Effect of Crowding by Dextrans and Ficolls on the Rate of Alkaline Phosphatase–Catalyzed Hydrolysis: A Size-Dependent Investigation

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Abstract: The cell cytosol is crowded with macromolecules such as proteins, nucleic acids, and membranes. The consequences of such crowding remain unclear. How is the rate of a typical enzymatic reaction, involving a freely diffusing enzyme and substrate, affected by the presence of macromolecules of different sizes, shapes, and concentrations? Here, we mimic the cytosolic crowding in vitro, using dextrans and Ficolls, for the first time in a variety of sizes ranging from 15 to 500 kDa, in a concentration range 0–30% w/w. Alkaline phosphatase–catalyzed hydrolysis of p-nitrophenyl phosphate (PNPP) was chosen as the model reaction. A pronounced decrease in the rate with increase in fractional volume occupancy of dextran is observed for larger dextrans (200 and 500 kDa) in contrast to smaller dextrans (15–70 kDa). Our results indicate that, at 20% w/w, smaller dextrans (15–70 kDa) reduce the initial rate moderately (1.4- to 2.4-fold slowing), while larger dextrans (>200 kDa) slow the reaction considerably (>5-fold). Ficolls (70 and 400 kDa) slow the reaction moderately (1.3- to 2.3-fold). The influence of smaller dextrans was accounted by a combination of increase in viscosity as sensed by PNPP and a minor offsetting increase in enzyme activity due to crowding. Larger dextrans apparently reduce the frequency of enzyme substrate encounters. The reduced influence of Ficolls is attributed to their compact and quasispherical shape, much unlike the dextrans. © 2006 Wiley Periodicals, Inc. Biopolymers 83: 477–486, 2006

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INTRODUCTION

The aqueous phase of cytoplasm is crowded with macromolecules such as soluble proteins, nucleic acids, and membranes that often approach total concentrations in the range of 300–400 mg/mL, which corresponds to occupation of a substantial volume fraction in the cell interior.1,2 As a consequence of such macromolecular crowding, the thermodynamic activity of the macromolecule increases manifold. In the crowded milieu, biochemical equilibria strongly favor association of macromolecules.3,4

Another outcome of macromolecular crowding is the decrease in molecular diffusion, a major determinant of many cellular processes. The translational diffusion of a fluorescent probe such as carboxyfluorescein is slowed by approximately fourfold inside the cytoplasm relative to aqueous medium.5 while for a macromolecule like green fluorescent protein, it is reduced by three- to fivefold under the same condition.6 The diffusion of FITC–dextran and FITC–Ficoll of molecular size 500 kDa or less have been shown to be equally slow inside the cytoplasm.7 Molecular diffusion of enzymes and their substrates play a key role in metabolism where the encounter of the free substrate with the active site of the freely diffusing enzyme can often be the rate-determining step. Quite a few studies have however explored the consequences of crowding on enzyme catalysis in vitro. Minton has analyzed the effect of macromolecular crowding on the different kinetic steps of enzyme catalysis namely, 1) the enzyme (E)–substrate (S) encounter and formation of ES complex, 2) the formation of enzyme–product complex from ES complex, and 3) release of product from the enzyme–product complex.8 It is predicted that under the condition when the encounter between the substrate and enzyme is rate-limiting, the rate of an enzyme-catalyzed reaction will experience a monotonic decrease with increase in the fractional volume occupancy (\(\phi\)) of the crowding agent. The influence of different concentrations of Ficoll 70 on the rate of EcoRV-catalyzed cleavage of pBR 322 was investigated by Wenner and Bloomfield.9 They concluded that Ficoll 70 had little effect on the overall reaction velocity of EcoRV in the concentration range 0–20% g/dL owing to offsetting increases in \(V_{\text{max}}\), \(K_m\), and stronger nonspecific binding between enzyme and substrate/product. The influence of PEG 8000 on the kinetics of trypsin-catalyzed hydrolysis of p-nitrophenyl acetate was investigated recently.10 Although the \(K_m\) was invariant, an \(-2.7\)-fold decrease in \(k_{\text{cat}}\) was observed for the enzyme in the PEG concentration range 0–395 gL\(^{-1}\). Several other studies in the past have also reported only a moderate influence of high concentrations of neutral polymers on enzyme reactions.11,12

