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Slowed Diffusion and Excluded Volume Both Contribute to the Effects of Macromolecular Crowding on Alcohol Dehydrogenase Steady-State Kinetics

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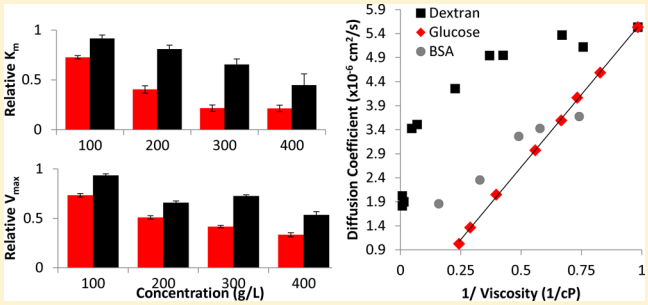
Supporting Information

ABSTRACT: To understand the consequences of macromolecular crowding, studies have largely employed *in vitro* experiments with synthetic polymers assumed to be both pure and "inert". These polymers alter enzyme kinetics by excluding volume that would otherwise be available to the enzymes, substrates, and products. Presented here is evidence that other factors, in addition to excluded volume, must be considered in the interpretation of crowding studies with synthetic polymers. Dextran has a weaker effect on the Michaelis–Menten kinetic parameters of yeast alcohol dehydrogenase (YADH) than its small molecule counterpart, glucose. For glucose, the decreased V_{max} values directly correlate with slower translational diffusion and the decreased K_m values likely result from enhanced substrate binding due to YADH stabilization. Because dextran is unable to stabilize YADH to the same extent as glucose, this polymer's ability to decrease K_m is potentially due to the nonideality of the solution, a crowding-induced conformational change, or both. Chronoamperometry reveals that glucose and dextran have surprisingly similar ferricyanide diffusion coefficients. Thus, the reduction in V_{max} values for glucose is partially offset by an additional macromolecular crowding effect with dextran. Finally, this is the first report that supplier-dependent impurities in dextran affect the kinetic parameters of YADH. Taken together, our results reveal that caution should be used when interpreting results obtained with inert synthetic polymeric agents, as additional effects from the underlying monomer need to be considered.



Cells are packed with proteins, nucleic acids, sugars, and many other large molecules, which can occupy 20–30% of the available volume in the cytosol.[1] This phenomenon, known as macromolecular crowding, results in two main effects. First, at the high concentrations encountered within cells, these macromolecules repel and attract one another through chemical interactions. Second, two structures cannot physically occupy the same region of space. Consequently, the presence of macromolecules will decrease the volume available to other molecules in the same solution. This excluded volume effect scales significantly with molecule size.[2] As a result, the effective concentration of a cell, from the perspective of a protein, is much greater than what is implied by the actual concentration of 200–300 g/L.[1]

The ramifications of macromolecular crowding have been well characterized and are described at length in a number of reviews.[2−5] In short, theory predicts excluded volume to increase thermodynamic activities. As a larger fraction of the solution volume is occupied, the entropy of the system decreases such that compact states become more thermodynamically favored. Consequently, crowded systems entropically drive proteins to fold and adopt configurations that minimize the occupied volume. Consistent with theory, experiments have documented crowding to stabilize proteins, promote aggregation, enhance binding, and alter other equilibria.[6]

Macromolecular crowding also slows diffusion of both small molecules and proteins because of the high viscosities of these solutions.[7,8] Generally, the translational diffusion coefficient (D) of a spherical particle (tracer) in a homogeneous solution can be related to the solution's dynamic viscosity (η) using the Stokes–Einstein equation: $D = \kappa T/6\pi\eta r$, where κ is the Boltzmann constant, T is the temperature, and r is the particle's radius. Crowded solutions, however, often deviate from this relationship, especially when the size of the tracer is much smaller than the radii of the macromolecule crowders.[9,10] Because of this deviation, experts question the extent to which large macromolecules, like crowding agents, have the ability to affect small molecule diffusion.[11]

Despite these well-established implications of macromolecular crowding, the trends observed with enzyme kinetics are more complex. Crowding enhances thermodynamic activities but slows diffusion. Because these two factors have opposing

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consequences, the effects of crowding on enzymatic rates are difficult to predict. Some enzymes exhibit enhanced activity[12−15] in the presence of crowding, while the rates of others are decreased[16−18] or unaffected.[19] Even for a single enzyme, crowding can either increase or decrease the reaction rate, depending on the macromolecular concentration.[20] In theory, crowding should slow the rates of diffusion-controlled enzymes, while enhancing the rates of enzymes limited by the activity of their transition state.[1] In reality, however, this generalization is often complicated by other factors such as enzyme conformation,[12,21] oligomeric state,[13,22] product inhibition,[18] and the nature of the substrate[23] or the macromolecule used to exclude volume.[22] For example, urease activity can be increased or decreased by crowding depending on the specific macromolecule used,[22] illustrating the complex and not yet predictable nature of the effects of crowding on enzyme kinetics.

