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Macromolecular Crowding and the Steady-State Kinetics of Malate Dehydrogenase

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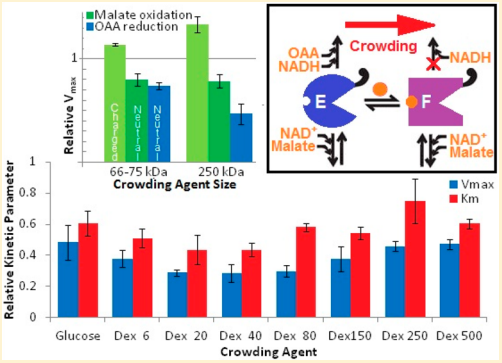
Supporting Information

ABSTRACT: To understand how macromolecular crowding affects enzyme activity, we quantified the Michaelis–Menten kinetics of mitochondrial malate dehydrogenase (MDH) in the presence of hen egg white (HEW), lysozyme, bovine serum albumin (BSA), gum arabic, poly(vinylpyrrolidone) (PVP), and dextrans of various molecular weights. Although crowding tended to decrease K_m and V_{max} values, the magnitude depended on the crowding agent, reaction direction, and isozyme (mitochondrial porcine heart or thermophlic TaqMDH from *Thermus flavus*). Crowding slowed oxaloacetate reduction more significantly than malate oxidation, which may suggest that mitochondrial enzymes have evolved to function optimally under the crowded constraints in which they are immersed. Since direct comparisons of neutral to charged crowders are underrepresented in the literature, we performed these studies and found that neutral crowding agents lowered V_{max} values more than charged crowders of similar size. The exception was hen egg white, a mixture of charged proteins that caused the largest observed decreases in both K_m and V_{max}. Finally, the data provide insight about the mechanism by corroborating MDH subunit dependence.



The inside of a cell is a heterogeneous mixture of macromolecules that reaches concentrations of 300–400 g/L.[1,2] This macromolecular crowding can influence many cellular functions by altering protein stability, activity, binding, and mobility.[3−6] Specific compartments, such as the mitochondria, are even more densely packed with macromolecular concentrations as high as 560 g/L.[7] Mitochondrial enzymes are highly regulated, but we do not yet know the role that crowding plays in regulation. A growing body of research indicates that crowding influences kinetics,[8−10] yet, to our knowledge, only one mitochondrial enzyme, creatine kinase, has been assessed under crowded conditions.[11]

Theory and experiment have revealed several themes.[5] Briefly, macromolecules tend to exclude volume such that crowded systems favor the most compact configuration. Thus, for an enzyme with multiple conformations, excluded volume will shift the equilibrium toward the smallest conformation. Likewise, if an enzyme is in equilibrium between a more compact dimer and individual monomers, crowding will favor the dimer.[12] Crowding typically decreases the Michaelis–Menten constant, K_m, presumably by enhancing the activity of water or increasing the ratio of activity coefficients between the enzyme and enzyme–substrate complex.[13−17]

Despite these trends, there remain more questions than answers. For example, synthetic polymeric crowding agents elevate the enzymatic activity of some proteins, such as glucosidase II[18] and ADP-sugar phosphatase,[15] but decrease the activity of others, like hexokinase[16,17] and alkaline phosphatase.[19] Even for a single enzyme, the effects can depend on macromolecule concentration in a nonlinear fashion.[13] For the multicopper oxidase, Fet3p, an increase in crowding initially increases the rate, but above a certain macromolecule concentration, the rate decreases.[20] Often, the nature of the macromolecule used for crowding plays a critical role. For example, one study found that low concentrations of protein crowding agents increased enzymatic activity, whereas the same concentrations of synthetic crowding polymers, such as dextran, decreased activity of the same enzyme.[13]

These conflicting observations are most likely due to the fact that crowding has differing effects on the variables contributing to the enzymatic rate. Diffusion, binding, thermodynamic activity, and enzyme stability all impact rates; yet, crowding influences each of these factors in a unique manner.[21] Typically, crowding by synthetic polymers stabilizes enzymes and promotes protein folding, self-association, and binding, while hindering diffusion.[5] As a result, the effects of crowding on kinetics may depend on the mechanism of an individual enzyme and require a case-by-case analysis. In addition, the nature of the crowding agent is likely to play a significant role; yet, to our knowledge, only one previous kinetic study has directly compared the effects of neutral and charged crowders for a single enzyme.[13] Measuring the way an enzyme responds to a range of crowded conditions could provide important insight about the kinetic mechanism, conformation, or oligomeric state of that enzyme.

