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Macromolecular crowding effects on the kinetics of opposing reactions catalyzed by alcohol dehydrogenase

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ABSTRACT

In order to better understand how the complex, densely packed, heterogeneous milieu of a cell influences enzyme kinetics, we exposed opposing reactions catalyzed by yeast alcohol dehydrogenase (YADH) to both synthetic and protein crowders ranging from 10 to 550 kDa. The results reveal that the effects from macromolecular crowding depend on the direction of the reaction. The presence of the synthetic polymers, Ficoll and dextran, decrease V_{max} and K_m for ethanol oxidation. In contrast, these crowders have little effect or even increase these kinetic parameters for acetaldehyde reduction. This increase in V_{max} is likely due to excluded volume effects, which are partially counteracted by viscosity hindering release of the NAD^+ product. Macromolecular crowding is further complicated by the presence of a depletion layer in solutions of dextran larger than YADH, which diminishes the hindrance from viscosity. The disparate effects from 25 g/L dextran or glucose compared to 25 g/L Ficoll or sucrose reveals that soft interactions must also be considered. Data from binary mixtures of glucose, dextran, and Ficoll support this "tuning" of opposing factors. While macromolecular crowding was originally proposed to influence proteins mainly through excluded volume effects, this work compliments the growing body of evidence revealing that other factors, such as preferential hydration, chemical interactions, and the presence of a depletion layer also contribute to the overall effect of crowding.

1. Introduction

Macromolecular crowding significantly impacts the thermodynamic and kinetic properties of protein structure and dynamics [1]. In highly occupied solutions, like the cytoplasm, steric repulsions between macromolecules exclude volume available to other macromolecules thereby stabilizing proteins, slowing diffusion, and enhancing associations [2]. While both computational [3,4] and experimental [1] approaches agree that crowding shifts thermodynamic equilibria of reactions towards products, the influence of crowding on reaction kinetics, especially those involving enzymes, is not as well understood. Crowding usually decreases the catalytic activity for diffusion-limited reactions due to impeded enzyme-substrate encounters [5–8]. For transition-state-limited reactions, though, crowding effects are less predictable due to the complex, often opposing, interplay of excluded volume, viscosity, soft interactions, and hydration effects [9]. These trends are further complicated by the fact that crowding has differing effects on steps within an enzymatic process and can thereby alter the rate-limiting step of a reaction [10]. Excluded volume can increase enzyme activity by improving substrate binding [11], stabilizing the enzyme [12], or promoting the proper oligomerization state [13]; yet, crowding can also decrease enzyme activity by enhancing product inhibition [14], altering thermodynamic activities [15], impeding a critical conformational change [16], aggregating the enzyme [17], interacting with the substrate [18], or dehydrating the protein [19,20].

The effects of crowding on enzyme kinetics has been extensively studied in the context of gene expression. Both experimental [21–24] and computational analysis [25–28] reveal a complex, non-linear relationship between the rate of gene expression and the extent of macromolecular crowding. For example, Matsuda et al. employed Brownian dynamics and Monte Carlo simulations to reveal that crowding effects on the rate of mRNA production are non-linear and can be modulated by factors such as the binding affinity of RNA polymerase for transcription factor and reactant concentrations [28]. This model predicts that mRNA

Abbreviations: dex, dextran (number afterward represents the molecular weight of the polymer in kDa); K_D, dissociation constant; kDa, kilodaltons; K_m, is Michaelis constant; PEG, polyethylene glycol; PVP, polyvinylpyrrolidone; V_{max}, maximal rate under Michaelis-Menten kinetics.

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synthesis is optimized at crowding levels typically observed in living cells. Other computational efforts show that macromolecular crowding reduces the inherent noise in transcription [29], regulates intracellular molecular motion [30], improves the robustness of gene expression [23]. and partitions cellular space into smaller spatially-distinct sub regions [26]. Many of these effects are size dependent, with larger crowders exerting more influence than smaller crowders [23]. Crowding in cells is not uniform, as the cytoplasm contains distinct regions of high and low macromolecular density [31]. Computational models predict that this heterogeneity results in irregular diffusion, which deviates significantly from standard diffusion equations [30]. As such, the kinetics of diffusion-limited reactions are slower in heterogeneous environments than corresponding homogeneous ones due to crowding-induced anisotropic transport [27]. Furthermore, molecules are directed towards and then remain in less densely packed regions of this heterogeneous environment [30], thereby promoting co-localization of necessary machinery (mRNA and ribosomes) [10,26]. Taken together, this information suggests that cells may use crowding a means to regulate gene expression and to direct molecules to specific cellular locations [25].

The generally accepted method for analyzing excluded volume effects *in vitro* involves exposing the enzyme to high concentrations of synthetic polymers (Ficoll, dextran, PEG) or protein (BSA, lysozyme) crowding agents [1]. This strategy is based on the assumption that crowding agents are inert, having no interaction with the test protein, and thus behave as hard spheres [2]. However, studies continue to uncover evidence of both repulsive and attractive interactions between crowders and the protein system [32–36]. These non-specific "soft" interactions, though weak and transient, become significant at the concentrations employed for crowding studies and can diminish or even overpower excluded volume effects [37,38]. As such, there has been a progressive shift towards trying to quantify and better understand these contributions [39,40]. One method involves using low concentrations of crowder, so that the influence from excluded volume is negligible [34]. A separate strategy is to compare effects of similar sized crowding agents. Crowders consuming comparable hydrodynamic volumes should elicit similar excluded volume effects but have different chemical interactions with proteins. In contrast, polymers and their small molecule counterparts (such as dextran and glucose) should have similar chemical interactions with a protein at a given g/L concentration, while their excluded volume effects will differ [35,39,41].

In addition to chemical interactions between crowder and protein, macromolecular crowding can indirectly alter a protein's biophysical properties by perturbing the structure of water in the hydration shell of the protein [42]. Crowders experience steric repulsion [43] and are preferentially excluded from the surface of proteins, creating a cavity or depletion layer directly surrounding the protein [44]. The low concentration of crowder in this layer allows the protein to diffuse [45] and associate with other proteins [46] significantly faster than would be expected in the bulk crowding solution. A depletion layer is only observed with crowders larger than the protein of interest and the thickness of this layer is directly correlated with the size of the crowder [47]. At the same time, the high concentration of crowder beyond the depletion layer lowers the activity of water [8], creating an osmotic force that dehydrates the protein surface [48]. Since unfolded proteins have a higher solvent accessible surface areas than the native state, the unfolded form experiences more dehydration, thereby stabilizing the folded protein [39]. Small sugars also stabilize proteins by means of preferential hydration, but the mechanism is thought to involve changes in surface tension or optimization of the hydrogen-bonding network [49].