To better understand crowding, experiments have frequently been performed in simple buffer solutions consisting of a high concentration (50−300 g/L) of a purified macromolecule called a crowding agent. This bottom-up approach provides a well-controlled environment for validation of theoretical predictions because crowded and dilute solutions can be directly compared. While proteins such as hemoglobin[22] and bovine serum albumin (BSA)[12,13,21] have served as crowding agents, the vast majority of crowding studies use synthetic neutral polymers. These highly soluble polymers are available in a range of sizes, which allows for a systematic investigation of individual factors such as dimension and concentration. Two of the most prevalent synthetic crowders are dextran, a glucose polymer, and ficoll, a sucrose polymer, although the nonsugar polymers polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) are commonly used, as well. Ficoll is loosely spherical, whereas dextran assumes a linear, cylindrical form.[4] Because of its inherent compressibility, ficoll is reported to have a weakened influence on biological systems relative to a similarly sized dextran polymer at equivalent concentrations.[6] Nonetheless, any effects observed in the presence of ficoll, dextran, and PVP are assumed to be due to excluded volume, because these polymers are typically treated as "inert", having no interaction with the protein of interest.[3,4]

On the basis of the extensive use of these supposedly inert polymers, the chemical effects due to crowding have been largely ignored.[2] In fact, "excluded volume" is commonly yet imprecisely used synonymously with "macromolecular crowding".[2] However, evidence is beginning to emerge suggesting that chemical interactions, in addition to excluded volume, may play a significant role in the effects of crowding.[24−27] In fact, the synthetic crowding agent, polyethylene glycol (PEG), has been known to interact favorably with proteins for some time.[3] While repulsive interactions tend to increase the chemical activity of a solvent and can augment excluded volume effects, attractive interactions typically counteract excluded volume to destabilize proteins.[28] Finally, a recent study that focused on horseradish peroxidase reveals the need to consider substrate−crowder interactions, as well.[23]

Presented here is a systematic investigation into the effects of synthetic crowding agents on the kinetics of yeast alcohol dehydrogenase (YADH) and horse liver alcohol dehydrogenases (HLADH). YADH is a 150 kDa tetramer that catalyzes the reversible oxidation of ethanol to acetaldehyde using the cofactor NAD^+. This particular system was chosen because the enzyme is well-studied[29] and commercially available and obeys Michaelis−Menten kinetics. HLADH, a 80 kDa dimer, has also been extensively characterized and shares many similarities with YADH. For example, the enzymatic rate of both enzymes is limited by the release of the NADH cofactor, which involves a conformational change.[30] Using these dehydrogenases, we have begun to elucidate the kinetic differences between excluded volume and chemical effects by employing spectroscopic and electrochemical techniques. Together, these findings indicate a complex interrelationship among excluded volume, viscosity, and other intermolecular forces involved in catalytic dynamics.

EXPERIMENTAL PROCEDURES

Chemicals. Alcohol dehydrogenase (EC 1.1.1.1) from *Saccharomyces cerevisiae* (YADH, 310 units/mg) and horse liver (HLADH, 1.99 units/mg) were purchased as purified lyophilized powders from Sigma-Aldrich. Dextran polymers from *Leuconostoc mesenteroides* (∼9−11, 150, and 200 kDa) and *Leuconostoc* spp. (∼40 and 450−650 kDa) were obtained from Sigma-Aldrich. Dextran polymers from *L. mesenteroides* were also purchased from Alfa Aesar (∼20, 40, 150, and 500 kDa), Spectrum Chemicals (∼250 kDa), and Molecular Probes (∼86 kDa). For polyvinylpyrrolidone (PVP), the ∼10 kDa polymer was from Sigma-Aldrich and the ∼40 kDa polymer was from Molecular Probes. Ficoll (400 kDa) was from Sigma-Aldrich. Bovine serum albumin (BSA) fraction V was from Fisher Scientific. The pH of all crowding agent solutions was corrected to 8.9 before use.

Kinetic Assays. YADH activity was monitored spectrophotometrically at 25 °C in a 96-well plate with a Biotek Eon or a Tecan Infinite M200Pro spectrophotometer. Each of 16 wells contained a total volume of 200 μL consisting of 1.5 mM NAD^+, 1.25−75 mM ethanol, 0.067 μg/mL YADH, and a crowding agent in 100 mM pyrophosphate buffer (pH 8.9). The YADH stock was prepared by adding 8 μL of a 1 g/L enzyme solution to 5986 μL of 1 g/L BSA, which served to stabilize and maintain enzyme activity.[31] Immediately following the addition of the YADH stock solution to initiate the reaction, the rate of NADH formation was measured at 340 nm every 40 s (Biotek) or 12.5 s (Tecan) for 6 min while the sample was being shaken. The HLADH assay was performed under the conditions described above except that the reaction mixture contained 50 μg/mL HLADH and 0.025−6 mM ethanol. In addition, NAD^+ was added to initiate the reaction instead of enzyme.

Kinetic Data Analysis. Both kinetic assays were analyzed as previously described.[32] In short, enzymatic rates for 16 different ethanol concentrations were collected in triplicate and fit to a single Michaelis−Menten curve using SigmaPlot to yield K_m and V_{max} values. These initial enzymatic rates were determined from the maximal slope of five data points on the absorbance versus time graph via Magellan software. K_m and V_{max} values obtained under crowded conditions were normalized to the values obtained in buffer only, yielding relative kinetic values. Three independent K_m and V_{max} values were averaged to generate a single set of relative kinetic parameters for each crowded condition.