Conspicuously, most of the studies highlighted above have utilized crowding agents of a fixed size or narrow size range only. It is well known that the living cell is crowded by macromolecules in a wide variety of shapes and sizes.13 It is imperative to investigate the influence of crowding as exerted by macromolecules in a range of sizes. The role of obstacle shape in crowding is another aspect that deserves attention. Will dextran and Ficoll of identical molecular weight exert similar influence during crowding? Again this remains to be explored. The influence of larger obstacles (>200 kDa) has NEVER been investigated. These are relevant, for example, to interpret biochemical events in the densely crowded mitochondrial matrix, the site for TCA cycle and fatty acid oxidation pathway. Consequently, the influence of crowding by large macromolecules of size 200 kDa and above is much needed, but is as yet unexplored. Finally, theoretical simulations have predicted that classical Michaelis–Menten kinetics may not apply to enzyme reactions in crowded media.14–16 This needs to be tested experimentally. We seek to address all of the issues highlighted above in this report.

We investigate how the kinetics of an enzymatic reaction is dependent on i) the concentration of the crowding species, ii) the size of the crowding species, and iii) the shape/compactness of the crowding species. We have employed the alkaline phosphatase–catalyzed hydrolysis of p-nitrophenyl phosphate (PNPP) as the model reaction for the above-mentioned purpose. Macromolecular crowding inside the physiological media was mimicked using inert polymers such as dextrans and Ficolls in a range of molecular weights from 15 to 500 kDa. The validity of Michaelis–Menten kinetics in enzyme reactions crowded with dextrans in a range of sizes from 15 to 500 kDa was also investigated.

Our results reveal a steeper decrease in the rate (in logarithmic scale) as a function of the fractional volume occupancy with larger dextrans (200 and 500 kDa) compared with smaller dextrans (15–70 kDa). In the crowded presence of 20% dextran (\(w/w\), a concentration typical of the total concentration of macromolecules inside the cytoplasm, the reaction rates are slowed \(-2\)-fold among smaller dextrans (15–70 kDa), \(-5\)-fold for 200 kDa dextran, and \(-7\)-fold for 500 kDa dextran. Ficolls of size similar to dextran (40 and 400 kDa) had a comparatively smaller influence on the reaction rates (\(-2\)-fold). We also observe that classical Michaelis–Menten kinetics is not obeyed in enzymatic reactions crowded by 40, 200, and 500 kDa dextran (each at
20% w/w) in contrast to that observed in the absence of crowding.

**EXPERIMENTAL**

**Materials**

Alkaline phosphatase (from bovine intestinal mucosa), Ficoll 70 and 400, and glycine were purchased from Sigma–Aldrich Chemicals Pvt. Ltd., New Delhi, India. Dextran (from *Leuconostoc mesenteroides*) of molecular weight 15, 40, 70, 200, and 500 kDa was purchased from Fluka. The polydispersities of the dextrans were typically less than 2.0 as reported by the manufacturer. *Para*-Nitrophenyl phosphate disodium salt was bought from Sisco Research Laboratories, Mumbai, India. All other chemicals employed were of analytical grade.

**Hydrolysis of PNPP**

A typical reaction mixture contained alkaline phosphatase (2 μM equivalent to 5 U/mL) and PNPP of desired concentration dissolved in an aqueous solution of 100 mM glycine buffered at pH 9.5. The substrate concentration was kept at 1 mM, which is well above the measured *K*~m~ under the reaction conditions employed (0.4 mM). A high substrate to enzyme molar ratio is also in agreement with similar ratios inside living cells. However, to rule out situations, such as substrate being trapped/bound in dextrans, a few experiments were also carried out with 20 mM substrate as described later. The concentration of dextran or Ficoll in the medium was varied between 0 and 30% (w/w). The total weight of the reaction medium was kept constant at 0.50 g. For dextrans, the concentrations were expressed in terms of the fractional volume occupancy (fvo) to simplify quantitative analysis later. The fractional volume occupancy, φ, was calculated as follows: The dextran concentration in % w/w was converted to w/vol (expressed as g/mL) using the measured values of solution density. The concentration of dextran expressed in g/mL was multiplied by the specific exclusion volume for dextran (0.75 cm³/g) reported earlier by Minton and coworkers to arrive at the fractional volume occupancy of dextran in solution.