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To investigate the connection between kinetic parameters and macromolecular crowding, we chose the mitochondrial enzyme malate dehydrogenase (MDH). MDH is a homodimer that catalyzes the reversible reduction of oxaloacetate to malate by oxidizing NADH to NAD^+ for use in both the citric acid cycle and the malate/aspartate shuttle. MDH has been extensively characterized because of its central role in metabolism and biotechnological applications.[22] The rate-limiting step has been determined to be the release of NAD^+ or NADH, which involves a conformational change.[23,24] Nevertheless, investigators are divided as to whether the subunits act independently since a stable MDH monomer has not been isolated.[22] One school of thought suggests a reciprocating compulsory ordered mechanism in which both subunits are necessary.[25] This mechanism is supported by studies showing reduced activity upon forced dissociation to the monomer.[26]

The contrasting model, which assumes subunit independence, is corroborated by observed enzymatic activity in an immobilized monomeric form of MDH.[27] Gelpi et al. apply a modified version of this model to explain MDH regulation involving malate activation and oxaloacetate inhibition. Their model proposes two conformations: the normal enzyme, E, and a conformation, F, with lower substrate affinity but a higher catalytic efficiency.[28] The conformations are in equilibrium, but binding of malate or oxaloacetate to a regulatory site favors the conversion of E to F. Although E binds NAD^+ or NADH and catalyzes the forward and reverse reaction (as required by microscopic reversibility), F cannot bind NADH and thus catalyzes only malate oxidation. Their idea is supported by several independent studies suggesting that NAD^+ and NADH bind different conformations of the enzyme.[29,30] Studying the effect of crowding on catalysis may provide insight because crowding often enhances oligomerization.

Here, we elucidate the influence of crowded conditions on the kinetics of a mitochondrial enzyme to gain insight into its mechanism. More specifically, we focus on how neutral, inert polymers and charged proteins of various sizes affect K_m and V_{max}. MDH is an ideal model for several reasons. First, its activity can be monitored in both directions with UV spectroscopy because NADH absorbs at 340 nm but NAD^+ does not. Second, MDH obeys Michaelis–Menten kinetics and has been well-characterized.[22,31] Third, there are no known interactions between dehydrogenases and the dextrans used as crowding agents.[9]

The size of MDH, 70 kDa, is also advantageous. Several studies focus on how the relative size of the enzyme and crowder contribute to crowding effects.[8] Generally, the activity of smaller enzymes like α-chymotrypsin (25 kDa)[32] and horseradish peroxidase (42 kDa) is unaffected by the molecular weight of the crowding agent;[33] yet, the activity of large enzymes such as lactate dehydrogenase (150 kDa)[9] and alkaline phosphatase (105 kDa)[19] is decreased more dramatically as dextran size increases. This trend arises from competing factors. Large dextrans reduce enzyme–substrate encounters, which subsequently decrease rates. This effect is offset by caging effects for smaller dextrans. Experiments with MDH are especially interesting because its size is between that of enzymes previously studied and between the sizes of crowding agents that are commercially available.

EXPERIMENTAL PROCEDURES

Chemicals. Chemicals were analytical or reagent grade. Mitochondrial porcine heart malate dehydrogenase (mMDH, E.C. 1.1.1.37, 250 U/mg) from EMD Chemicals and *Thermus flavus* malate dehydrogenase (TaqMDH, 50 U/mg) from Sigma-Aldrich were received as purified lyophilized powders. Dextran polymers (6, 20, 40, 75, 150, and 500 kDa) were purchased from Alfa Aesar, except for 250 kDa dextran, which was from Spectrum Chemicals. For poly(vinylpyrrolidone) (PVP), the 10 kDa polymer was from Sigma-Aldrich, and the 40 kDa polymer was from Molecular Probes. The pH of all crowding agent solutions was corrected to 8.0.

Hen Egg White. Hen eggs, from Kreher's farm (Clarence, NY), were prepared by mechanically separating the white from the yolk.[34] Subsequent filtering of the hen egg white (HEW) with cheesecloth to eliminate thick membranes provided a solution at a pH of 9.0. Thus, for any assays involving HEW or buffer assays run concurrently for normalization, a 100 mM Trizma buffer (pH 9.0) was used. To ensure that HEW did not contain MDH or MDH substrate, control assays were run as described below except with MDH or substrate omitted. These controls yielded flat absorbance versus time graphs.