Viscosity Measurements. Calibrated Cannon−Fenske viscometers (sizes 50, 100, 200, and 400) were used in a 22.0 °C water bath to determine the efflux times (t), in seconds, of each crowding agent solution in triplicate. Weighing 10 mL of each solution with an analytical balance yielded the solution densities (ρ). Finally, dynamic viscosities (η) in centipoise were then calculated from the equation

$$\eta = t(\text{viscometer calibration constant})\rho$$

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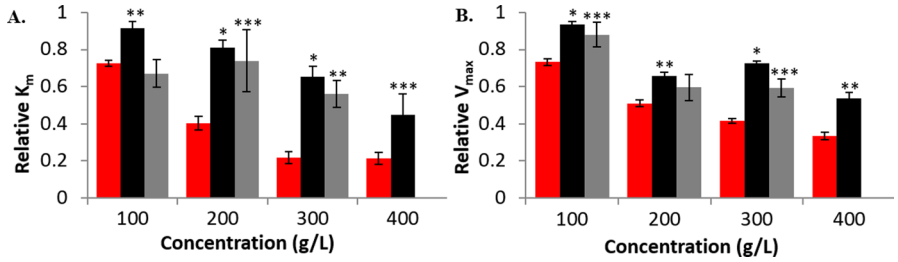

Figure 1. Both glucose and dextran decrease YADH kinetic parameters. Assays containing 0.067 μg/mL YADH, 1.5 mM NAD^+, and 1.25−75 mM ethanol in 100 mM pyrophosphate buffer (pH 8.9) were performed in the presence of increasing concentrations of glucose (red), 86 kDa dextran (black), or 150 kDa dextran (gray). (A) K_m and (B) V_{max} values from the resulting 16-point Michaelis−Menten curves were normalized to values acquired in the absence of dextran to yield the relative kinetic constants shown above (y-axes). The 150 kDa dextran (gray) was not soluble at the concentration necessary to perform the YADH assay at 400 g/L. Error bars represent standard errors ($n = 3$). Asterisks indicate a significant difference in effect between glucose and dextran: *$p < 0.001$, **$p < 0.005$, and ***$p < 0.08$ (Student's two-tailed t test).

Diffusion by Chronoamperometry. Crowding agent solutions were prepared with 500 mM KCl and 1.00 mM potassium ferricyanide in 100 mM pyrophosphate buffer (pH 8.9). Using a model AFRDE4 Pine potentiostat, all electrochemical experiments were conducted with a Au BAS macroelectrode polished immediately before use with 0.05 μm alumina (Buehler) and rinsed copiously with distilled water. Cyclic voltammetry, which was used to select a voltage for chronoamperometry experiments and to confirm solution purity and diffusion control, was conducted at an initial voltage of 0.40 V with sweep rates of 0.05−0.20 V/s. For chronoamperometry, currents were measured from 1 to 5 s to obtain multiple values of current and to confirm diffusion control with a constant value of $(i_t)^{1/2}$, an initial voltage of 0.40 V, and an applied voltage of −0.50 V. The measured current allowed for the calculation of $Fe(CN)_6^{3-}$ diffusion coefficients using the Cottrell equation:

$$D = \left(\frac{i_t t^{1/2} \pi^{3/2}}{\eta FAC}\right)^2$$

where n is the number of moles of electrons transferred (1 mol), F is Faraday's constant (96486 C/mol), A is the area of the electrode (0.0209 cm^2), C is the concentration of $Fe(CN)_6^{3-}$ (0.90−1.10 mM), i_t is the current in solution at time t (coulombs per second = 1 A), and D is the diffusion coefficient (square centimeters per second).

Denaturation Studies. For detection by kinetic activity, YADH assays were performed as described above at the highest concentration of ethanol (75 mM), except in the presence of 0−1.3 M guanidine hydrochloride (GdHCl). For fluorescence detection, solutions containing 0.5 g/L YADH and 100 g/L dextran (80, 150, or 550 kDa) or glucose were incubated for 2 h at 25 °C with 0−4.0 M GdHCl before being analyzed with a Tecan M200 Pro instrument. Using excitation at 295 nm, YADH denaturation was quantified by I_{360}/I_{321}, where I_{321} and I_{360} are the fluorescence intensities of tryptophans buried in a nonpolar environment and solvent-exposed, respectively.[33]

For the modified version of the denaturing kinetics experiment, a 10 μL solution containing 0.5 M GdHCl, 1 g/L YADH, and 300 g/L dextran (150 or 550 kDa), glucose or buffer, was incubated for 1.5 h before denaturation was stopped by the addition of 7.5 mL of 1 g/L BSA. The resulting solution was used as the YADH stock solution to perform the YADH assays previously described.

RESULTS

Crowding Agent Effects. YADH kinetic assays were performed with increasing concentrations of glucose or dextran polymers of varying size. The day-to-day variability in the assay was minimized by normalizing the resulting Michaelis−Menten parameters, K_m and V_{max}, to values obtained concurrently in the absence of a crowding agent. Concentration-dependent decreases in K_m and V_{max} were observed for both glucose and the dextran polymers (86 and 150 kDa), but glucose exhibited effects more drastic than those of either dextran polymer (Figure 1). Similarly, ficoll (400 kDa) affected YADH kinetics less than its small molecule counterpart, sucrose, which yielded results comparable to those with glucose (Figure S1).

