. It is not surprising that $k_{\text{cat}}$ contributes significantly to $K_m$ for ethanol oxidation, because the chemistry is not slow. In fact, a more complex model derived by Northrop is helpful for analyzing this system in which $k_{\text{cat}}$ is separated into a rate constant for catalysis ($k_1$) and release ($k_3$):

$$E + S \rightleftharpoons_{k_{-1}}^{k_1} ES \rightarrow EP \rightarrow E + P$$

where ethanol is in excess, $E$ is the YADH enzyme, $S$ is NAD$^+$, $P$ is NADH, and $K_m$ is defined by

$$K_m = \frac{k_3(k_{-1} + k_2)}{k_1(k_2 + k_3)}$$

The Michaelis constant for this system can be further simplified to $K_m \approx k_3/k_1$ because product release is slow. Leskovac et al. showed that catalysis is faster than release ($k_2 \gg k_{-1}$). Consequently, the decrease in $K_m$ observed in the presence of glucose or dextran (Figure 2B) is most likely due to impeded product release, $k_3$. This argument is also supported by the fact that crowding has similar effects on the NAD$^+$ $K_m$ values (Figure 2B) and the ethanol $K_m$ values previously reported for the same system, which would not necessarily be the case if $K_m$ were a reflection of substrate binding.

In contrast to ethanol oxidation, the NAD$^+$ $K_m$ value more closely corresponds its $K_p$ value for isopropanol (Table 1). For larger alcohol substrates, like isopropanol and benzyl alcohol, the hydride transfer that occurs during YADH catalysis becomes limited by NADH release. Thus, the decrease in $K_m$ for the $K_p$ to begin approximating $K_D$. This claim that the Michaelis constant reflects NAD$^+$ binding is supported by the similar $K_m$ and $K_p$ values obtained in dilute solution (Table 1). Furthermore, the binding curve obtained in glucose from the fluorescence experiment yielded a relative $K_p$ value.
that is within error of the relative NAD+ $K_m$ value, both of which were close to 1 (Figure S). Thus, the negligible change in $K_m$ with the addition of glucose or dextran (Figure 2B) for the isopropanol oxidation can be ascribed to the sugars having little to no effect on YADH–NAD+ binding. Because the Michaelis constant does appear to approximate the $K_P$ value for isopropanol oxidation, the fact that the relative $K_m$ values for glucose and dextran are similar suggests that macromolecular crowding has little effect on the binding of YADH to NAD+.

**Crowder Size.** At first glance, our observation that the effect of dextran on YADH kinetics is independent of polymer size contradicts previous trends with enzymes of similar dimensions.15,24,31 An extensive study by the Mas group reported larger decreases in both $V_{\text{max}}$ and $K_m$ values with larger dextran polymers, revealing that obstacle size plays a major role in the magnitude of crowding effects for >100 kDa enzymes.32 The authors explain that large crowders reduce the frequency of enzyme–substrate encounters, but this decrease is partially counterbalanced by caging effects with smaller crowders. Unlike the enzymes used in the Mas study, our current and previous data15 suggest that YADH is not limited by substrate–enzyme encounters, even under crowded conditions.

Our previous work with malate dehydrogenase (MDH) also reveals a nontrivial dextran size dependence for kinetic crowding effects,29 even though this enzyme shares the same rate-limiting step as YADH, the release of NADH. Importantly, the decrease in $V_{\text{max}}$ in the presence of glucose is similar for both MDH and YADH (∼50%), further confirming our theory that the presence of this small molecule, which cannot exclude volume, likely impedes product release. In addition to slowing diffusion, the presence of dextran introduces an additional excluded volume effect on MDH that further decreases the $V_{\text{max}}$. Such crowding effects are usually size-dependent. In fact, in the presence of dextrans comparable in size to MDH (70 kDa), the relative $V_{\text{max}}$ was 40% lower than in the presence of glucose. In contrast, while the YADH relative $V_{\text{max}}$ values in the presence of dextran are slightly greater than for glucose, the values never differ by >15%. Thus, the reason the effects of dextran on YADH kinetics are size independent is because our results indicate that excluded volume plays only a minor role. Rather, as evidenced by the similar effects of dextran and glucose, our work reveals that factors other than excluded volume may be at play. This finding is consistent with a recent computational study revealing that excluded volume is less important than typically assumed.33 Similarly, a surge of recent reports have emphasized the importance of considering soft interactions and the chemical effects introduced by crowding.62–65 Thus, it is not surprising that these trends observed with YADH would be independent of dextran size.

**CONCLUSION**

The results presented here directly highlight the critical role that enzyme mechanism plays in macromolecular crowding effects. For a single enzyme, dextran was able to increase or decrease $V_{\text{max}}$ values depending on the rate-limiting step of the mechanism as controlled by the substrate. Second, comparison of the $K_m$ and $K_P$ values for ethanol oxidation provides a cautionary warning against interpreting changes in the Michaelis constant simply as binding effects. Rather, $K_m$ contains a kinetic component, such that the decrease in $K_m$ with ethanol oxidation is likely due to impeded product release. Finally, this work contributes to the growing body of evidence that suggests other aspects of macromolecular crowding, in addition to excluded volume, must be considered.33,62–66 Soft interactions have begun to be characterized in the context of protein binding and stability,62–65 but there is still a critical need to understand these interactions in the context of enzyme kinetics.

**AUTHOR INFORMATION**

Corresponding Author
Address: 300 Pulteney St., Geneva, NY 14456. E-mail: slade@hws.edu. Phone: (315) 781-3613. Fax: (315) 781-3860.

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**ABBREVIATIONS**

NAD+ and NADH, oxidized and reduced β-nicotinamide adenine dinucleotide, respectively; YADH, yeast alcohol dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; BSA, bovine serum albumin; $K_m$, Michaelis constant; $V_{\text{max}}$, maximal rate under Michaelis–Menten kinetics.

**REFERENCES**