While most *in vitro* crowding experiments test high concentrations of a single crowding agent, the cytoplasm contains relatively low amounts of any individual macromolecule. Instead, cellular crowding is a result of the sum total of many molecules. Even simple prokaryotic cells contain more than 4000 proteins [50]. As such, recent efforts have begun exposing biophysical measurements to multi-component solutions, such as mixtures of two crowding agents [51–53]. Studies consistently report that binary mixtures stabilize proteins to a greater extent than the sum of the individual crowders and that the effects depend on the size, shape and the ratio of the two components [17,53–57]. In contrast, the effects from small sugar mixtures are additive [58,59], meaning that osmolytes in a mixture operate independently of one other and thus the contributions of each component sum to the overall effects of the mixture [58,59]. One study using fluorescence correlation spectroscopy (FCS) to monitor small molecule diffusion found that the microviscosity of binary crowding mixtures was greater than the sum of the microviscosities for the individual crowding agents. Since deviations between mixture effects and the sum of its parts was greatest for the largest crowders (70 kDa dextran and 70 kDa Ficoll), the authors attributed the increased microviscosity to crowder entanglement [60]. Finally, this same work showed that binary mixtures containing 6 kDa dextran had the similar effects on the domain movements of a protein regardless of the identity of the other dextran (40 or 70 kDa) or Ficoll (70 kDa), suggesting that the smaller component in a binary mixture dictates the overall effects.

Here, we focus on how macromolecular crowding effects the steady-state kinetics of the enzyme yeast alcohol dehydrogenase (YADH), a 147 kDa tetramer that catalyzes the reversible conversion of ethanol and NAD^+ to acetaldehyde and NADH. YADH is of wide scientific interest due to its far-reaching applications in biotechnology [61], food [62], fuel [63], and chemical industry [64]. This enzyme has served as a biological catalyst for stereo-selective redox reactions [65], continuous regenerations of NAD(P)H [66], synthesis of pharmaceutical compounds [67], and generation of many other starting materials [68]. As such, significant resources have been devoted to improving YADH protein stability and catalytic efficiency [68–72]. Our efforts to understand the effects of crowding on YADH activity will provide further insight for these industrial applications.

YADH is so well-characterized that it has served as a model to develop experimental strategies for elucidating enzyme mechanism [73]. For ethanol oxidation, the release of the NADH product is rate-limiting [74], and YADH mutations that increase coenzyme affinity also decrease catalytic activity [75]. Similarly, since YADH has a greater affinity for NADH than NAD^+, acetaldehyde reduction is a faster reaction than ethanol oxidation [76]. Furthermore, crystallographic [75] and pressure perturbation studies [77] show that binding of NAD^+ or NADH coenzyme to YADH initiates an isomerization from an open to closed conformation [75]. Evidence suggests that coenzyme binding and subsequent conformational changes occur before substrate can bind in both directions of the reaction [78]. However, the mechanism for acetaldehyde reduction is generally accepted to be ordered, while ethanol oxidation is modeled as random order with a preference for NAD^+ binding [79]. Finally, isotopic studies have revealed that the hydride transfer step required for YADH catalysis occurs via a quantum mechanical tunneling process, which has been extensively studied by the Klinman and Northrop groups [74,80,81]. The efficiency of this tunneling process depends on the distance between the donor and acceptor molecules within the active site [82].

Given the complex intricacies of metabolic regulation, understanding which intracellular factors influence the directionality of an enzyme-catalyzed reaction is of great importance. In yeast, acetaldehyde reduction is essential for fermentation of glucose during anaerobic growth, while horse liver alcohol dehydrogenase predominately catalyzes the opposing reaction. In vitro comparisons of the kinetic constants for these two isomers reveals that enzymes have evolved to have a greater affinity for their substrate than their product, and thus the binding affinity has more of an influence on the directionality than the maximal rates of each reaction [83]. Furthermore, situations may arise when it is advantageous for a cell to alter the relative rates of the opposing reactions or switch the net direction [84].

The work presented here focuses on comparing the effects of

macromolecular crowding on the rates of the opposing reactions catalyzed by YADH. Our lab has previously characterized the effects of dextran on ethanol oxidation [85,86]. The increased viscosity from crowders and small molecule sugars impede release of the NADH product, thereby slowing catalysis. In contrast, acetaldehyde reduction has not yet been exposed to crowding agents nor has its mechanism been as extensively studied as ethanol oxidation. In fact, the rate-limiting step of acetaldehyde reduction is still debated. Stopped-flow and product inhibition studies suggest that dissociation of the NAD^+ product is rate-limiting [87,88], while isotopic kinetics point to the hydride transfer step [89]. Both steps are likely to be partially rate-limiting [90] as the rate constants are similar [91]. Our data shows that crowding can decrease enzyme activity in one direction, while enhancing the rate of the opposing reaction. In addition, the use of chloroacetaldehyde (CAA) as an alternative substrate for YADH reveals that factors other than excluded volume contribute to the crowding effects on enzyme kinetics.

2. Materials and methods

2.1. Chemicals

Alcohol dehydrogenase (EC 1.1.1.1) from *Saccharomyces cerevisiae* (YADH, 361 units/mg) as a lyophilized powder, bovine serum albumin (BSA) and lysozyme both as purified lyophilized powders, β-Nicotinamide adenine dinucleotide disodium salt trihydrate in its reduced form (NADH), acetaldehyde, chloroacetaldehyde, D-(+)-glucose, 70 kDa Ficoll (GE healthcare), and dextran polymers from *Leuconostoc mesenteroides* (~9–11, 40, 150, 60) and *Leuconostoc* spp. (450–550 kDa) were purchased from Sigma-Aldrich. Absolute anhydrous ethanol was obtained from Pharmco-Aaper. Glucose and sodium pyrophosphate were purchased from Acros Organics. Trehalose from Swanson was used for all experiments because trehalose from Sigma-Aldrich contained impurities that interfered with the kinetic assay (see negative control below). All solutions were prepared in 100 mM sodium pyrophosphate buffer (pH = 8.9). The pH of the crowding agent solutions was corrected to match the pH of the buffer before use.