To deconvolute the chemical and excluded volume effects of dextran, the YADH assay was repeated with polyvinylpyrrolidone (PVP), another supposedly inert crowding agent. If the effects of these crowding agents are due mainly to exclusion of volume, one would expect inert polymers of equivalent size and concentration to yield similar effects regardless of differences in their chemical structure. Despite the fact that PVP is not a sugar polymer, the relative V_{max} values for the 10 and 40 kDa PVP solutions are not statistically different from those of the equivalent size dextran solutions (Figure 2), suggesting that excluded volume plays a major role in the observed effects on YADH rates.

Given the importance of excluded volume on YADH kinetics, it is necessary to account for the consequent changes in solute

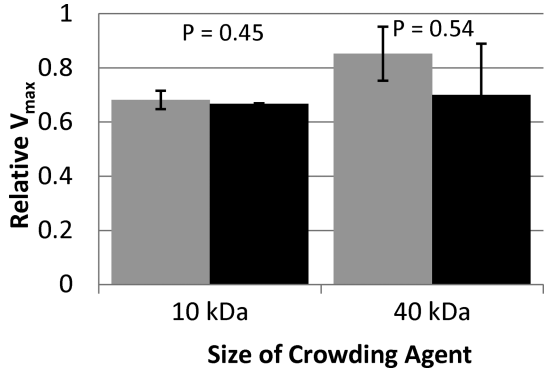

Figure 2. PVP has the same effect on YADH kinetics as dextran. The relative V_{max} for the YADH-catalyzed reduction of ethanol was studied using the conditions described in Figure 1, except in the presence of 300 g/L PVP (gray) or dextran (black). Error bars represent standard errors ($n = 3$). p values were calculated with a two-tailed Student's t-test comparing dextran and PVP V_{max} values.

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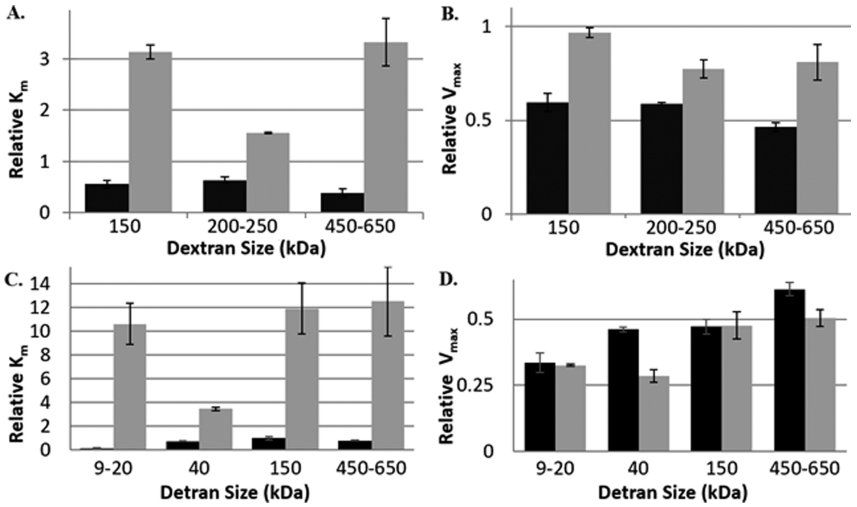

Figure 3. Source of dextran matters for its effects on YADH and HLADH kinetics. (A and B) YADH assays performed under the conditions described in the legend of Figure 1 and (C and D) HLADH assays containing 50 μg/mL HLADH, 1.5 mM NAD^+, and 0.025−6 mM ethanol in 100 mM pyrophosphate buffer (pH 8.9) were performed in the presence of 300 g/L "impure" (gray; Alpha Aesar, Spectrum) and "pure" (black; Sigma-Aldrich) dextran polymers to yield relative (A and C) K_m and (B and D) V_{max} values. Error bars represent standard errors (n = 3).

concentration.[3] Crowding decreases the volume available to other solutes in solution, thereby increasing their effective concentrations. This increase could significantly alter enzyme kinetics, because the Michaelis−Menten constant, K_m, depends on substrate concentration and the catalytic constant, k_{cat}, depends on enzyme concentration. As such, concentrations of the ethanol substrate and YADH enzyme were corrected by subtracting the volume occupied by the crowders from the total solution volume, as addressed in the Supporting Information and previously described.[34] The relative kinetic parameters were then recalculated with these new effective concentrations. Overall, these corrected values (Figures S2 and S3) did not deviate significantly from the relative kinetic parameters originally presented (Figure 1), which is consistent with previous reports.[34,35]

It is important to note that surprisingly different results were observed with dextran polymers purchased from different suppliers (Figure 3). While dextran polymers from Sigma-Aldrich and Molecular Probes generated colorless solutions and decreased both YADH kinetic parameters, dextran polymers from Alpha Aesar and Spectrum yielded yellow solutions that increased K_m values. To determine why the sources of dextran generated disparate results, the absorbance and fluorescence spectra of these polymers were compared (Figure S4). The significant absorbance between 270 and 400 nm exhibited by the dextran samples from Alpha Aesar and Spectrum suggests that the differences in YADH kinetics may be caused by trace impurities. The specific purification methods chosen by each supplier likely impact the trace amounts of small molecules present in the dextran, which become more significant at the concentrations required in crowding studies. As such, the lack of a trend in K_m values with dextran size (Figure 3A) could be a result of supplier-dependent amounts of impurity present, a hypothesis that is supported by the matching trends between the K_m values and the absorbance intensities. For example, the 250 kDa dextran sample has the lowest absorbance and fluorescence intensity (Figure S4), suggesting the smallest amount of impurity, and generates the lowest relative K_m and V_{max} values (Figure 3A,B) in comparison to those of the other more impure samples. Furthermore, this impurity has a more drastic effect on horse liver alcohol dehydrogenase (HLADH) than on YADH (Figure 3C,D), yet for the related enzyme, malate dehydrogenase (MDH), the same relative K_m and V_{max} values were obtained regardless of the source of the dextran (data not shown). As a result of these supplier differences, all other experiments were performed with dextran from Sigma-Aldrich or Molecular Probes. To the best of our knowledge, this is the first report of discrepancies between crowding agent sources, and caution should be used when interpreting results with supposedly inert crowding agents.