Assays. mMDH activity was monitored spectrophotometrically at 25 °C in a 96-well plate with a Tecan InfiniteM200 Pro spectrophotometer. Each of 16 wells contained a total volume of 200 μL consisting of 1.5 mM oxaloacetic acid, 20–300 μM NADH, 2.5 μg/mL mMDH, and various crowding agents (100 or 300 g/L) in 100 mM Trizma buffer (pH 8.0). The reaction was initiated by the addition of oxaloacetic acid, and the absorbance was monitored at 340 nm every 12.5 s for 4 min with shaking. The above assay was repeated, but 0.25 μg/mL TaqMDH was substituted for mMDH. The reverse reaction with mMDH also was performed with the above conditions except that NAD^+ concentrations ranged from 0.08 to 2.0 mM, and 30 mM malic acid was added to initiate the reaction. All assays were repeated in triplicate.

Data Analysis. The reduction of oxaloacetate by NADH and the oxidation of malate by NAD^+ can be treated as single-substrate reactions following the scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \quad (1)$$

because the oxaloacetate and malate were both used at saturating levels. E is the enzyme, S is the substrate (NAD^+ or NADH), and k_1, k_{-1}, and k_{cat} are rate constants. Thus, the enzymatic activity can be studied with the Michaelis–Menten equation

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

where V_{max} is the maximum rate defined as $k_{cat}[E]_t$ and K_m is the Michaelis–Menten constant defined as $(k_{-1} + k_{cat})/ k_1$.

Absorbance at 340 nm versus time plots (Figure S1 of the Supporting Information) were analyzed for maximal initial rate via Magellan software. A single Michaelis–Menten curve was then constructed by averaging three initial rates, v_o, per NADH concentration, [S], for 16 concentrations. Each curve was fit to eq 2 using SigmaPlot to determine K_m and V_{max} values. K_m and V_{max} values under crowded conditions were normalized to the values obtained in buffer alone.

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RESULTS

The mitochondrial MDH-catalyzed reaction was performed under crowded conditions. In most cases, a concentration of 300 g/L dextran was used to mimic the macromolecular concentrations in a typical cell.[1,2] Initial rates were measured in triplicate for each concentration of NADH and averaged to generate a 16-point Michaelis–Menten curve (Figure 1). To account for day-to-day variability, crowding K_m and V_{max} values were normalized to the K_m and V_{max} values obtained concurrently in buffer

$$\text{relative kinetic parameter} = \frac{\text{kinetic parameter with crowding agent}}{\text{kinetic parameter in buffer only}} \quad (3)$$

The relative values from three independent curves were averaged to provide relative K_m and V_{max} values.

Histograms comparing these normalized values reveal that exposure to any size dextran results in relative V_{max} values statistically less than 1, with the lowest values corresponding to intermediate-sized polymers (Figure 2A). Glucose also causes a decrease in V_{max} and K_m (Figure 2A,B). In fact, most of the kinetic parameter values in the presence of dextran are not statistically different than those for glucose. Exposure to poly(vinylpyrrolidone) (PVP), another polymer commonly used as a crowding agent, resulted in trends very similar to those with dextran (Figure S2 of the Supporting Information).

The MDH assay also was conducted at multiple concentrations of glucose, dextran, or PVP. For polymers similar in size to MDH (70 kDa) and smaller, assays with 300 g/L crowding agent resulted in V_{max} values significantly lower than those with 100 g/L of crowding agent (Figure 2C). In contrast, altering the concentration of a larger dextran polymer (250 kDa) did not affect the V_{max} significantly. Unexpectedly, the concentration of glucose did not affect the MDH kinetic parameters (Figure S3 of the Supporting Information).

To more closely mimic intracellular conditions, the MDH assay was repeated with charged crowding agents. V_{max} is

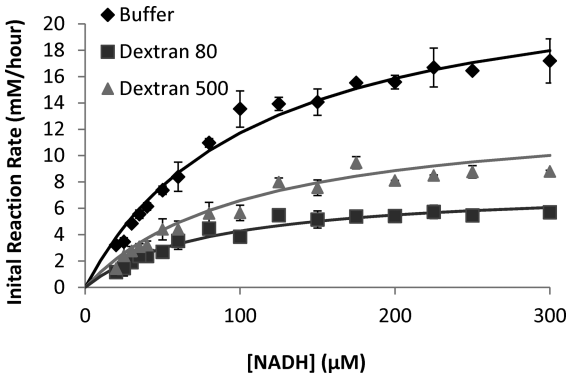

Figure 1. Michaelis–Menten curves for the MDH-catalyzed reduction of oxaloacetate. Assays comprising 1.5 mM oxaloaceate, 20–300 μM NADH, and 2.5 μg/mL mitochondrial porcine heart MDH in 100 mM Trizma buffer (pH 8.0) were run in the presence of 300 g/L dextran 500 kDa, dextran 80 kDa, or buffer only and monitored spectrophotometrically at 340 nm. Initial reaction rates were determined from the slope of the linear portion of the absorbance versus time plot. Error bars represent sample standard deviations (n = 3).

