Macromolecular Crowding Effect upon *in vitro* Enzyme Kinetics: Mixed Activation-Diffusion Control of the Oxidation of NADH by Pyruvate Catalyzed by Lactate Dehydrogenase

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ABSTRACT

Enzyme kinetics studies have been usually designed as dilute solution experiments, which differ substantially from *in vivo* conditions. However, cell cytosol is crowded with a high concentration of molecules having different shapes and sizes. The consequences of such crowding in enzymatic reactions remain unclear. The aim of the present study is to understand the effect of macromolecular crowding produced by Dextran of different sizes and at diverse concentrations in the well-known reaction of oxidation of NADH by pyruvate catalyzed by L-Lactate Dehydrogenase (LDH). Our results indicate that the reaction rate is determined by both the occupied volume and the relative size of Dextran obstacles in respect to the enzyme present in the reaction. Moreover, we analyzed the influence of macromolecular crowding on the Michaelis-Menten constants, v_{max} and K_m. The obtained results show that only high concentrations and large sizes of Dextran reduce both constants suggesting a mixed activation-diffusion control of this enzymatic reaction due to the Dextran crowding action. From our knowledge, this is the first experimental study that depicts mixed activation-diffusion control in an enzymatic reaction due to the effect of crowding.

Keywords: enzyme kinetics, macromolecular crowding, LDH, Dextran, mixed activation-diffusion control.

INTRODUCTION

It is well known that up to a 40% of the volume of the cytosol is occupied by a wide variety of macromolecules and solutes[1]. For this reason, any solute moving through the intracellular environment will see its diffusion rate affected with respect to diffusion in aqueous solution[2-8], either being reduced or presenting anomalous diffusion at short times.

Macromolecular crowding inside the cell, does not only affect diffusion processes, but also biochemical reaction processes by inducing the enzyme to undergo protein folding, self-association or protein binding processes[9-22], which in turn alter enzymatic activity. Thus, in order to obtain more accurate rates for enzymatic reactions, it is important to perform studies of biochemical processes in nature-like microenvironments that try to mimic this effect. Indeed, there are an important number of works which have studied how enzyme kinetics is affected by crowded environments, even in vitro[23-41].

The best approach for this sort of studies would certainly be using cell extracts. However, the experimental data collection and their interpretation would be challenging because cell extracts are complex media that present a high heterogeneity in their geometrical and physical properties. Therefore, the major experimental studies on crowding effect have used purified macromolecules as crowding agents. Dextran is one of them and its use is widely spread due to its lack of reactivity and high solubility in water. Moreover, its flexibility and random coil shape in solution are suitable for modelling many macromolecules present in the natural state of the cell. It is also readily available in various sizes and large quantities.

In recent years, the effects of crowding on enzyme catalysis have been explored

by different works, excellently depicted by Zhou et al[8] and Noris and Malys[34]. Most of them indicate that under Michaelis-Menten conditions in crowded media, excluded volume is the major player in modulating enzymatic behavior. Moreover, gathering all the contributions published so far, some general trends are encountered. For instance, a slight reduction in the apparent substrate-binding affinity constant, K_m is usually reported, despite of the characteristics of the crowding agent[9, 24, 26-32]. In contrast, in crowded media experiments k_{cat} can increase in some cases[24-25, 30-33], or it can also decrease[9, 25, 27-28].

Previously, we reported how the kinetic behavior of two enzymatic reactions are influenced by crowding. Our first study, the hydrolysis of N-succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by alpha-chymotrypsin[37], showed that the total volume excluded by Dextrans , but not its size, is the property that makes significant changes on the reaction rates. We obtained a v_{max} decrease and an K_m increase when the concentration of Dextran in solution is heightened. The slower diffusion of the alpha-chymotrypsin in presence of Dextrans was responsible of the rise of K_m. The obtained diminish in v_{max} could only be explained by a mixed inhibition by product, enhanced by crowding. In our second study, the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by Hydrogen Peroxidase (H_2O_2) catalyzed by Horseradish Peroxidase (HRP) [41], results revealed that the reaction rate was also significantly influenced by the excluded volume effect, but it was independent of the Dextran size. Both v_{max} and K_m decay when increasing the concentration of the crowder in solution, suggesting an activation control. In other words, the catalytic constant (k_{cat}) brings a significant contribution as a result of the environmental surroundings influence. This contribution may be the consequence of one or the additive effect of the following aspects: the rise of the ratio of the activity coefficients between the enzyme and the complex formed by enzyme and

substrate as a consequence of a crowded microenvironment; the boost of water chemical activity favored by dense solution; and the conformational change of the enzyme active site induced by crowding.

Despite the range of experimental conditions covered by previous studies, in our opinion, more information and experiments in crowded media are required to elucidate the key factors that govern the enzyme kinetics in these conditions. Therefore, in this paper we study another enzymatic system in order to obtain more information about how enzymatic reactions could be affected by macromolecular crowding.