Viscosity and Diffusion. To elucidate why the presence of glucose causes drastic decreases in YADH activity, chronoamperometry was used to measure the translational diffusion coefficients of the small anion, ferricyanide $\{[Fe(CN)_6]^{3-}\}$, at various concentrations of glucose and dextran 150 kDa solutions. Diffusion measurements were also taken in solutions containing the commonly used protein crowding agent bovine serum albumin (BSA). While this 66 kDa protein is large like dextran, BSA solution viscosities are more comparable to those of glucose (Figure 4A). Ferricyanide was chosen because it displays well-established reproducible electrochemical properties, whereas electrochemical diffusion studies with the YADH substrate NAD^+ are more complicated.[36] Fluorescence and nuclear magnetic resonance have served as the major means for determining translational diffusion in crowded environments, and to the best of our knowledge, this is the first use of an electrochemical technique. Nonetheless, our orthogonal approach yielded results consistent with previous studies, showing that a 20% (w/v) solution of dextran results in a 4-fold decrease in the extent of diffusion.[17,37] A direct linear correlation was observed when these diffusion coefficients were plotted against the relative YADH V_{max} values collected at the same concentration of glucose (Figure 4B). While some correlation also exists between diffusion coefficients and the relative V_{max} values collected in dextran 150 kDa solutions, this plot deviates more from linearity (with an R^2 of <0.8) than the corresponding glucose plot. This difference may be related to the fact that the glucose solutions obey the Stokes−Einstein relationship, while the dextran 150 kDa solutions do not (Figure 4C). BSA solutions

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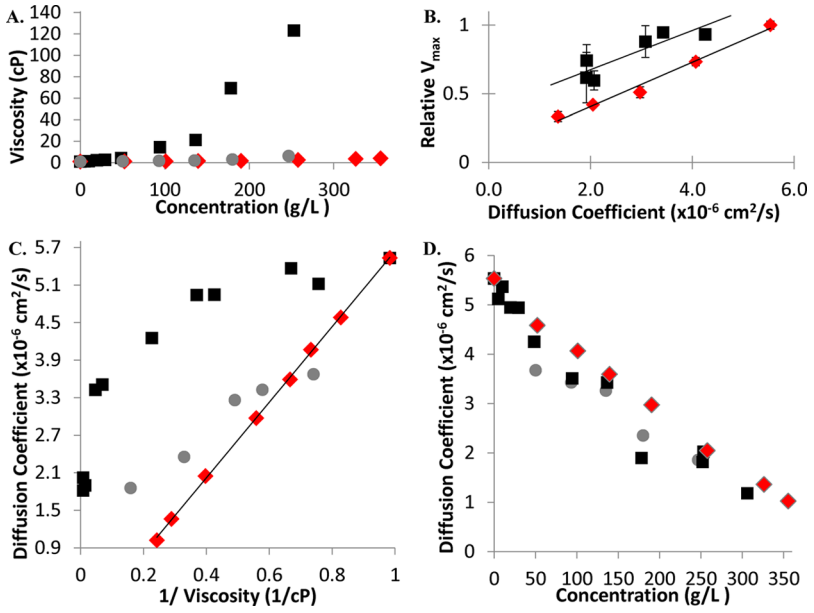

Figure 4. Comparison of viscosities and diffusion coefficients in glucose, dextran, and BSA solutions. A Cannon−Fenske viscometer provided viscosities, and chronamperometry was used to obtain translational diffusion coefficients of potassium ferricyanide in the 0−400 g/L glucose (red diamonds), dextran 150 kDa (black squares), and bovine serum albumin (gray circles) solutions. (A) Viscosity vs concentration plots depend on the solute. (B) The relative YADH V_{max} values described in Figure 1 were plotted against ferricyanide diffusion coefficients obtained in the corresponding glucose and dextran 150 kDa solutions. Error bars represent the standard deviation (n = 3). For glucose, R^2 = 0.98. (C) Diffusion coefficients are plotted vs inverse viscosity or (D) the concentration of each corresponding solution.