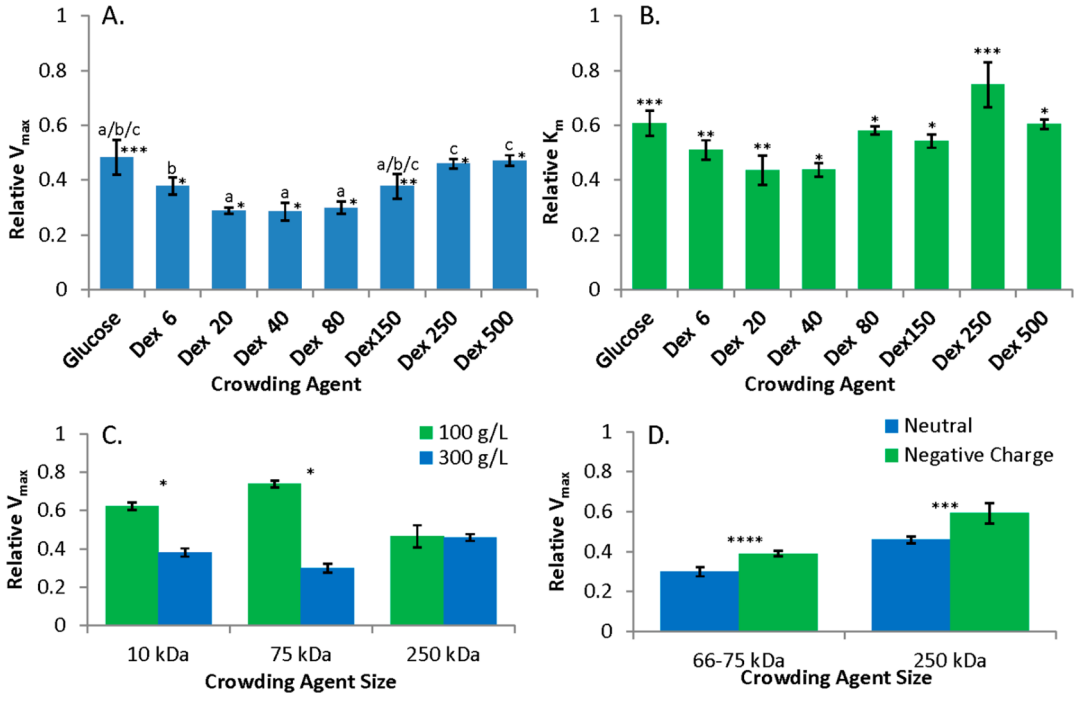

Figure 2. Crowding affects reduction of oxaloacetate. Assays containing 2.5 μg/mL mitochondrial MDH, 20–300 μM NADH, and 1.5 mM oxaloacetate in 100 mM Trizma buffer (pH 8.0) were performed in the presence of 300 g/L glucose or crowding agent unless otherwise stated. The crowding agents were (C) PVP (10 kDa), Dex (75 kDa), and Dex (250 kDa); (D) Dex (75 kDa, blue), BSA (66 kDa, green), Dex(250 kDa, blue), and gum arabic (250 kDa, green). K_m and V_{max} values from the resulting 16-point Michaelis–Menten curves were normalized to values acquired in the absence of crowding agent to yield the relative kinetic constants shown above (y axes). (A) Bars that do not share a common letter (a, b, c) differ significantly ($P < 0.05$). The kinetic parameter values in the presence of dextran are not statistically different than those for glucose (two-tailed Student's t test $P > 0.1$ except for K_m with Dex 250, $P = 0.02$). Error bars represent standard errors (n = 3). Astericks indicate a significant difference from unity (A, B) or between charged and neutral crowders (C, D): *$p < 0.005$, **$p < 0.01$, ***$p < 0.05$, ****$p < 0.08$ (Student's two-tailed t test).

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significantly higher in the presence of both anionic crowding agents tested (gum arabic, GA, and bovine serum albumin, BSA) compared to that with the neutral dextran polymer of similar size (Figure 2D). In contrast, no trend in K_m is observed when comparing charged and neutral crowding agents (Figure S4A,C of the Supporting Information). Cationic crowding agents were not used at 300 g/L because they require lower pH environments to dissolve and often aggregate at high concentrations.[35] The MDH assay performed in 100 g/L lysozyme (pI 11.3) resulted in a relative V_{max} that was not statistically different than the value for dextran (Figure S4B of the Supporting Information). The assay was repeated with hen egg whites, which contain lysozyme as well as 40 other proteins.[34] Interestingly, the egg white decreased the MDH kinetic parameters more than any other crowding agents tested, with a relative K_m of 0.26 ± 0.03 and a relative V_{max} at 0.34 ± 0.04.