2.2. Michaelis-Menten kinetics assays

YADH activity was monitored spectrophotometrically by following the appearance (for ethanol oxidation) and disappearance (for acetaldehyde reduction) of NADH at 340 nm every 18 s for 6 min with shaking using a Tecan Infinite M200Pro spectrophotometer. Addition of YADH initiated the reaction at 25 °C, but adding coenzyme last instead, did not affect the resulting rates. Each crowding assay was performed in parallel with a non-crowding assay (crowder omitted) with the same stock reagents on the same 96-well plate, to minimize daily fluctuations in enzyme activity. The original YADH concentration for acetaldehyde reduction was 0.13 μg/mL. When NADH was varied at saturating acetaldehyde, the presence of dextran resulted in sigmoidal curves (Fig. S1C). Decreasing the enzyme concentration to 0.067 μg/mL yielded hyperbolic Michaelis-Menten curves under all conditions. The one exception was the chloroacetaldehyde assays, which require a higher enzyme concentration of 3.8 μg/mL YADH to generate an observable slope in the absorbance vs. time plot. Nonetheless, all Michaelis-Menten curves for the chloroacetaldehyde assay were hyperbolic even in the presence of crowder.

2.2.1. Acetaldehyde reduction

A single reaction contained 0.067 μg/mL YADH stabilized in BSA solution, 480 μM NADH, varying concentrations of acetaldehyde (0–10 mM), 300 g/L crowding agent and enough buffer for a final volume of 200 μL per well. YADH stock solution was prepared by adding 8 μL of 1 g/L YADH to 6.00 mL of 1 g/L BSA solution, which stabilizes the enzyme.

2.2.2. Choloroacetaldehyde (CAA) reduction

Since chloroethanol cannot be oxidized by NAD^+, the reduction of CAA is truly irreversible [92]. Each well contained 3.8 μg/mL YADH stabilized in BSA solution, 480 μM NADH, varying concentrations of chloroacetaldehyde (0–40 mM), crowding agent, and enough buffer for a final volume of 200 μL per well. YADH stock solution was prepared by adding 456 μL of 1 g/L YADH solution to 5.54 mL of 1 g/L BSA.

2.2.3. Varying NADH for acetaldehyde reduction

Each well contained 0.067 μg/mL YADH stabilized in BSA solution (described above), 15 mM acetaldehyde, varying concentrations of NADH, crowding agent, and enough buffer for a final volume of 200 μL per well. While YADH was typically added last to initiate this reaction, the assay was also repeated with a 1 min pre-incubation of YADH and NADH, which was then added to the well to initiate the reaction.

2.2.4. Ethanol oxidation

Assays were performed as previously described [86]. Each of 16 wells contained 0.067 μg/mL YADH stabilized in BSA solution, 1.5 mM NAD^+, varying concentrations of ethanol (1.25–75 mM) and enough buffer for a final volume of 200 μL per well.

2.3. Data analysis

Initial enzymatic rates, v_o were obtained by taking the maximum slope of 8 data points from absorbance versus time plots using Magellan software. A single Michaelis−Menten curve was constructed from v_o values at 12 aldehyde or NADH concentrations, [S], each collected in triplicate (i.e. Three crowding and three buffer only assays were completed with a single set of reagents). Representative Michaelis-Menten plots are shown in Fig. S1 (A and B). SigmaPlot was used to determine the Michaelis constant (K_m) and the maximum rate (V_{max}) from best fits to the equation:

$$v_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

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 relative kinetic parameters for each crowding condition (i.e. each substrate concentration was sampled 9 times: 3 sets of 3).

2.4. Negative controls

The specific crowding agents were chosen because they passed a "negative control". When the YADH assay was performed in the presence of crowder with one reagent omitted (enzyme, acetaldehyde, or NADH), no enzyme activity should be detectable. Dextran 150 and PEG 20 were not used for acetaldehyde reduction because their absorbance versus time slopes were significant (>5%) even without acetaldehyde compared to the slopes of the complete assay. Dextran 150 passed the negative controls for ethanol oxidation. The presence of trehalose from Sigma or BSA from Bio Basic resulted in a non-zero slope when YADH was omitted.

3. Results

The effects of crowding on YADH activity depend on the direction of the reaction. The presence of 300 g/L dextran decreases the rate of ethanol oxidation, while having no effect or even enhancing the rate of acetaldehyde reduction (Fig. 1A). In contrast, the presence of 300 g/L glucose, sucrose, or Ficoll decrease YADH activity in both directions. With the exception of Ficoll and sucrose, crowding effects on the YADH K_m values parallel the V_{max} trends (Fig. 1B). Dextran elicits similar effects on the Km of NADH compared to the Km of acetaldehyde, as does glucose (Fig. S2).

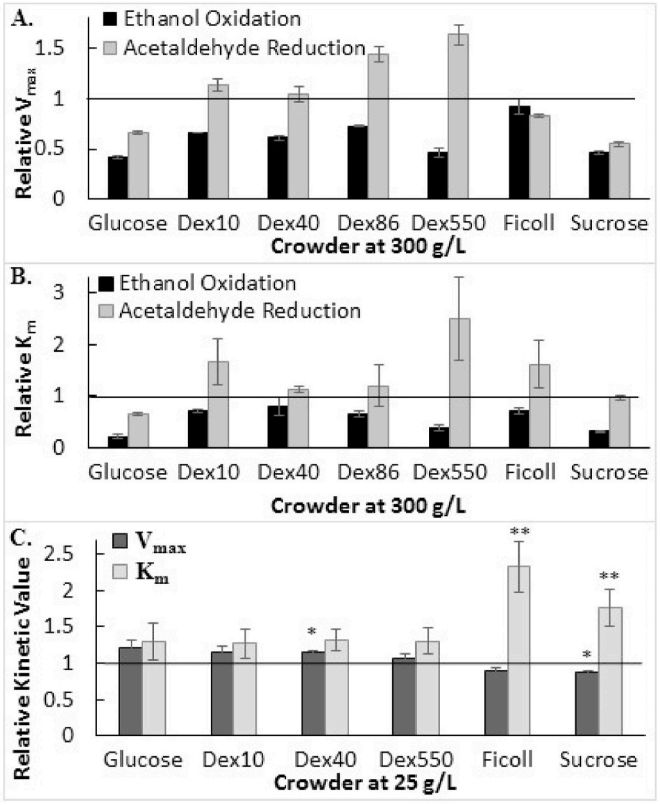

Fig. 1. Crowding effects on YADH depend on the direction of the reaction. YADH assays varying ethanol (black) or acetaldehyde (grey) at saturating NAD^+ (black) or NADH (grey) were performed in the presence of (A, B) 300 g/L or (C) 25 g/L dextran (10, 40, 86, or 550 kDa), Ficoll 70 kDa, glucose, or sucrose. (A) V_{max} and (B) K_m values from the resulting Michaelis– Menten curves were normalized to values acquired in buffer only to yield relative kinetic constants (y-axes). Error bars represent standard errors (n = 3). C) Asterisks (*p < 0.03, **p < 0.09) indicate relative kinetic values significantly different than one (Student's two-tailed *t*-test). P-values were calculated to determine if the difference in relative V_{max} was statistically significant for glucose compared to sucrose (p = 0.02) and Ficoll compared to dextran 10, 40 and 550 kDa (p = 0.05, 0.002, and 0.001) with a Student's two-tailed *t*-test.