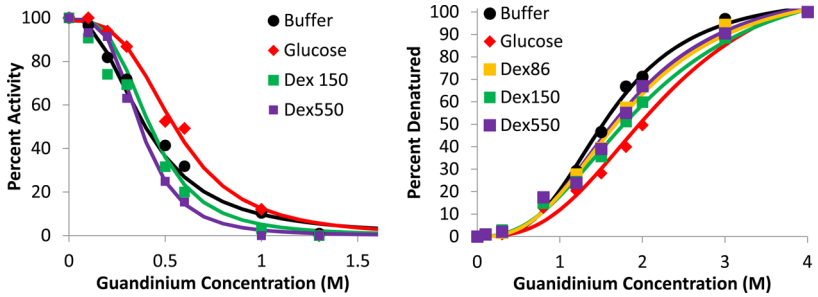

Figure 5. Glucose stabilizes YADH more than dextran. YADH was incubated for 2 h with various concentrations of guanidinium hydrochloride in the presence of 300 g/L glucose (red diamonds), dextran 86 kDa (yellow squares), dextran 150 kDa (green squares), dextran 550 kDa (purple squares), or buffer only (black circles). The percent denaturation was determined as described in Experimental Procedures using (A) YADH kinetic assays or (B) fluorescence (excitation at 295 nm, emission at 360 nm). Each data point represents an average of three trials.

deviate much less from this linear relationship between diffusion and the inverse viscosity than do the dextran 150 kDa solutions.

Finally, diffusion depends mainly on the solute concentration and not its identity. Ferricyanide diffusion coefficients were surprisingly similar for equal concentrations of BSA, glucose, dextran (Figure 4D), and ficoll, despite these solutions exhibiting very different viscosities (Table S1). One exception to this trend was the low diffusion coefficient observed in PVP solutions (Table S1). Similarly, diffusion is independent of the size of the surrounding crowder (Figure S5), which is consistent with previous experiments.[17,37]

Denaturing and Unfolding Assays. Upon comparison of the chemical effects of glucose and dextran on the YADH system, protein stabilization must be considered. It is well-documented that small sugars often stabilize proteins as a result of changes in the hydration shell, entropy, water-accessible surface area, surface tension, and hydrophobic interactions,[38−40] but less is known about the glucose polymer, dextran. To investigate if dextran is able to stabilize YADH to the same extent as glucose, the enzyme was denaturated with guanidinium hydrochloride in the presence of glucose or dextran and the percent denaturation was assessed both by enzymatic activity and by fluorescence (Figure 5). YADH inactivation as detected by kinetic activity occurs at concentrations of denaturant lower than those used for the spectroscopically detected changes in the tryptophan environment.[41,42] At the same time, because the effect of dextran on YADH kinetics is already in question, fluorescence analysis provides a simple, orthogonal means to assess YADH stability that is unrelated to its kinetics. Consistent with previous studies,[42] glucose clearly stabilizes YADH as evidenced by the shifts in the denaturation curves relative to those for buffer alone. In contrast, the denaturation curves in the presence of dextran are less conclusive, but dextran does not appear to have much of a stabilization effect (Figure 5A). By fluorescence detection, a slight shift in the denaturation curve is observed with dextran relative to buffer only, but not to the same extent as glucose (Figure 5B). To further verify these observations and remove ambiguity, a modified version of the denaturing kinetic experiment was performed. First, YADH was incubated with the denaturant guanidinium chloride in the presence of dextran,

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glucose, or neither. The solution was then diluted 750-fold before a kinetic assay was used to assess the percent denaturation. This extreme dilution ensured that glucose and dextran did not have a direct effect on YADH kinetics because their concentrations were only significant during the denaturation portion of the assay. The results show that glucose was able to protect YADH from chemical denaturation, with an ∼2-fold greater activity than either dextran or buffer solutions (Figure S4). In contrast, little difference exists between the Michaelis−Menten curves from YADH samples with and without dextran present during the denaturation period. This observation indicates that dextran does not preserve YADH activity to the same extent as glucose.

DISCUSSION

The altered YADH kinetic parameters in the presence of glucose and sucrose complicate the macromolecular crowding analysis. Interestingly, the ficoll and dextran polymers have significantly less influence on the K_m and V_{max} of YADH than their small molecule counterparts. A potential explanation for this finding is that ficoll and dextran are inert, as suggested by previous reports.[43,44] Ficoll exhibited a particularly negligible effect (Figures S1 and S2), so we turned our focus to glucose and dextran. To understand why the small molecules alter YADH kinetics more drastically than their macromolecule counterparts (Figure 1 and Figure S1), it is first necessary to determine which factors contributing to this decrease are shared by both glucose and dextran. One possibility is that dextran polymers are too big to exert the same effects as glucose. Alternatively, glucose and dextran could share the same properties that result in the reduced kinetic parameters, but because of its size, dextran may exert additional macromolecular crowding effects that partially offset this decrease. Because a better understanding of the differences in physical properties between dextran and glucose would aid in distinguishing between these possibilities, we compared small molecule diffusion in the two solutions.

Viscosity and Diffusion. Unexpectedly, the concentration of the crowder has a much greater influence on small molecule diffusion than the identity of the crowder (Figure 4D). The similar diffusion coefficients for glucose and dextran are intuitively surprising provided the vastly different solution viscosities (Figure 4A) and particle sizes of the two solutes. In addition, different diffusion coefficients were anticipated for the BSA and dextran solutions, given the growing body of evidence that chemical interactions must be considered in crowded environments.[23] Nevertheless, the concentration, not the size, shape, or chemical properties of the crowder appears to govern diffusion (Figure 4D).