Next, the effects of crowding were investigated for the reverse reaction: malate and NAD^+ conversion to oxaloacetate and NADH. All of the relative K_m values in this direction are greater than one regardless of crowding agent size or charge (Figure 3A, green bars). The relative V_{max} are affected

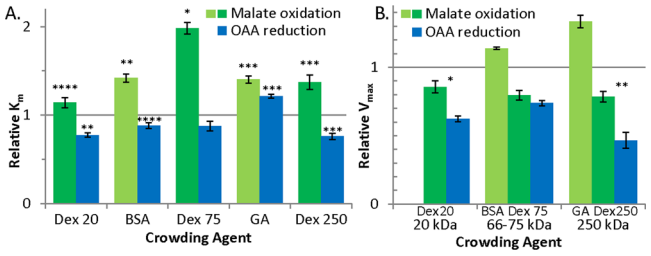

Figure 3. Comparing forward and reverse reactions. The relative kinetic parameters (A) K_m and (B) V_{max} for converting malate and NAD^+ to oxaloacetate (OAA) and NADH (green) or oxaloacetate and NADH to malate and NAD^+ (blue) were obtained in the presence of crowding agents at 100 g/L. Dark green represent the neutral dextrans (Dex) (20, 75, and 250 kDa), and light green denotes the charged crowding agents: bovine serum albumin (BSA, 66 kDa, pI 4.9) and gum arabic (GA, 250 kDa, anion). Error bars represent standard error (n = 3). Astericks indicate (A) a significant difference from unity or (B) a significant difference between malate oxidation and OAA reduction V_{max} values: *p < 0.005, **p < 0.01, ***p < 0.05, ****p < 0.08 (Student's two-tailed t test).

differently by neutral and charged crowders such that charged crowding agents increase reaction rates, but neutral polymers hinder the rates (Figure 3B). Thus, for both malate oxidation and oxaloacetate reduction, V_{max} values were significantly lower with neutral crowders than those with charged crowders. Interestingly, though, dextran had more of an effect on the rate of oxaloacetate reduction than on malate oxidation.

To understand how crowding affects isozymes, the assays were repeated with thermophilic TaqMDH from *T. flavus*. The relative K_m values for the TaqMDH assay (Figure 4A) are statistically greater than one in the presence of neutral polymers, but they are not statistically different than those in the presence of charged crowding agents (lysozyme and gum arabic). Interestingly, TaqMDH V_{max} values were significantly greater in the presence of neutral crowders than those with charged ones, which is the opposite trend from that observed for mitochondrial porcine heart MDH (mMDH). Overall, the presence of neutral polymers lowered the kinetic parameters of

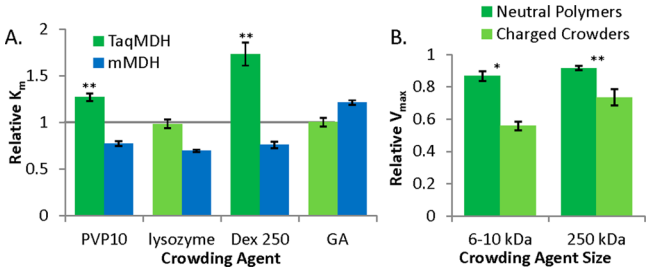

Figure 4. Crowding effects depend on isozyme. The relative kinetic parameters (A) K_m and (B) V_{max} for the reduction of oxaloacetate were studied using 0.25 μg/mL thermophilic TaqMDH from *T. flavus* (green) or 2.5 μg/mL mitochondrial porcine heart malate dehdrogenase, mMDH (blue), and the conditions described in Figure 2, except with 100 g/L crowding agent. Light green denotes the charged crowding agents lysozyme (14 kDa, pI 11.3) and gum arabic, GA (250 kDa, anion). Error bars represent standard errors (n = 3). Astericks indicate (A) a significant difference from unity for the TaqMDH data or (B) a significant difference in effect between neutral and charged crowders on V_{max} values: *p < 0.01, **p < 0.05 (Student's two-tailed t test).

mMDH significantly lower than TaqMDH (Figures 4A and S4D of the Supporting Information).

DISCUSSION

Macromolecular crowding effects on MDH depend on the isozyme, reaction direction, and crowding agent (Table 1). Crowding increases K_m of mitochondrial MDH for malate oxidation and of TaqMDH for oxaloacetate reduction, but it decreases the K_m value of mitochondrial MDH in the oxaloacetate reduction direction. All crowding conditions decrease the MDH-catalyzed rate of converting oxaloacetate and NADH to malate and NAD^+, but the effect on the reverse reaction depends on the charge of the crowding agent. In fact, for mitochondrial MDH, relative V_{max} values are always larger in charged crowding agents than those in equivalent-sized neutral polymers.