Given the differing effects of dextran and Ficoll, it was important to identify if these polymers have non-specific interactions with YADH or its substrates. To this end, acetaldehyde reduction was exposed to low concentrations of each polymer and its corresponding monomer. While 25 g/L Ficoll or sucrose decreases the relative V_{max} of this reaction, 25 g/L glucose or dextran slightly increase both kinetic parameters (Fig. 1C). P-values from Student's t-test indicate that the relative V_{max} values for dextran are significantly different than the values for Ficoll, and glucose effects significantly differ from sucrose. While the K_m values with 25 g/L dextran or glucose are not statistically different than in buffer, the presence of 25 g/L Ficoll doubles the K_m of acetaldehyde.

To determine which step of the YADH mechanism was affected by glucose and dextran, the assay was repeated with a different substrate, chloroacetaldehyde (CAA). In the presence of dextran and low concentrations of glucose, the relative V_{max} values are consistently lower when CAA is the substrate as compared to acetaldehyde (Fig. 2). With CAA, no crowder was able to significantly increase the V_{max} of the reaction. High concentrations of glucose or its dimer, trehalose, elicited similar effects on the YADH kinetics regardless of the substrate.

In efforts to create more cell-like conditions, the YADH assay was exposed to the protein crowders lysozyme and bovine serum albumin (BSA), as well as binary mixtures of crowders. At 25 g/L lysozyme, the relative V_{max} was 1.4 ± 0.09 and relative K_m was 2.3 ± 0.6, but higher concentrations of lysozyme resulted in protein aggregation. The aggregation is likely due to the high pH of the YADH assay (8.9), especially given that the isoelectric point of lysozyme is 11. Exposure to BSA resulted in similar relative V_{max} and K_m values at both concentrations tested (Fig. 3). Binary mixtures consisted of 150 g/L of each component for a total concentration of 300 g/L. When acetaldehyde reduction was exposed to these mixtures, each kinetic parameter was in between the corresponding kinetic values of the mixture's individual components (Fig. S3). For example, when the YADH assay was exposed to a 1:1 mixture of glucose and any size dextran, the resulting relative V_{max} values were less than with 150 g/L dextran only, but not as low as the relative V_{max} values in glucose (Fig. 4A and B). In contrast, the binary mixtures decrease the V_{max} of ethanol oxidation less than each of the individual components at the same total crowder concentration (Fig. 4C and D). The effects on the K_m for ethanol oxidation depend on the binary mixture (Fig. S4).

It is important to note that only hyperbolic Michaelis-Menten curves were analyzed throughout this work. At high concentrations of YADH (0.13 μg/mL), the presence of dextran, but not glucose, altered the Michaelis-Menten curves from hyperbolic to sigmoidal when NADH was

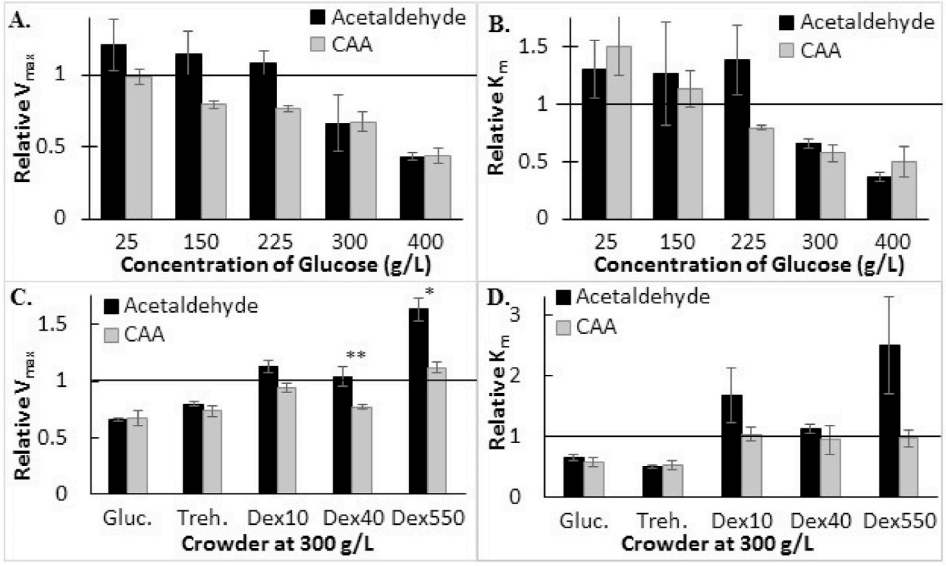

Fig. 2. Crowding effects on YADH kinetics are substrate-dependent. Assays varying acetaldehyde (black) or chloroacetaldehyde, CAA, (grey) at saturating NADH were performed in the presence of (A, B) varying concentrations of glucose or (C, D) 300 g/L glucose (gluc), trehalose (Treh) or crowder. V_{max} and K_m values from the resulting Michaelis–Menten curves were normalized to values acquired in buffer only to yield the relative kinetic values. Error bars represent standard errors (n = 3). (C) Asterisks (*p < 0.005, **p < 0.03) indicate a significant difference between the relative kinetic values for acetaldehyde and CAA (Student's two-tailed *t*-test).

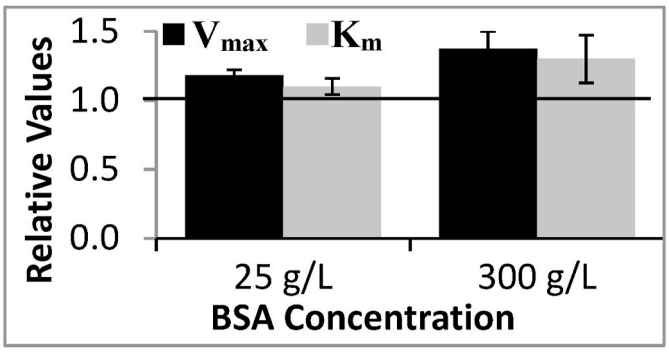

Fig. 3. BSA effects on YADH kinetics. YADH assays varying acetaldehyde at saturating concentrations of NADH were performed in the presence of 25 or 300 g/L bovine serum albumin (BSA). V_{max} (black) or K_m (grey) values from the resulting Michaelis–Menten curves were normalized to values acquired in buffer only to yield relative kinetic values (y-axes). Error bars represent standard errors (n = 3).

varied for saturating acetaldehyde (Fig S1 C-E). When NADH was pre-incubated with the enzyme or if lower enzyme concentrations (0.067 μg/mL) were employed, the Michaelis-Menten curves were again hyperbolic. When varying ethanol, acetaldehyde, or CAA, Michaelis-Menten curves were hyperbolic for all conditions.