These unanticipated observations are better explained in the context of the recent work by Holyst et al.[45] Their study reveals that crowded solutions do not deviate from the Stokes−Einstein equation as previously described.[44,46] Rather, in crowded solutions, the tracer experiences a different viscosity, called the nanoviscosity, which can be orders of magnitude below the macroscopic viscosity measured by standard techniques. This nanoviscosity deviates from the macroscopic viscosity only when the tracer diameter is smaller than that of the surrounding solute molecules, as is the case of ferricyanide in dextran 150 kDa solutions. As such, the supposed deviations from the Stokes−Einstein equation observed in dextran and BSA solutions (Figure 4C) can be attributed to a difference in the measured macroviscosity compared to the actual nanoviscosity that the tracer experiences. The similar diffusion coefficients obtained for a given concentration of glucose and dextran 150 kDa suggest that the macroviscosity of glucose is nearly equivalent to the nanoviscosity experienced by ferricyanide in dextran 150 kDa solutions. In short, a small molecule experiences similar nanoviscosities in both glucose and dextran. Furthermore, the observation that small molecule diffusion is independent of crowding agent size (Table S1), in contrast to protein diffusion,[10,46] further illustrates the importance of comparing the tracer size to that of the surrounding solution, supporting Holyst's theory.[9]

Effects of Crowding on V_{max}. In previous crowding studies, correlations between slowed diffusion and impeded rates have frequently been attributed to reduced substrate−enzyme encounters.[17,22,47,48] However, this explanation is unlikely in our system for two reasons. First, under saturating substrate conditions, the value of V_{max} should not be affected by substrate−enzyme encounters. Second, YADH is limited by product release of NADH,[49] though it is possible that enzyme−substrate encounters become rate-limiting at the higher viscosities encountered in the glucose solutions. In fact, partial diffusion control of YADH was previously observed in polyethylene glycol solutions.[50] This same work also documented that the slower horse liver alcohol dehydrogenase (HLADH)-catalyzed reaction exhibits no diffusion limitations even in the presence of polyethylene glycol. As such, we analyzed the effects of glucose on HLADH kinetics to determine if reduced substrate−enzyme encounters in viscous glucose solutions contribute to the decrease in YADH activity. In the presence of 300 g/L glucose, the resulting relative V_{max} value for HLADH was 0.32 ± 0.01 compared to 0.41 ± 0.05 for YADH. If YADH becomes limited by substrate−enzyme encounters in viscous solutions, glucose should decrease the relative V_{max} of YADH more than that of HLADH, which is too slow to be diffusion-limited, even in viscous solutions. The fact that glucose imposed similar effects on both enzymes suggests that the decrease in V_{max} is not due to reduced enzyme−substrate encounters.

Rather, the low relative V_{max} values observed in the presence of glucose (Figure 1B) are most likely a result of impeded NADH product release caused by increased solution viscosity and slowed diffusion. Previous studies have documented that viscous solutions can hinder product release by restricting essential enzyme dynamics.[51−53] Both structural and kinetic experiments have confirmed that coenzyme binding induces a major conformational change in HLADH.[49] Recent evidence suggests that YADH may also undergo a structural change because of NADH/NAD^+ association.[54] As such, the high solution viscosity from glucose may likely impede the conformational changes necessary for YADH to release NADH in the rate-limiting step. In a previous study with hexokinase, the decreased enzymatic activity in the presence of crowding was attributed to impeded diffusion of product away from the enzyme complex.[21] This justification is especially likely for YADH because the rate-limiting step involves release of the NADH product. Consequently, slowed diffusion in glucose is likely to obstruct clearance of NADH from the active site, thereby decreasing YADH activity. This hypothesis is supported by the direct linear correlation between the small molecule translational diffusion and the relative YADH V_{max} values for the corresponding glucose concentrations (Figure 4B).

Assuming glucose slows YADH activity as a result of impeded product diffusion, similar V_{max} values should be also be obtained in dextran, because small molecules diffuse at comparable rates in both solutions (Figure 4D). However, the observed V_{max} in the presence of dextran is much greater than that in glucose (Figure

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Biochemistry 2015, 54, 5898−5906

1), suggesting that an additional factor or factors must also be involved. Because a better correlation between diffusion and V_{max} is observed with glucose than with dextran (Figure 4B), we speculate that the effects for glucose are due solely to impeded diffusion, whereas both diffusion and macromolecular crowding contribute to the effects observed with dextran. That is, crowding enhances the YADH rates to partially offset the drastic decrease in activity expected from impeded product diffusion. A similar argument was previously employed to justify why dextran did not decrease the enzymatic activity of alkaline phosphatase as much as expected.[17] The authors concluded that the decrease in rates due to viscosity was partially offset by crowding, which enhanced enzyme activity.

To understand why macromolecular crowding enhances the activity of YADH, one must consider the YADH mechanism and the corresponding conformational change, because crowding has been demonstrated to impact protein conformation.[48] Whent the coenzyme binds to the open conformation of the apoenzyme, the YADH complex isomerizes to a closed state.[55] After catalysis, YADH returns to the open state to release NADH in the rate-limiting step. A recent computational simulation revealed that crowding favors the more compact, closed state of the seven proteins studied and can reduce the population of protein in the open conformation by up to 79%.[56] Crowding also affects the rate of transitioning between the two conformations. Unlike most enzymes, the closed state for YADH is less compact than its open conformation.[54] Consequently, macromolecular crowding is likely to favor the YADH open conformation and to enhance the transition rate from the closed to open form, thereby augmenting the rate-limiting release of NADH.