The reaction was initiated by forcefully mixing the enzyme (typically ~25 mg in buffered aqueous medium inside a cuvette) with an aqueous buffered mixture containing PNPP and crowding agent (typically ~475 mg), using a syringe to ensure complete mixing. The progress of the reaction was conveniently monitored using a spectrophotometer (Hitachi, Model U2001, Japan) by recording the absorbance of the product *p*-nitrophenol at 450 nm. The completeness of the mixing was assessed and supported by the following observations: i) The absorbance of product formed initially increased steadily with time from the start, maintaining a linear (monophasic) profile. ii) The initial slope of the absorbance/time plot obtained above was reproducible when the experiment was repeated subsequently a multiple number of times under identical conditions.

The initial velocity, *V*, was obtained by linear regression of the first 10 s of the absorbance/time data, so that inhibition from appreciable build up of the product, inorganic phosphate, is negligible. The initial velocity observed under identical conditions, but in the complete absence of the crowding species, was referred to as *V*~0~. The normalized rate, *V*~norm~, was calculated from the following equation:

\[
V_{\text{norm}} = 100 \left( \frac{V}{V_0} \right)
\]

A value of 100 was chosen, so as to avoid negative numbers in the logarithmic scale. The points depicted in the figures are averages of at least four independent experiments done on different days. Blank solutions containing 25% (w/w) of the crowding agent employed and 1 mM PNPP showed negligible change in the absorbance in the complete absence of the enzyme under identical conditions, proving that all of the crowding agents employed in the study are indeed chemically inert (data not shown). All samples were made in deionized water. All experiments were carried out at 25°C.

**Fitting Enzyme Kinetic Data**

When the rate at which enzyme and substrate encounter each other in solution becomes rate limiting, the dependence of the observed initial velocity, *V*, on the fractional volume occupancy, φ, of the crowding agent, may be written as follows:

\[
V = V_0 \exp(-g\phi)
\]

where *V*~0~ is the initial velocity observed in the absence of the crowding agent.

This can be expressed in terms of *V*~norm~ as follows:

\[
V_{\text{norm}} = A \exp(-g\phi)
\]

Here *V*~norm~, the normalized rate is a function of φ, while A is a constant and g is another constant that is a function of the relative sizes and shapes of the enzyme, substrate, and crowding macromolecule. The dependence of *V*~norm~ on φ for dextrans of different sizes (see Figure 2) was fitted to Eq. (3).

**Dynamic Fluorescence Quenching of Fluorescein by Iodide**

Fluorescence lifetime was measured by nanosecond time-domain fluorimetry, using the “Fluorocube” supplied by IBH (Glasgow, UK). Samples of fluorescein were excited by light pulses at 495 nm from a nano-LED (pulse width of lamp profile at FWHM was ~1.32 ns) with a pulse repetition rate of 1 MHz. Fluorescence intensity (in counts) was detected at magic angle emission polarizer setting through a OG 550 long-pass filter using a cooled Model TBX-04-D photon detection module (IBH) operating in the time-corre-
lated single-photon counting mode.\textsuperscript{20} The output from the
detector was fed to a multichannel analyzer card interfaced
to a personal computer. The fluorescence peak counts were
not less than 10,000 and the time interval between success-
itive points in the raw intensity/time data were 0.11295 ns.
The raw intensity/time data (typically 200 points or more)
were fitted to a single exponential using the method of non-
linear least squares with iterative reconvolution of the
measured lamp profile. The goodness of the fit was eval-
uated from the randomness of the residuals and the reduced
$\chi^2$. The fluorescence lifetime of fluorescein ($0.5\text{ m}\text{M}$)
was measured (in a medium buffered at a pH of 9.5) in the pres-
ence of a series of concentrations of iodide (0–300 mM).
The medium also contained 0.1 mM Na$_2$S$_2$O$_3$ to avoid the
formation of I$_3$. The bimolecular quenching constant,
$k_q$, was calculated from the Stern–Volmer equation given
below:\textsuperscript{21}