Here, we study how the crowding of Dextran at several concentrations and sizes affects the catalytic reaction of LDH (oxidation of NADH by Pyruvate). We chose this enzymatic reaction as a model process for several reasons. First, it is a well-known reaction in which small substrates and products lead to the occurrence of the reaction without a significant variation in excluded volume. Second, there are no interactions between the enzyme and the Dextrans used as crowding agents. And third, the LDH size (Mw = 140 kDa) is intermediate between those of the selected Dextrans (from 50 to 410 kDa).

Therefore, here we illustrate how the reaction catalyzed by Lactate Dehydrogenase is strongly influenced by different amounts of neutral polymers of different dimensions, mimicking the known intracellular crowding levels. Specifically, we study the dependence of v_{max} and K_m parameters on the total volume of the solution excluded by the crowder and the individual volume occupied by each of the polymeric coils.

MATERIALS AND METHODS

Chemicals

Rabbit muscle L-Lactate Dehydrogenase (E.C. 1.1.1.27) (140 Umg^{-1}), received as a purified and lyophilized powder, sodium pyruvate and β-NADH were acquired from Sigma-Aldrich Chemical (Milwaukee, WI, USA). Four Dextrans (Fluka) of 410, 275, 150 and 50 molecular wight were use without further purification. All the chemicals were of analytical or spectroscopic reagent grade.

Oxidation of NADH

The oxidation of NADH was made at 25ºC in Imidazole·Acetic Acid buffer (30 mM, pH = 7.5, containing 60 mM of CH_3COOK and 30 mM of $MgCl_2$). Each sample contains the same concentration of 8.2 10^{-13} M of LDH and 1.17 10^{-4} M of NADH. Michaelis-Menten plots were obtained by measuring initial velocity of the reaction at different pyruvate concentrations, in a range between 7.1 10^{-5} and 5.4 10^{-4} M. This process was first done without the addition of crowding agent. After the incorporation and homogenization of the enzyme into a sample, which contains NADH and pyruvate, the reaction starts. Subsequently, we dissolved into the same sample mixture different Dextran concentrations (25, 50 and 100 mg/mL) for each Dextran size mentioned above: 410, 275, 150 and 50 kDa, which will be referred as D410, D275, D150 and D50, respectively. The reaction progress and the data analysis was described in detail in Pastor el al.[37]. Briefly, in this case we follow the reaction by the absorbance change that occurs as NADH is oxidized into NAD^+, which no longer absorbs at 320 nm. Initial reaction velocity, v_0, was obtained by linear fitting of the initial data points in the absorbance-time plot. Blank solution containing substrate and Dextran in the same concentrations than the sample was measured in each case.

The oxidation of NADH by Pyruvate catalyzed by LDH can be treated as a single substrate reaction and its kinetic study can be performed using the scheme proposed by Henri[42]:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1)$$

where k_1, k_{-1}, and k_2 are rate constants. This system can be described using Michaelis-Menten equation[42]:

$$v_0 = \frac{v_{max}[S]_0}{K_m + [S]_0} \qquad (2)$$

where v_{max} is the maximum velocity and K_m is the Michaelis-Menten constant. With the combination of (1) and (2) some definitions can be stated: K_m can be defined as $K_m = (k_{-1} + k_2)/k_1$, and for v_{max}, $v_{max} = k_{cat}[E]_T$, and $k_{cat} = k_2$.

Since Michaelis and Menten studies, it has been assumed that enzyme activity can be studied applying equation (2), obtaining data to fit that equation by performing measurements of the initial rate of product formation at different substrate concentrations.

RESULTS AND DISCUSSION

For a suitable evaluation of the influence exerted by macromolecular crowding on the kinetic parameters, one must consider at least two important features of the study. First, the reactions under investigation should be processes whose kinetic behavior has an established and generally accepted interpretation. In our case, the

reaction between NADH and pyruvate catalyzed by LDH is a well-known process following a Michaelis-Menten mechanism and it can easily be monitored by UV-spectroscopy. Second, the crowding induced effect should be accounted only by the changes incurred by the crowding polymers with regard to its dimensions and concentration. Besides, as stated above, the selected reaction is accompanied by an insignificant variation of excluded volume during the progression of the reaction, owing to the small size of NADH, pyruvate, NAD^+ and L-lactate.

We studied how the selected system presents a different kinetic response than in dilute solution when different concentrations and sizes of Dextran are added to the reaction media. An initial linear decrease followed by a plateau is observed when measuring the absorbance of NADH at 320 nm in all the studied cases (results not shown). As explained in Material and Methods, initial velocity (v_0) values were acquired by the fitting of the linear part of the absorbance-time plot for each sample.

Figure 1 shows v_0 values as a function of the substrate concentration. Each data point is obtained from the mean between 3 to 5 samples with the same reaction conditions in terms of substrates, enzyme and Dextran concentrations. Each figure, A to D, represents a given Dextran size, from the smallest to the biggest Dextran. Dextran concentration in mg/mL and excluded volume are directly proportional. A significant dependence of v_0 on both the excluded volume and the obstacle dimension is clearly found. Figure 2 reveals that reaction rate decreases as a function of Dextran size when high Dextran concentrations (> 50 mg/mL) are used. In this case, the process behavior depends not only on the excluded volume but also on the dimension of the obstructive particles present in the reaction media.