The larger relative V_{max} values for YADH compared to those of HLADH in the presence of dextran can also be explained by this hypothesis (Figure 3B,D). While NADH product diffusion would be slowed equally for YADH and HLADH, the aforementioned computational study revealed that larger proteins typically experience stronger crowding effects.[56] Consequently, the macromolecular crowding effect opposing diffusion would increase the V_{max} more for YADH (150 kDa) because it is twice as large as HLADH (80 kDa).

Effects of Crowding on K_m. Most kinetic experiments have documented macromolecular crowding to decrease K_m values to a similar extent as observed with our YADH system, but different reasons have been used to explain these decreases.[12,16,19−21,32,47,57,58] One common justification is an increase in effective concentrations in crowded solutions.[20] For YADH, however, decreased K_m values are still observed even after correcting the parameter for effective concentration (Figure S3). Instead, a more likely explanation is that dextran promotes conformational changes that decrease K_m. A recent study used fluorescence to show that macromolecular crowding elicits structural changes in enzymes, presumably because of domain repacking.[57] On the basis of the similar results obtained with four unrelated enzymes, the study concluded that these conformational changes may be a general phenomenon of crowding. Alternatively, the observed decrease in K_m has often been attributed to the nonideality of the crowded media.[48] Crowding may, for example, increase the ratio of activity coefficients between the free and substrate-bound enzyme, thereby decreasing K_m.[21] Crowding can also alter the activity of water, which plays a significant role in the thermodynamics of enzyme−substrate binding.[58] Thus, the decrease in YADH K_m with an increasing dextran concentration could result from the nonideality of the solution, a crowding-induced conformational change at the active site of the enzyme, or both.

The ability of glucose to decrease the K_m values of YADH (Figure 1A) is most likely due to an enhanced binding affinity of the enzyme for the ethanol substrate. In fact, decreases in the YADH K_m values in the presence of glycerol have been correlated with similar decreases in the binding constant, K_D.[59] This previous study performed numerous controls to confirm that the decrease in K_m with glycerol was not due to changes in subunit associations, secondary structure, viscosity, dielectric constant, or chemical activity. It is likely that the binding enhancement observed with glucose results from the ability of this sugar to stabilize YADH. In fact, for YADH alone, there are numerous accounts of stabilization from small sugars.[42,60,61] Our results corroborate that glucose can stabilize YADH (Figure 5). In contrast, dextran does not have the same ability to protect the enzyme from denaturation (Figure S6). Consequently, the observed decreased K_m values in the presence of dextran are likely rooted in a cause different from that of glucose (Figure 1A).

Dextran Impurity. To the best of our knowledge, this is the first report of discrepancies between crowding agent sources. The extent to which the dextran impurity affects the Michaelis−Menten kinetic parameters inversely correlates with enzyme specificity for its substrate. In the case of malate dehydrogenase (MDH), which is unaffected by these impurities, the high substrate specificity for the two carboxylate moieties of malate may prevent binding of the crowding agent contaminant.[62] In contrast, the alcohol dehydrogenases have greater substrate promiscuity and can catalyze the reaction with a wide range of alcohols or ketones.[49] In addition, YADH is more selective than HLADH,[63] which can oxidize secondary alcohols that are not substrates for YADH. As such, the dextran impurity is likely to bind to the nonspecific active site of HLADH as a competitive inhibitor, resulting in a drastic increase in the K_m (Figure 3C), while V_{max} remains unaffected (Figure 3D). In contrast, the dextran impurity is likely to affect YADH by a different mechanism because both K_m and V_{max} values are altered. One possibility is that the impurity stabilizes the open conformation of YADH, resulting in weaker binding (increased K_m), but quicker NADH release (increased V_{max} relative to that of pure dextran). Regardless of the mechanism, caution should be used when interpreting results with supposedly inert crowding agents.

CONCLUSION

Through a combination of kinetic assays and physical characterization, we have begun to differentiate between the effects of glucose and dextran on YADH kinetics. The decrease in V_{max} for YADH in the presence of glucose is due to slowed diffusion during product release. With dextran solutions, this effect is partially offset by macromolecular crowding, which augments the necessary conformational changes required for catalytic turnover. The decreased K_m values observed with glucose result from stabilization of the native YADH conformation, which enhances substrate binding. In contrast, the decreased K_m values observed with dextran must be caused by a different effect because dextran is unable to stabilize YADH to the same extent as glucose.

It has become increasingly apparent that the assumed inert properties of crowding agents must be carefully reevaluated to improve our understanding of the effects of macromolecular crowding. Furthermore, continuing research using this bottom-up approach will require that crowding agent supplies be assessed for potential impurities as evidenced by our results with several commercial dextran sources. Although these effects may be

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Biochemistry 2015, 54, 5898−5906

largely enzyme specific, at the sufficiently high concentrations required for crowding studies the specific chemical effects may be significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00533.

YADH relative kinetic parameters in the presence of sucrose and ficoll (Figure S1), influence of the effective concentration of ficoll (Figure S2) and dextran (Figure S3), absorbance and fluorescence spectra of the dextran solutions from different suppliers (Figure S4), ferricyanide diffusion coefficients in varying sized-dextran polymers (Figure S5), diffusion and viscosity data for various crowding agents (Table S1), and kinetic assays to assess YADH stability (Figure S6) (PDF)

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Notes

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■ ABBREVIATIONS

BSA, bovine serum albumin; HLADH, horse liver alcohol dehydrogenase; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide, respectively; PVP, polyvinylpyrrolidone; YADH, yeast alcohol dehydrogenase.

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