Table 1. Summary of Crowding Effects[a]

MDH	direction	crowder	K_m	V_{max}
Mito	oxaloacetate + NADH → malate + NAD^+	neutral	↓	↓↓
		charged	↓	↓
	malate + NAD^+ → oxaloacetate + NADH	neutral	↑	↓
		charged	↑	↓
Taq	oxaloacetate + NADH → malate + NAD^+	neutral	↑	↓
		charged	--	↓↓

[a]↑ denotes increase, ↓ denotes decrease, and -- denotes no change for crowded solutions relative to buffer alone. Double arrows are used to emphasize statistically different V_{max} values for charged and neutral crowder (Mito, P < 0.08; Taq, P < 0.05). Mito denotes mitochondrial porcine heart MDH, and Taq denotes MDH from the thermophile, *T. flavus*.

The most significant finding from this work is that the nature of the crowding agent plays such a crucial role in influencing MDH kinetics. This observation is not surprising since differing effects with charged globular proteins and synthetic neutral polymers have been well-documented in protein stability studies.[36−41] Nonetheless, for kinetic studies, the charge of the crowding agent has been largely ignored. One study that did directly compare globular proteins and synthetic neutral

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polymers found very different kinetic effects with each type of crowder.[13] Specifically, the activities of three enzymes decreased with increasing polymer concentration but increased with globular protein concentration. Thus, consistent with our findings, the previous study observed enzyme rates to be higher in the presence of charged proteins than those in the presence of neutral polymers. The enhanced activity with protein crowders was attributed to increased enzyme oligomerization, which provides a logical explanation for our data as well. MDH is a dimer, and its forced dissociation to monomer has been shown to decrease activity.[26] Thus, if charged crowders enhance dimerization, then V_{max} values would be greater in their presence than with neutral polymers (Figures 2D and 4B). In contrast, K_m would not be affected (as observed in Figures 3A and S4 of the Supporting Information) because each MDH subunit independently binds substrate. With the enzymes studied thus far, both in this and previous work, the increased enzymatic activity in the presence of charged crowders can be explained by enhanced oliogomerization relative to neutral crowders. However, this theory is still speculative and further experiments are necessary to corroborate it. In future work, we plan to compare the kinetic effects of charged and neutral crowders on dehydrogenases with similar mechanisms to MDH that are not influenced by oligomerization.

Given the importance of crowding agent properties and the fact that a cell is a mixture of various charged macromolecules, we exposed the MDH assay to hen egg white (HEW). HEW is arguably a better mimic of intracellular conditions than any single protein or polymeric crowding agent because it contains a high concentration of macromolecules (over 12% by weight) with about 40 different proteins.[34] Ovalbumin (45 kDa), the most abundant protein in HEW (54% of the protein), has similar properties as those of BSA (66 kDa), including its isoelectric point and amino acid content. Lysozyme is another abundant protein in HEW; yet, HEW affected MDH kinetics very differently than did BSA and lysozme. While most charged crowders increased V_{max} relative to the corresponding neutral polymer, HEW decreased the V_{max} more drastically than any of the neutral polymers. This result may be due to the fact that HEW is a mixture. Evidence suggests that mixtures of crowding agents have more significant effects than a single crowding agent.[42−44] Another possibility is that HEW may contain a protein or small molecule inhibitor that alters MDH kinetics in a manner unrelated to crowding. This perplexing observation reveals that although HEW may be more cell-like than single crowding agents, its intricate, uncharacterized nature complicates interpretation of any results. Thus, we still have much to learn about the complex crowded environment of the cell, and a need still exists for experiments with simple crowding agents or mixtures of crowders.

To begin to comprehend which properties of macromolecules have the most effect on enzyme kinetics, we focused on understanding the relationship between crowding agents and enzyme size. Contrary to earlier work, no size dependence was observed at low dextran concentrations (100 g/L).[8,9,32,33] At 300 g/L, MDH activity was most affected by intermediate-sized polymers, rather than by the largest ones (Figure 2A). Furthermore, unlike lactate dehydrogenase (LDH) and alkaline phosphatase, the effect of crowding on MDH was independent of concentration for 250 kDa dextran (Figure 2C, compare blue and green bars). These differences are most likely due to two factors. First, at 70 kDa, MDH is smaller than LDH (150 kDa) and alkaline phosphatase (105 kDa). Thus, it is not unexpected that it would be more affected by smaller dextrans that more closely reflect its size. Second, crowding effects for LDH and alkaline phosphatase were attributed mainly to inhibition of substrate−enzyme encounters.[8] In contrast, other factors for MDH are likely to be influenced by crowding, such as the multiple conformational changes involved in catalysis.[23] It is surprising that crowding would not affect MDH and LDH more similarly since they share parallel mechanisms and the same rate-limiting step.[24,45] However, the differences may be attributed to size or additional MDH conformational changes resulting from allosteric regulation.[28]