$$\frac{\tau_0}{\tau} = 1 + k_q[Q]$$

Here, $\tau_0$ is the fluorescence lifetime in the absence of the
quencher, while $\tau$ is the lifetime in the presence of the
quencher. $Q$ refers to the molar concentration of the
quencher in the medium. Our experiments on the dynamic
quenching of fluorescein by iodide in aqueous buffer
yielded a $k_q$ of $\sim 2.7 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ at 298 K. The above
experiment was repeated in the presence of 20\% w/w of
each of the following dextrans: 15, 70, 200, and 500 kDa
dextran. The fluorescence lifetime of fluorescein in the ab-
sence of iodide ($\sim 4$ ns) was unaffected by the added pres-
ence of dextrans mentioned above. The Stern–Volmer plots
were linear in the added presence of dextrans (see Figure 5),
indicating a single uniform environment for fluorescein in
solution in the midst of dextrans.

**RESULTS AND DISCUSSION**

The alkaline phosphatase–catalyzed hydrolysis of $p$-
nitrophenyl phosphate to $p$-nitrophenol and inorganic
phosphate was chosen as a model enzymatic reaction
to investigate the influence of macromolecular crowd-
ing on biochemical reaction rates. The reasons for this
choice were as follows: i) The reaction is accompanied by minimal change in the excluded volume. In fact, the
substrate, PNPP ($M_w \sim 220$ Da), and product, $p$-
nitrophenol ($M_w \sim 140$ Da), are tiny compared with
surrounding macromolecules ($M_w \sim 15,000$ Da or
more) that excluded volume effects on substrate, and
product can be safely neglected. ii) The progress of the
reaction can be easily monitored by UV-visible spec-
troscopy. iii) Alkaline phosphatase, which is a homodi-
meric enzyme ($M_w \sim 105,000$ Da), diffuses relatively
less compared with the substrate ($M_w \sim 220$ Da). The
substrate initially is 500-fold in excess compared with the
enzyme. Thus, effectively it is the diffusion of the
substrate in the midst of relatively immobile enzyme
and background macromolecules that is relevant for
the enzyme–substrate encounter. A tiny molecule like
PNPP is ideal for this purpose.

Figure 1A and B reveals the typically observed initial
rise and subsequent plateau in the absorbance owing
to formation of the yellow product, $p$-nitrophenol, dur-
ing the hydrolysis of PNPP in the presence of increasing
amounts of 40 and 500 kDa dextran in the reaction
medium, respectively. In Figure 1A, we observe
only a marginal decrease in the initial velocity of the
reaction with increasing amounts of 40 kDa dextran
as revealed by the initial slope of the absorbance/time
data. In contrast, Figure 1B shows that there is a

![FIGURE 1] The typical progress of an alkaline phospha-
tase–catalyzed hydrolysis of PNPP (at 1 mM) monitored by
measuring the absorbance at 450 nm is shown. (A) In the pres-
ence of 40 kDa dextran. From top to bottom, the curves corre-
spond to fractional volume occupancy, $\phi = 0$, 0.038, 0.078,
0.161, 0.207, and 0.253. (B) In the presence of 500 kDa dex-
tran. From top to bottom, the curves correspond to $\phi = 0$,
0.038, 0.078, 0.119, 0.161 and 0.207. Other conditions are
similar to those described under Experimental. For time pro-
files of reactions in the presence of 15, 70, and 200 kDa dex-
trins and 70 and 400 kDa Ficolls, please see supplementary
figures.

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major drop in the initial velocity when increasing amounts of 500 kDa dextran are added to the medium. At a fvo = 0.207, which is equivalent to 25% w/w, we notice that the reaction slows appreciably. Similar data were collected with dextrans 15, 70, and 200 kDa and Ficoll 70 and 400 kDa (see supplementary material).