4. Discussion

4.1. Effects on V_{max}

The disparities in crowding effects on the YADH forward and reverse reactions (Fig. 1) can be attributed to differences in the relative rates of the chemistry (k_2) compared to the product release (k_3):

$$E + S \underset{k\text{-}1}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E + P$$

E is the enzyme YADH, S is the substrate, and P is the coenzyme product, which depends on the direction of the reaction:

$$\text{ethanol} + NAD^+ \rightleftharpoons \text{acetaldehyde} + NADH$$

For ethanol oxidation, release of the NADH product is rate-limiting, as k_3 is 10-fold lower than k_2 (Table 1). Our previous work revealed that crowding decreases YADH catalytic activity by impeding release of the NADH product [85]. The focus of this work is the effect of crowding on acetaldehyde reduction, which is complicated by the fact that k_2 and k_3 are similar in magnitude (Table 1) and thus both influence the overall reaction rate [90]. Consequently, at least three factors contribute to the macromolecular crowding effects on YADH kinetics for acetaldehyde reduction: 1) impeded release of the NAD^+ product, 2) soft chemical interactions between crowder and YADH, and 3) excluded volume effects.

For acetaldehyde reduction, the decreases in V_{max} observed with the small osmolytes, glucose, sucrose, and trehalose (a glucose dimer) are most likely due to increased viscosity impeding the release of the NAD^+ product (Figs. 1 and 2). It is well documented that viscosity can hinder essential enzyme motions thereby slowing product release [93–95]. For YADH, early studies from the Jakoby lab suggested that the high viscosity of glycerol interferes with a structural change essential for coenzyme binding [96]. Later efforts from both high pressure experiments and crystallographic studies revealed that YADH requires a global conformational change, requiring a 10° rotation of its subunits from a closed state to an open one in order to release NAD^+ or NADH [75,97]. For lactate dehydrogenase, an enzyme in the same family as YADH, Demchenko et al. showed that sucrose, glycerol, and ethylene glycol impede a crucial conformational change thereby decreasing catalytic activity [95]. Furthermore, crowding-induced decreases in enzyme activity of hexokinase have been attributed to impeded product diffusion [98]. Thus, the increased viscosity from small osmolytes likely decreases k_3 of YADH acetaldehyde reduction either by obstructing the conformational changes necessary for product release or by restricting diffusion of NAD^+ away from the active site. This claim is supported by the corresponding decrease in V_{max} with increasing concentrations of glucose (Fig. 2A). Under dilute conditions, the hydride transfer step and the NAD^+ release have rate constants of the same magnitude (Table 1) and thus crowding effects on both steps must be considered [78]. As the glucose concentration and thus viscosity is increased, impeded product release eventually becomes rate-limiting, and crowding effects on the

Table 1
YADH rate constants [76].

	EtOH Ox	Acet Red
k_2	4000 s^{-1}	35,000 s^{-1}
k_3	388 s^{-1}	21,000 s^{-1}

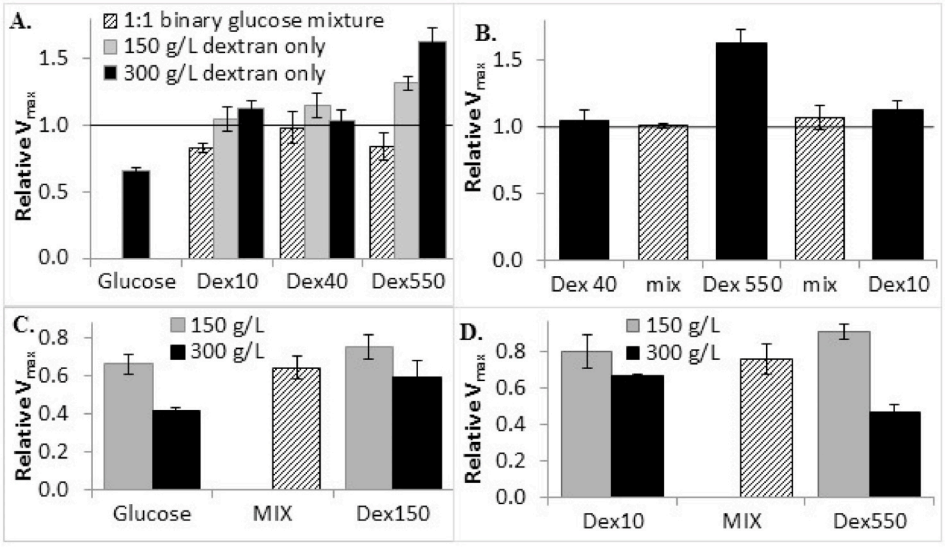

Fig. 4. Effects of binary mixtures on YADH kinetics. Assays varying (A,B) acetaldehyde at saturating NADH or (C, D) ethanol at saturating NAD^+ were performed in the presence of 150 g/L (grey), 300 g/L (black) or a 1:1 binary mixture (striped) of dextran (10, 40 or 550 kDa) or glucose. The total concentration in the binary mixture was 300 g/L with 150 g/L of each component. V_{max} values from the resulting Michaelis–Menten curves were normalized to values acquired in buffer only to yield the relative kinetic values. Error bars represent standard errors (n = 3).

hydride step are less influential. Thus, high concentrations of glucose or trehalose have similar effects on YADH kinetics regardless of the substrate (Fig. 2). If these osmolytes decreased V_{max} through chemical interactions with the system or by hindering the hydride transfer step, then the osmolyte effects should be substrate-dependent. In contrast, a sugar's ability to restrict the release of the NAD^+ product should be independent of the substrate used.