Often, studies with polysaccharide crowding agents use small sugars to differentiate between excluded volume and chemical effects.[13,14,19,20] Both dextran and glucose have similar chemical properties because dextran is a polymer of glucose, but glucose is not able to exclude volume like its larger counterpart. Thus, small molecule sugars can help to isolate the effects of macromolecular crowding from other factors like viscosity, polarity, and ionic strength. Our findings with glucose (Figure 2A) are consistent with the previous observation that 40% glycerol decreases K_m and k_{cat} values of MDH.[23] In that study, the decreased K_m was explained by a dielectric constant induced increase in electrostatic interactions. The authors also concluded that the decreased k_{cat} was evidence that the rate-limiting step of the MDH mechanism involved a conformational change. Their rationale was that conformational changes are slower in more viscous solvents. This interpretation, however, does not fully explain our data because the decrease in K_m and V_{max} is independent of glucose concentration (Figure S3 of the Supporting Information). On the basis of the explanations above, higher glucose concentrations should affect both K_m and V_{max} because of increased viscosity and lower dielectric constants.

Alternatively, the decreased K_m could be due to glucose stabilizing the optimal MDH conformation for coenzyme binding because MDH undergoes multiple conformational changes[24] and because it is well-known that sugars stabilize proteins,[46,47] including alcohol dehydrogenase.[35] In addition, it is likely that the observed decrease in V_{max} is a consequence of the decreased K_m. If MDH has a greater binding affinity for NADH in the presence of glucose (decreased K_m), then MDH will most likely have a greater affinity for NAD^+ as well. The enhanced affinity of MDH for the coenzyme could result directly in decreased enzymatic activity because the rate-limiting step of this reaction is NAD^+ release.

Regardless of the specific reason that glucose decreases both K_m and V_{max}, the important component for the scope of this article is to compare the effect of glucose and dextran on MDH kinetics. The fact that the relative K_m for glucose is similar to that of the dextrans suggests that the K_m effects are most likely a chemical phenomenon and not due to macromolecular crowding. Similarly, the V_{max} values for dextran cannot be shown to be statistically different than the glucose V_{max} value. Since the lack of statistical significance is likely due to the large error associated with the glucose V_{max} (Figure 2A), a different approach was necessary to determine if the decreased V_{max} values in the presence of dextran are due to exclusion of volume or chemical interactions with the dextran. The MDH assay was repeated in the presence of the polymer poly(vinylpyrrolidone) (PVP). PVP is another widely accepted crowding agent with different intermolecular forces and viscosity (in aqueous solution) than those of dextran.[37] The relative kinetic parameters of PVP were consistent with the values obtained

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in the presence of similar sized dextran polymers (Figure S2 of the Supporting Information), suggesting that the effect is a physical one due to crowding rather than a chemical manifestation.

These complex effects from macromolecular crowding may be explained in the context of the E and F conformations from the model proposed by Gelpi et al.[28] We speculate, based on our results, that crowding may favor the F conformation of MDH, either directly or by increasing the effective concentration of malate or oxaloacetate (Figure 5). If dextran does

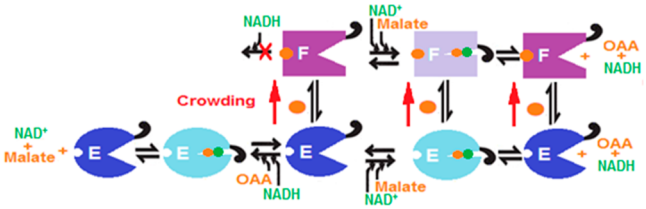

Figure 5. Schematic representation of the effects of macromolecular crowding on the MDH kinetic mechanism. E and F are MDH conformations where E (blue circle) binds both coenzymes (green circles) but F (purple square) binds NAD^+, not NADH. F binds NAD^+ with lower affinity than E. Both crowding and allosteric effectors (orange circle) citrate, oxaloacetate (OAA), and L-malate promote conversion of E to F. Once both substrates bind, the open configuration (dark colors) undergoes an external loop closure to form the closed configuration (light colors).

promote F, then the V_{max} of oxaloacetate reduction would decrease, as observed, since F does not bind NADH. The decreased substrate affinity exhibited by F for NAD^+ also impedes V_{max} in the opposite direction (but not as drastically) because crowding inhibits enzyme−substrate encounters. This decreased NAD^+ affinity also explains the fact that the relative K_m values are all above 1 for this opposite direction (Figure 3A). In contrast, the K_m values for NADH are not affected by F because this conformation cannot bind NADH. Rather, the decreased K_m is due to the same chemical effects observed with glucose (Figure 2A).