The initial velocity of the reaction was selected as the experimental parameter to measure in this study. This approach is simple and unambiguous because i) the substrate to enzyme density ratio is sufficiently large during this interval (10 s) and ii) inhibition of the enzyme by the product (P*), which is 100% present during this part of the reaction, can be neglected. Other kinetic parameters such as Vmax, Km, and kcat were avoided for reasons that are described later in this report.

The effect of differing concentrations of 15, 40, 70, 200, and 500 kDa dextran, taken individually, on the normalized reaction rate is depicted in Figure 2. The data were fitted to Eq. (3). The difference in the profile of smaller dextrans (15–70 kDa) and larger dextrans (>200 kDa) is clearly visible. While the smaller dextrans (especially 15 kDa) do not appreciably influence the rate until reaching a concentration of 0.20 fvo, the larger dextrans reveal a steep monotonic decrease in normalized rate in a logarithmic scale. This aspect is also reflected in the slopes of the fitted straight lines for individual dextrans. The slopes by themselves are indicative of the size of the individual dextrans. The profile observed with 200 and 500 kDa dextrans is similar to that expected when the rate of the reaction is limited by the diffusional encounter between alkaline phosphatase and PNPP.8,22

The alkaline phosphatase reaction, in the absence of crowding, is not diffusion limited (kcat/Km = 1.4 × 104 M⁻¹ s⁻¹) under the conditions employed by us; however, crowding by large macromolecules (200 kDa and higher) appears to reduce the frequency of enzyme substrate encounters resulting in the observed profile shown in Figure 2.22 The plateau type profile observed with smaller dextrans (15–70 kDa) in Figure 2 appears to resemble a situation in which the reaction is partly transition state–limited and partly diffusion-limited (Ref. 22, see Figure 3C). Clearly, smaller dextrans in contrast to larger dextrans do not affect the enzyme substrate encounters unless they are present in high concentrations. Taken together, it is obvious that size of the crowding dextran (15–70 versus 200–500 kDa) plays a major role in influencing the enzymatic rate. A more detailed explanation on the role of larger dextrans in affecting enzyme substrate encounter requires mathematical modeling.

A noticeable feature that deserves mention is that dextrans in the size range 15–70 kDa possess an almost similar influence on the enzyme reaction rate. This is interesting. We know that proteins constitute the majority of soluble macromolecules that crowd the cytoplasm.2,17 It is widely believed that the average molecular weight of proteins that are present in the cytosol (typically at concentrations ~0.15 fvo) is around ~40 kDa.13 Thus it appears that the crowding by dextran of size comparable to that of a protein having average molecular mass in the cytosol slows the reaction by only ~2-fold.

A best estimate for g in Eq. (3) was obtained from the fitted parameters. These are stated in the Figure 2 legend. A pronounced increase in the value of g from 5.4 with 15 kDa dextran to 14.5 with 500 kDa dextran is observed. Since the size of the enzyme, substrate, and product are invariant in this study; the increase in g reflects the increase in size of the dextran.

We now proceed to investigate in deeper detail the factors that could account for the mild influence of smaller dextrans (15–70 kDa). In Figure 3, the un-filled bars show the effect of different macromolecules that were used in this work on the fold decrease in the rate of alkaline phosphatase–catalyzed hydrolysis of PNPP. To enable comparison, the concentra-
tion of the crowding agent was maintained constant at 20% w/w (equivalent to fractional volume occupancy of 0.16 for dextrans), a value that comes close enough to the concentration of soluble macromolecules in the cytoplasm. As observed in the bar chart, dextrans in the range 15–70 kDa slow the reaction by 1.4- to 2.4-fold (*2-fold) only. A similar influence is also exerted by sucrose. The presence of 200 kDa dextran slows the reaction by *5-fold while the same in the presence of 500 kDa dextran is slowed by *7-fold. In contrast, solutions containing Ficoll 70 and Ficoll 400 slow the reaction by *1.3- and *2.3-fold, respectively. The influence of Ficolls on the reaction rate is discussed later in the paper. The decrease in enzymatic rate can also occur if PNPP gets trapped or adsorbed or stuck inside the dextran, leading to a decrease in free substrate concentration and consequently the rate of hydrolysis. To test this possibility, we carried out experiments with 20 mM PNPP. The gray-filled bars show the fold decrease observed when PNPP concentration is raised by 20-fold. A marginal increase is observed in the degree of slowing compared with 1 mM data (unfilled bars) for 40, 200, and 500 kDa dextrans, but the trend observed with 1 mM PNPP against increasing dextran size is maintained with 20 mM PNPP also. Thus the above possibility is ruled out.