At lower glucose concentrations, V_{max} values deviate for the two substrates, acetaldehyde and chloroacetaldehyde, CAA (Fig. 2A), because the rate of hydride transfer (k_2) is now a significant contributor to the overall reaction rate. As the concentration of glucose is increased, the release of NAD^+ (k_3) is eventually impeded enough that it becomes rate-limiting. Since k_2 is substrate-dependent, but k_3 is not, the glucose concentration at which k_3 becomes rate-limiting will differ for acetaldehyde and CAA. Hence acetaldehyde and CAA V_{max} values differ at intermediate glucose concentrations (Fig. 2A). This observation is consistent with a previous study showing that crowding had distinct effects on isoenzymes of β-galactosidase with different rate-limiting steps [99]. Furthermore, there is precedent gene regulation studies that crowding can alter the rate-limiting step of a reaction [10].

The most likely source of osmolyte influence on k_2 is soft interactions either through direct attractive interactions with YADH, or indirectly via repulsive interactions, aka preferential hydration. Separating these sources from others such as viscosity is complicated by the fact that these sources increase with cosolute concentration. One strategy for parsing these sources is to expose the system to low concentrations of a macromolecular crowder so that excluded volume and viscosity effects are negligible, leaving only attractive chemical interactions [34,100]. For acetaldehyde reduction, the V_{max} values in 25 g/L glucose or dextran are different than those acquired in sucrose and Ficoll, and the differences are likely to increase at the higher concentrations. Glucose has reducing ends and has been proposed to interact with the amines of YADH [72]. In addition, glucose stabilizes proteins by preferential hydration [49]. Either of these possibilities would alter the conformation of YADH and thus its catalytic activity, explaining the slight increase in V_{max} observed at low glucose concentrations. Trehalose, sucrose, and glucose stabilize YADH [70], but sucrose stabilizes YADH more than glucose [72]. While some restriction of a protein's motion helps to preserve the native state and promote stabilization, enzymes require flexibility to function, and too much rigidity impedes catalysis [101]. For example, the Klinman group showed that alcohol dehydrogenases (ADH) require flexibility for efficient hydride transfer, especially in their inner domains [80]. Mutations of horse liver ADH that impede the internal dynamics of its inner domains slow hydride transfer, decreasing catalytic activity [82]. The same mechanism by which sucrose stabilizes YADH is also likely to restrict protein motion, compromising the flexibility necessary for catalysis, and thereby decreasing V_{max}. After all, molecular dynamics simulations from the Basso lab reveal that sucrose can compress an enzyme to a greater extent than glucose, thereby lowering its catalytic activity [102].

While older studies identify synthetic crowders as inert, our finding is consistent with recent work showing that Ficoll and dextran can interact with proteins and that these interactions differ for the two polymers [7,51,103]. A recent study highlighted the difference in the soft interactions of these crowders, showing that 70 kDa Ficoll destabilizes myoglobin to a greater extent than 70 kDa dextran [36]. This study also revealed that both Ficoll and dextran interact with myoglobin through hydrogen-bonding to specific residues on the protein. A separate study reported that 200 g/L sucrose decreases catalytic activity of the enzyme 2-trans-enoyl-ACP (CoA) reductase (InhA), while glucose had no effect [102]. Thus, the most likely cause of the different effects between dextran and Ficoll or sucrose and glucose (Fig. 1A) is soft interactions with the YADH system. Similarly, the increase in V_{max} from the protein crowders, BSA (Fig. 3) and lysozyme appear to be a result of chemical interactions with YADH, as judged by an effect even at 25 g/L. Over 200 proteins have been documented to interact with serum albumins [104,105], and thus it is reasonable to believe that YADH will interact with BSA, thereby altering its kinetics.

Ficoll and sucrose should exhibit similar attractive interactions because Ficoll is a sucrose polymer [106–108]. As such, similar effects are observed in 25 g/L solutions of Ficoll and sucrose (Fig. 1C). Thus, the differences in V_{max} values between 300 g/L sucrose or Ficoll likely arise from the macromolecular excluded volume effect exerted by the polymer (Fig. 1A). Similarly, 300 g/L dextran results in greater V_{max} values than glucose (Fig. 2C). Excluded volume effects often, but not always, alter protein conformation [2]. Our previous work shows that dextran influences tryptophan fluorescence of YADH, but glucose does not [85]; and the Klinman group revealed that the efficiency of YADH catalysis depends on the donor-acceptor distance during the hydride transfer [82]. Taken together these results suggest that excluded volume effects likely alter the YADH conformation, optimizing the distance for hydride transfer and increasing V_{max} for acetaldehyde reduction. However, the optimal distance is substrate specific [109]. We speculate that dextran optimizes the donor acceptor distance for acetaldehyde, but not for a different substrate, CAA, whose kinetics are less affected by crowding (Fig. 2C and D).

The larger gap in V_{max} values between glucose and dextran compared to sucrose and Ficoll (Fig. 1A) is likely due to differences in polymer shape and compressibility. Dextran is more rod-shaped, while Ficoll is more spherical [110]. Furthermore, Ficoll is more compressible and thus excludes less volume than comparable sizes of dextran [103]. In fact, the hydrodynamic radius (R_h) of 70 kDa Ficoll is 34.5 Å [102], while those of 10 kDa and 40 kDa dextran are 26 Å and 58 Å, respectively (Table S1) [111]. Indeed, the gap in V_{max} for small dextrans and glucose is similar to the gap between Ficoll and sucrose (Fig. 1A), supporting the idea that this gap is due to macromolecular excluded volume effects.

Dextrans significantly larger than YADH (R_h of 50 Å) [112] increase V_{max} more than smaller polymers. A likely explanation is that 86 kDa and 550 kDa dextran, which are bigger than YADH (Table S1), generate a depletion layer surrounding the enzyme and therefore do not slow diffusion to the same extent as glucose. In contrast, smaller dextrans and Ficoll likely impede NADH release to a similar extent as glucose or sucrose. Both small molecule and protein diffusion studies reveal that the concentration of crowder, and not polymer size, influences probe diffusion [86,113,114]. One study, using fluorescence quenching showed that translational diffusion of iodide and fluorescein are unaffected by the dextran size (15–500 kDa) in crowded solutions [5]. Another study using electrochemical techniques reports that small molecule diffusion coefficients are not statistically different in solutions of glucose and 150 kDa dextran at equal g/L concentrations [7]. It is important to differentiate that these diffusional studies are for the bulk crowding solution and thus no depletion layer is present. When diffusion was measured directly above the surface of a protein by fluorescence correlation spectroscopy in a PEG 35 kDa solution, the time for diffusion was only 20% of that in bulk solution [44]. This same study showed that larger crowders can actually speed up association rates of protein subunits due to the depletion layer.