To further investigate crowding effects on mechanism, a second isozyme, TaqMDH, also was examined.[24] Most MDH isomers contain an aspartic acid at the coenzyme binding site that forms hydrogen bonds with the hydroxyl groups of the adenine-ribose, but TaqMDH has a glutamic acid in the equivalent position.[45] This substitution enhances the affinity of TaqMDH for the coenzyme and in turn reduces the enzymatic rate.

Although crowding decreases K_m for mMDH, the K_m values of TaqMDH are unaltered in the presence of lysozyme and gum arabic (Figure 4A). As a thermophilic enzyme, TaqMDH has evolved to function at high temperatures, presumably by optimizing its stability.[48] Thus, unlike mMDH, addition of crowders has no additional effect to stabilize TaqMDH or decrease K_m. Given the lysozyme and gum arabic results, the increased K_m for TaqMDH in the presence of neutral polymers is more puzzling. Crystallographic studies reveal that TaqMDH compensates for the bulky glutamic acid by contracting its peptide backbone to optimize the hydrogen-bonding distance.[45] Viscous polymeric solutions perhaps impede the ability of TaqMDH to contract its backbone and thus decrease its binding affinity. After all, solutions of synthetic polymers tend to be much more viscous[13] than charged crowder solutions, and previous studies have documented that viscous solutions can impede conformational changes.[49−51] This justification also explains why the relative V_{max} values for TaqMDH are significantly larger in the presence of neutral polymers compared to that with charged crowders. The increased V_{max} could result from neutral polymers decreasing the TaqMDH binding affinity for NADH and NAD^+. In fact, Alldread et al. previously demonstrated a correlation between these two kinetic parameters by mutating glutamic acid-41 of TaqMDH to an aspartic acid, which resulted in increasing both the K_m and the k_{cat} by 2.5-fold.[23]

Finally, MDH is of particular interest because it catalyzes a reversible reaction, but it functions primarily in the malate oxidation direction within the mitochondria. Thus, comparing the way that macromolecular crowding affects the two directions of this reaction may provide some insight as to how biological systems have evolved to optimize effective enzyme function under the constraints of crowding. One might expect malate oxidation, which occurs in the densely packed mitochondria, to be positively influenced by crowding. Indeed, our results show that, while crowding decreases V_{max} for all other conditions (Table 1), protein crowding agents, like those found in the mitochondria, increase MDH activity in the direction of malate oxidation (Figure 3B).

At the same time, the increased K_m for malate oxidation (NAD^+ reduction) and decreased K_m for oxaloacetate reduction (NADH oxidation) are consistent with the fact that intracellular concentrations of NAD^+ are high relative to those of NADH.[52,53] In fact, evidence suggests that bidirectional metabolic enzymes tend to have K_m values close to intracellular substrate concentrations to allow efficient response to changes in metabolite flux.[54] For malate oxidation, the slightly increased K_m from crowding (Figure 3A) suggests that higher concentrations of NAD^+ substrate are required to reach V_{max}. This change would allow the mitochondria to exert more regulation over MDH activity by controlling substrate concentration.

■ CONCLUSIONS

Macromolecular crowding effects are complex. Even isoforms show different kinetic responses to crowding. It is concerning that most kinetic crowding studies to date have used neutral polymers.[8,9,14,20,32,33,54,55] Our work shows that charge plays a major role. In fact, egg white, which may be a better mimic of cellular conditions than traditional crowding agents,[34] exhibited the most dramatic effect. Thus, the reality of the intracellular matrix may be quite different than even our best attempts to simulate it. As such, we hope our results will prompt others to create better means to understand the crowded cellular environment. Finally, we speculate that our results are consistent with the E and F conformational model of MDH, providing an example of how closely macromolecular crowding effects are tied to enzymatic mechanism. Due to this connection, future crowding studies could be helpful in providing insight about the mechanism of less established enzymes.

■ ASSOCIATED CONTENT

Supporting Information

Sample raw data of absorbance vs time (S1) and effect of PVP (S2), glucose (S3), and charge crowders (S4) on MDH kinetics. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

BSA, bovine serum albumin; HEW, hen egg white; GA, gum Arabic; LDH, lactate dehydrogenase; mMDH, mitochondrial porcine heart malate dehydrogenase; PVP, poly(vinylpyrrolidone); TaqMDH, thermophilic malate dehydrogenase from *Thermus flavus*

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