The effect of sucrose on the reaction rate may be explained by the proportional increase in the solution viscosity. This is confirmed by the dependence of reaction rate on bulk solution viscosity as shown in Figure 4. It is important to know the effect of solution viscosity on the translational diffusion of PNPP in the different dextran solutions employed here. For this purpose, we investigated the influence of 15, 70, 200, and 500 kDa dextran solutions on the dynamic fluorescence quenching of fluorescein by iodide employing the technique of time-resolved fluorescence. Dynamic fluorescence quenching requires that the fluorophore in the excited state and the quencher collide with each other for fluorescence quenching to

**FIGURE 3** Decrease in bimolecular encounter rates owing to crowding. The unfilled bars indicate the mean fold drop in rate \( V_0/V \), as observed from alkaline phosphatase–catalyzed hydrolysis of \( p \)-nitrophenyl phosphate (here [PNPP] = 1 mM) in the presence of sucrose/dextrans/Ficolls as indicated in the x-axis. The gray-filled bars show the mean fold drop in rate when [PNPP] = 20 mM. The dark filled bars highlight the mean fold drop in the bimolecular quenching constant, \( k_{q(0)} \) observed by dynamic fluorescence quenching of fluorescein by KI in the presence of sucrose/dextrans as indicated in the x-axis. The reduced \( \chi^2 \) obtained during the nonlinear least squares fitting for determining fluorescence lifetime during quenching were between 1.0 and 1.3 in all cases. For all cases above, the concentration of the background molecule (sucrose/dextran/Ficoll) was kept constant at 20% w/w to facilitate comparison.

**FIGURE 4** Dependence of the rate on solution viscosity is shown. The relative rate \( V/V_0 \) of hydrolysis of \( p \)-nitrophenyl phosphate by alkaline phosphatase is plotted against the relative viscosity \( \eta/\eta_0 \) in sucrose–water mixtures. Other conditions are similar to those described under Experimental.
occur. This demands diffusion of the fluorophore and quencher for the encounter. The bimolecular quenching constant, \( k_q \), for this reaction in purely aqueous medium is generally diffusion limited with a rate constant > \( 10^9 \) M\(^{-1}\) s\(^{-1}\). Hence, comparison of \( k_q \) in dextran solutions with that in aqueous buffer will give us information on the role played by viscosity in slowing the translational diffusion of small molecules in the dextran medium. It is assumed that the quenching efficiency of iodide is unlikely to be affected by an inert macromolecule such as dextran. As the root-mean-square distance \( [(6D\tau)^{1/2}, \text{where } D \text{ is the diffusion coefficient of the quencher (} \sim 2 \times 10^{-5} \text{ cm}^2/\text{s for iodide at 298 K)} \text{ and } \tau \text{ is the lifetime of the fluorophore (} \sim 4 \text{ ns for fluorescein) that is getting quenched]} \) over which iodide ion can diffuse during the lifetime of fluorescein is \( \sim 69 \text{ Å} \), it is unlikely that any parameter apart from viscosity will have a role to play in the translational diffusion. This was verified by measuring the \( k_q \) for the above quenching in the presence of different amounts of dissolved sucrose in the medium. The decrease in \( k_q \) was linear with solution viscosity (data not shown).