4.2. Mixtures

Taken together, the crowding results presented here show that YADH catalytic activity for acetaldehyde reduction is increased by excluded volume effects, which are partially counteracted by impeded release of the NAD^+ product (Fig. 5). Data from binary mixtures of glucose, dextran, and Ficoll support this "tuning" of opposing factors, because the V_{max} values of these mixtures are in between the V_{max} values of the individual components (Figs. 4 and S3). The 1:1 mixtures of glucose and dextran consistently had higher V_{max} values than glucose alone, due to the additional excluded volume effects. Excluded volume effects of the mixtures should be comparable to excluded volume effects in 150 g/L dextran, except that the mixtures have greater microviscosities [51] because both glucose and dextran impede diffusion. As such, the V_{max}

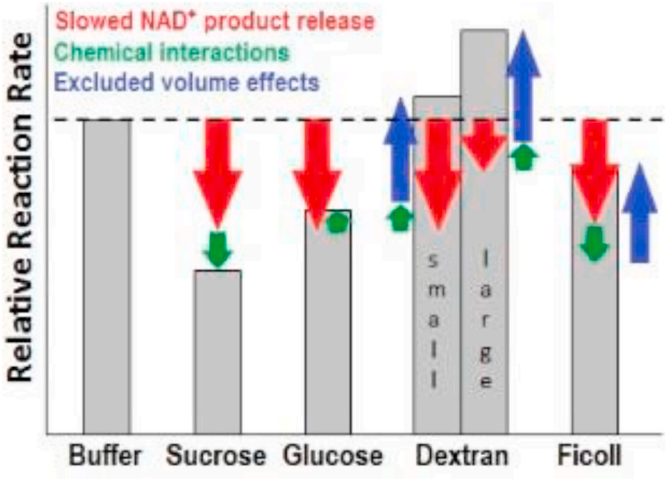

Fig. 5. Factors contributing to the effects of crowding on YADH.

values of the mixtures are below the corresponding dextran values (Fig. 4A). A bigger gap in V_{max} is observed between 150 g/L 550 kDa dextran and the glucose-dextran (550 kDa) mixture than with the other sizes of dextran (compare grey and striped bars in Fig. 4A). For this larger dextran, the presence of a depletion layer diminishes the microviscosity compared to the bulk solution. While this mixture contains a depletion layer void of dextran, glucose can occupy it and restrict product release. Consequently, the V_{max} value of this mixture is significantly lower than the V_{max} in 150 g/L dextran.

Mixtures of two sizes of dextran had no effect on the V_{max} value compared to buffer (Fig. 4B). Under these conditions, rate enhancement of hydride transfer from excluded volume effects is counteracted by slowed diffusion and impeded product release. These results support the claim of a previous study that the smaller crowder in a binary mixture dictates the overall effects of that mixture [60], because the relative V_{max} values of the mixtures were more similar to individual solutions of 10 and 40 kDa dextran, rather than 500 kDa dextran. Unlike previous work [17,53–57], our results suggest that effects from mixtures are comparable to the effects of the individual crowders. For example, mixtures of dextran and Ficoll had V_{max} values directly between those of pure dextran or Ficoll (Fig. S3A), again showing a balance of opposing factors (In this case, one of the factors is a decrease in V_{max} from the chemical effects of Ficoll). In contrast, most studies generally conclude that binary mixtures of crowders have a greater stabilization effect on proteins than the sum of the individual crowders [17,53–57]. This difference between our results and the literature is most likely due to the fact that previous reports of crowding mixtures focused on protein stability. Many additional factors including substrate binding, conformational changes, and thermodynamic activity influence the effects of crowding mixtures on enzyme kinetics.

For ethanol oxidation, the V_{max} effects from the binary mixtures were less drastic than the sum of the effects from the individual components of that mixture (Fig. 4C–D). In contrast, a recent study reports that fluorescein isothiocyanate diffusion is significantly slower in a binary mixture compared to the sum contributions from the two individual crowders [51]. Since crowding effects on V_{max} for ethanol oxidation have been directly correlated to diffusion [86], one would expect the binary crowding mixtures to decrease the V_{max} more than individual crowders. However, this same work explained that the slower diffusion in the binary mixtures was more extreme with combinations of similar-sized crowders because these polymers entangle. Perhaps the disparate results observed here are due to the two extreme sizes of crowders used: dextran 10 and 550. Alternatively, the differences in the results presented here from the literature may be due to the additional excluded volume effects that polymeric mixtures exert on YADH kinetics, opposing the diffusion effects. Regardless of the source, the multiple factors contributing to crowding mixtures ability to influence enzyme kinetics is more complex than their effects on protein stability or diffusion.

4.3. *Effects on K_m*

The presence of crowder decreased K_m for ethanol oxidation and increased K_m for aldehyde reduction. The only condition that decreased K_m in for aldehyde reduction was exposure to high concentrations of glucose (Fig. 2). As with ethanol oxidation, this decrease in K_m is most likely due to glucose slowing product release and thereby decreasing k_3. When product release is rate-limiting, K_m simplifies to k_3/k_1 [115], such that a decrease in k_3 would decrease K_m. This claim is supported by the fact that similar patterns are observed for the effects on both V_{max} and K_m (Compare Fig. 2 A and B) because both parameters are influenced by k_3 in viscous solutions. Furthermore, glucose has similar effects on the K_m of NADH and acetaldehyde. Finally, similar relative K_m values are observed for both acetaldehyde and CAA at high glucose concentrations. If K_m was instead a reflection of binding affinity, then the effects of crowding on different substrates would be more likely to differ, but the effects of crowding on k_3 should be substrate-independent.

The source of the K_m effects with dextran and Ficoll is less obvious. The fact that all crowders result in K_m values greater than their small molecule counterpart (compare dextran to glucose and Ficoll to sucrose), suggests that excluded volume is a major factor. Previous work has attributed increases in K_m with crowding to impeded diffusion limiting substrate-enzyme encounters [9]. However, this explanation is more relevant to diffusion-limited enzymes, and not to YADH. Since a conformational change is required for substrate binding to YADH, it is possible that excluded volume effects favor the open YADH conformation, thereby decreasing binding affinity. However, the fact the K_m for NADH and acetaldehyde are similarly affected by crowding (Fig. S2) makes this explanation less likely, since NADH binds before the conformational change and acetaldehyde binds after. An alternative explanation involves chemical activity, since volume exclusion increases thermodynamic activities of molecules [116]. However, further experiments are necessary to identify the actual source of these excluded volume effects.

In addition to excluded volume effects, the fact that low concentrations of dextran, glucose, Ficoll and sucrose increase K_m suggest that chemical interactions are also a contributing factor. This claim is further supported by the increase in the K_m for acetaldehyde in the presence of 300 g/L dextran, while the K_m for CAA is unaffected by dextran. In the presence of 300 g/L BSA, however, the K_m of CAA is greater than acetaldehyde. This observation is most likely due to interactions between the BSA and the CAA substrate.

A previous comparison of Michaelis-Menten parameters for alcohol dehydrogenases revealed that a lower K_m value is a better indicator of an isoenzyme's preferred directionality, instead of V_{max}, because intracellular concentrations of substrates are rarely near saturation [83]. Since YADH operates in the acetaldehyde reduction direction in yeast, it seems counter-intuitive then, that crowding increases the K_m of acetaldehyde, while lowering the K_m of ethanol. However, the ability to use crowding to alter the K_m values of opposing reactions could provide yeast with a defense mechanism against one of its environmental toxins. While yeast require acetaldehyde reduction for anaerobic growth, one of the products of this reaction, NAD^+, undergoes a consequent reaction with the environmental pollutant, allyl alcohol, to form the toxin, acrolein [117]. Thus, in the presence of allyl alcohol, yeast reduce YADH activity, even though it means slower growth [118]. Lower rates of acetaldehyde reduction result in an increased intracellular ratio of $NADH/NAD^+$, thereby protecting the cell against acrolein toxicity [119]. Through genetic sequencing, specific YADH mutants were identified in yeast with improved fitness in allyl alcohol due to decreased acetaldehyde reduction [118]. The W82R mutant had surprising similar *in vitro* K_m values to

wildtype YADH, considering that yeast containing this mutant exhibited drastically improved fitness in the presence of acrolein. This puzzling finding may be explained by the macromolecular crowding that cells impose on their enzyme thereby altering the kinetics constants of the mutant YADH inside cells compared to *in vitro*.

The crowding effects observed on the YADH K_m values in our work (Fig. 1B) suggests that, at the sub-saturating substrate conditions found in cells, the presence of crowding would increase the rate of ethanol oxidation relative to the rate of the opposing reaction, thereby maintaining a higher NADH/NAD^+ ratio. After all, evidence shows that cells can alter crowding levels as a means to maintain homeostasis and meet metabolic needs [120–123]. The data presented here shows that crowding has opposing effects on the kinetic parameters of ethanol oxidation and acetaldehyde reduction. Thus, macromolecular crowding could provide a way for yeast to regulate these intracellular NADH/NAD^+ levels depending on environmental stimuli.

4.4. Is acetaldehyde reduction an ordered mechanism?

At high concentrations of YADH, the presence of dextran altered the Michaelis-Menten curves from hyperbolic to sigmoidal when NADH was varied for saturating acetaldehyde (Fig. S1). Cooperativity in the YADH system has been considered based on the asymmetry of the subunit dimers and later refuted as artifacts in the crystallographic data [75]. In the presence of crowding, sigmoidal kinetics have also been observed as a result of the enzyme dimerizing, since excluded volume promotes oligomerization [99]. However, this explanation is unlikely to explain the results presented here because sigmoidal curves were only observed when varying NADH. If crowding altered the equilibrium between two quaternary structures of YADH, we would expect to see sigmoidal results for varying concentrations of acetaldehyde (at saturating NADH) and for ethanol reduction as well.

For a two-substrate enzyme, sigmoidal kinetics have been demonstrated to be due to a random order mechanism [102]. Early studies on YADH in both directions yielded controversial conclusions about whether the mechanisms are ordered or random [79]. Now, experts are generally in agreement that the acetaldehyde reduction is ordered and ethanol oxidation is a preferred ordered, with co-enzyme binding first in both directions [75,78]. The fact that the Michaelis-Menten curves were sigmoidal when NADH was varied, but not for acetaldehyde suggests that the mechanism for acetaldehyde reduction is not strictly ordered, as often described [76], but rather preferred-ordered: free YADH can bind either NADH or acetaldehyde, but the reaction rate is faster when NADH binds first. Since hyperbolic curves are observed in dilute solution, the enzyme most likely has a significant preference for binding NADH first. The presence of a crowder, however, slows enzyme-substrate encounters. At low NADH concentrations, free YADH appears to be able to bind acetaldehyde, which is in abundance. This order of binding leads to a slower initial reaction rate than if NADH were to bind first, and thus sigmoidal curves. Perhaps binding to acetaldehyde first requires YADH to undergo additional conformational changes to subsequently bind NADH and achieve the proper conformation for catalysis. Regardless of why, the reaction rate is faster at a given concentration of substrate if YADH binds NADH first, before acetaldehyde. This claim is supported by the fact that the Michaelis-Menten curves are hyperbolic when the NADH is pre-incubated with YADH, allowing the two reagents to bind even at low NADH concentrations, before acetaldehyde is present. Since the sigmoidal curve is only observed in the presence of dextran, it is possible that YADH is ordered, but that crowding alters the preferred conformational state of this enzyme to one that can bind acetaldehyde before co-enzyme, proceeding through a slower mechanism. It would be interesting to investigate if other osmolytes and crowders also alter the Michaelis-Menten curve to a sigmoidal shape at high enzyme concentrations.

5. Conclusion

The effects of crowding for aldehyde reduction are more complex than for ethanol oxidation because no single step is truly rate-limiting. For ethanol reduction, the slow step is release of the NADH product, which is impeded by crowding. For aldehyde reduction, excluded volume increases catalytic activity by optimizing the hydride transfer of YADH, but this effect is partially counteracted by impeded release of the NAD^+ product either due to slowed diffusion or a hindered conformational change. With dextrans larger than YADH, the presence of a depletion layer mitigates this impeded release, thereby leading to increases in V_{max}. At the same time, the effects of attractive interactions with YADH are crowder-dependent, with dextran and protein crowders enhancing the V_{max}, but Ficoll slowing this rate even at low concentrations.

While the magnitude of these crowding effects are small, they are physiologically significant. Minor opposing alterations to the rates of reactions in opposite directions can lead to an overall large change in the net flux. Given that synthetic polymers decrease the rate of ethanol oxidation while improving the rate of acetaldehyde reduction, it is possible that macromolecular crowding may serve as a means for cells to control the relative rates of opposing reactions and thereby regulate metabolism.

Author statement

Kristin Slade: Conceptualization, Visualization, Supervision, Validation, Funding acquisition, Project administration, Formal analysis, writing-original and draft, Xander Wilcox, Methodology, Investigation, writing-reviewing and editing, Charmaine Chung, Validation, Investigation, writing-reviewing and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.100956.

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