In Figure 3, the dark-filled bars show the influence crowding agents have on the fold decrease in the bimolecular quenching constant, \( k_q \), for the dynamic fluorescence quenching of fluorescein by iodide. In aqueous buffer we observed a \( k_q \) of \( \sim 2.7 \times 10^9 \) M\(^{-1}\) s\(^{-1}\) at 298 K during dynamic quenching of fluorescein fluorescence by iodide. Sucrose reduced \( k_q \) by \( \sim 2 \)-fold consistent with the 2-fold increase in solution viscosity for 20% (w/w) sucrose–water mixture at room temperature.\(^\text{24}\) In the presence of 15 or 70 or 200 or 500 kDa dextran, the decrease in \( k_q \) is confined to a narrow range between 3.2- to 3.8-fold only as revealed by the slope of the Stern–Volmer plots in Figure 5. This implies that the viscosity as sensed by a small molecule such as iodide or fluorescein during translational diffusion in crowded dextran solutions, for a constant dextran concentration (20% w/w), is independent of the size of dextran macromolecule. The above result is consistent with previous observations based on fluorescence photobleaching recovery of BCECF in dextran solutions, where it was shown that the \( D_0/D \) dependence on dextran concentration was independent of dextran size in the range 10–2000 kDa.\(^\text{5}\) Kao and coworkers\(^\text{5}\) observed an \( \sim 4 \)-fold decrease in diffusion of BCECF compared with aqueous medium in the presence of 20% w/w dextran (Ref. 5, see Figure 6), which is consistent with our results (3.2- to 3.8-fold) from dynamic fluorescence quenching on the diffusion of iodide and fluorescein. It is also in agreement with previous reports in literature on the diffusion of small solutes in solutions containing macromolecules.\(^\text{25,26}\) It is thus likely that the diffusion coefficient of PNPP is independent of the size of dextran crowding the solution at any given dextran concentration. We already know that increas-
ing the viscosity slows down the alkaline phosphatase reaction (Figure 4). Based on the facts above, the ~2-fold decrease in the enzyme-catalyzed rate of PNPP in dextran solutions of size range 15–70 kDa actually implies a small increase in the activity of the enzyme owing to crowding. A larger decrease in rate was anticipated here owing to the effect of viscosity on PNPP diffusion. One possible explanation for the increase in enzyme activity in the presence of crowding is likely to be the homodimeric nature of alkaline phosphatase. It has been shown recently that the dimeric alkaline phosphatase is 10,000 times more active than the monomer. It is well known that, due to volume exclusion, macromolecular crowding can favor the dimeric enzyme more strongly in a monomer–dimer equilibrium, especially when the monomeric enzyme is comparable in size to the crowding agent. Indeed, crowding agents have been shown to have an effect on the state of association of an enzyme, which may be linked to changes in specific activity of the enzyme. Another reason for the increase in enzymatic rate may be the increase in enzyme thermodynamic activity owing to crowding. We know that, under the conditions employed here, the alkaline phosphatase reaction is transition state limited. Crowding may therefore increase the thermodynamic activity of the transition state intermediate of the reaction.

The presence of different amounts of salts/ions in the different dextran size fractions bought commercially can also affect the $V_{\text{norm}}$ and create an illusion of a size effect. To rule out this possibility we measured the conductivity of dextran solutions (15, 200, and 500 kDa) in deionized water for the different concentrations employed in our study. We used 0.1 M KCl as the standard for reference. We observed negligible conductivity (<3% with respect to 0.1 M KCl) for the dextran sizes employed even at higher concentrations such as 20 and 30% w/w. Thus it is unlikely that salts are present in any significant amount to affect the results.

Now, we come to the influence exerted by different Ficolls on the reaction rate. It is interesting to observe in Figure 3 that Ficolls of size 70 (~1.3-fold slowing) and 400 kDa (~2.3-fold slowing) exert a minor influence on the reaction rate compared with dextrans of similar size. Figure 6 reveals the variation in the normalized reaction rate with the concentrations (in wt %) of 70 and 400 kDa Ficoll. The data obtained in the presence of 40 and 500 kDa dextran are also plotted in the same figure to facilitate comparison. These were plotted employing a concentration scale of % w/w as the specific excluded volume was not available for Ficolls. Clearly, the Ficolls show a reduced overall influence on the rate relative to dextrans of similar size. In fact, the trend observed with 400 kDa Ficoll is close to that observed with 40 kDa dextran as shown in Figure 6. The disparity of results between Ficoll and dextran of similar size may be explained by the fact that, in comparison to dextran, Ficoll is more compact, highly branched, and less flexible on a molecular weight basis (Ref. 28 and references cited therein). Dextran, a linear polysaccharide, is a flexible, ribbon-like, quasirandom coil molecule with few and short branches. Ficoll approximates a sphere much more closely than dextran. It has also been observed that, at higher concentrations (>100 g/L) of Ficoll 70, interpenetration and/or compression occurs. It is thus likely that the trajectory of the substrate is a more tortuous one if dextran is placed as the barrier in comparison to Ficoll. Increase in activity of alkaline phosphatase owing to excluded volume effects is also equally likely in the presence of Ficoll; however, more experiments are needed to establish this. Taken together, it is clear that the nature and shape of the barrier has a major implication in influencing the reaction rate.

Usually, changes in the kinetics of an enzymatic reaction are interpreted in terms of parameters such as $K_m$, $V_{\text{max}}$, and $k_{\text{cat}}$. However, these were not considered in this study for the reasons explained below. Figure 7 shows the initial rate of the reaction as a function of the substrate concentration in the presence (20% w/w) and absence of different crowding agents, namely 40, 200, and 500 kDa. In the absence of the crowding agent, the rate versus [S] curve displays the expected experimental profile for Michaelis–Menten kinetics. In the presence of a significant fraction of macromolecules in the reaction medium this is clearly not the case. A dip in the curve is evident in all three cases. Specifically, between [PNPP] = 2.5 and 5.0 mM for 40 kDa; [PNPP] = 3.5 and 5.0 mM for 200 kDa; and [PNPP] = 5 and 7.5 mM for 500 kDa. This discontinuity at the substrate concentrations above was consistently observed in multiple trials, but only in the presence of crowding agents. Accurate and reliable estimates of $K_m$, $V_{\text{max}}$, and $k_{\text{cat}}$ were not possible in the presence of crowding owing to scattered points in Lineweaver–Burk and Eadie–Hofstee plots. The large error bars at higher substrate concentrations is indicative of this trend. Such behavior indicates that conventional Michaelis–Menten kinetics cannot be applied here. Indeed, it has been argued previously that, in disordered media (as encountered in the crowded ambience here), the conventional equations of enzyme kinetics may no longer be valid, as the assumptions on which their derivation is based are no longer true. The equations need to be modified to account for fractal kinetics and anoma-
lous diffusion during the formation of the ES complex, especially in the regime of high obstacle densities. Another factor that can have an influence on the results above is the nonspecific binding of substrate (PNPP) to the crowding macromolecule. There are several reasons why such a possibility is unlikely. 1) We have observed the kinetics of the PNPP hydrolysis reaction in the presence of obstacles at 200 s or later. The absorbances at these times, in almost all cases (with the exception of 500 kDa) are greater than 90% of the value obtained in the absence of obstacles at the same time. This clearly implies that less than 10% of the substrate, if any, is likely to remain bound to macromolecules. 2) It has been shown in an earlier report that carbohydrate additives such as sucrose, fructose, lactose, etc. exert no nonspecific solute effects either on PNPP or alkaline phosphatase. 3) Figure 3 shows the fold decrease in the enzymatic rate at 1 and 20 mM PNPP concentrations. Only a marginal increase in the degree of slowing is observed at 20 mM PNPP compared with 1 mM PNPP. Significant binding of PNPP to obstacles would have resulted in a much larger disparity in the fold decrease. Thus, on the whole, the obstacles employed in the study are likely to be inert with relation to the enzyme and PNPP.

In conclusion, our results reveal that size, shape, and concentration of macromolecular barriers play a crucial role in influencing the rate of an enzymatic reaction. While large and irregular obstacles (such as 200–500 kDa dextrans) reduce the encounters between the enzyme and substrate, large but compact obstacles (such as 400 kDa Ficoll) exert only a minor influence on enzymatic rate. The effect of crowding by small obstacles (15–70 kDa) is partially offset by enhanced enzyme activity owing to excluded volume effects, resulting in a minor decrease in rate only.

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