This result is consistent with the results of Homchaudhuri et al.[29]. These authors stated that alkaline phosphatase makes the hydrolysis of p-nitrophenyl phosphate with a velocity which depends on the crowder size for a given excluded volume conditions. They showed that for samples of Dextrans of about 20% (w/w) the reaction rates decrease with the increase of Dextran size: a decrease of about 2, 5 and 7-fold was observed for Dextrans of 15-70, 200 and 500 kDa, respectively However, it is in contrast with the results showed in our previous works[37, 41] and with the results of Minton et al.[2, 8-12]. In all these works, only excluded volume but not obstacle size was presented as a major factor influencing enzymatic reactions occurring in reaction media crowded by macromolecules.

The major difference between the studies is the relative dimension of the protein in respect to the obstacle. It has to be acknowledged that the size of alkaline phosphatase (105 kDa) is comparable with that of LDH (140 kDa). As such, the rate of the reactions they catalyze has similar behavior in crowded media unlike the rate of the other investigated processes catalyzed by much smaller proteins: alpha-chymotrypsin (25 kDa) and HRP (42 kDa). As revealed by Homchaudhuri et al.[29], occasioned by the big size of the protein, large crowding agents make enzyme and substrates to come across less often. However, this effect is partially offset when using small obstacles and the decrease is less significant, due to a certain caging effect that causes an enhanced enzymatic activity. Our results underline the fact that the degree of crowding effect relies both on the size and concentration of the obstructive particles. In spite of that, the relative size of the enzyme in respect to the crowder seems to exert a significant influence when larger molecules are present in the system.

The values of Michaelis Menten constants, K_m and v_{max}, were estimated by fitting Eq. 2 to each data set in Figure 1, in order to get information about how the excluded volume could affect the oxidation of NADH by LDH in crowded media. Figures 3 and 4 and Table 1 show how K_m and v_{max} are dependent on the amount of volume excluded by macromolecular inert obstacles. In fact, we can perceive that only high concentrations and large sizes of Dextran affect both v_{max} and K_m values, whereas for smaller sizes of Dextran, the enzymatic system behaves in a similar way to our previous studies with α-Chymotrypsin[37].

Firstly, examining v_{max} values we see (Figure 3 and Table 1) that they diminishe with the increase of size and concentration of Dextran. In the Figure it is shown that the value of v_{max} in presence of D50, which size is lower than LDH, decreases slightly with respect to the value in diluted solution, but it is independent of the concentration of D50. Moreover, the presence of low concentrations (25 and 50 mg/mL) of D150 and D410, whose sizes are, respectively, equal and two times the LDH size, affects the value of v_{max} in the same sense, i.e. its value decays slightly with respect to the value in diluted solution, but it is independent of the concentration. However, high concentration of both Dextrans, D150 and D275, decreases hugely the value of v_{max}. In the same direction but even enhanced, the presence of a high concentration of D410, which size is three times the LDH size, causes biggest decreases of v_{max}.

As stated previously, v_{max} is defined as $v_{max} = k_{cat}[E]_T$, with $k_{cat} = k_2$. Therefore, its decrease with high concentration or large size of Dextrans could be due to two main reasons: the variation of the effective enzyme concentration or the deviation of the catalytic rate constant (k_{cat}). On the one hand, the excluded volume in crowded media is responsible for an increase of the effective enzyme concentration[2, 8-12]. In fact,

an increase of v_{max} in crowding situations is usually explained as a result of a raise in the effective enzyme concentration[24, 31, 33]. On the other hand, when a decrease in v_{max} is found in crowding situations, it is normally explained by the reduction of k_{cat} as a result of conformational changes of the catalytic centre of the protein derived from surrounding variations induced by the crowded media[27-28, 30, 33, 44]. In our case, it is reasonable to assume that k_{cat} would be unaffected by the presence of Dextran. Since the catalytic site is protected from the bulk solution, Dextran can not denaturize or somehow alter the inner cavities of the protein in contrast with other molecules[22, 23-24, 27-28, 30, 33, 43-44]. In fact, in this particular case we should also consider the comparable size between LDH and the obstacles to understand the decrease of v_{max} in crowded media. In presence of large obstacles, the comparable size between both, LDH and Dextrans, causes a reduction of the encounters between enzyme and substrates. On the contrary, a minor decrease in the v_{max} value is found in presence of small obstacles because the crowding effect is partially compensated by the improvement of enzymatic activity that a certain cage effect can induce. In our opinion, only this situation could explain our results.

Secondly, we analyzed the K_m behavior. As it is shown in Figure 4 and Table 1, only a high concentration of large Dextran, 100 mg/mL of D150, D275 or D410, decays the K_m value. As was explained in Materials and Methods section, the Michaelis-Menten constant is defined by $K_m = (k_{-1} + k_2)/k_1$ following the general chemical equation for enzymatic processes presented in Eq. 1. In a situation of diffusion control, an increase in K_m value can be expected since the bimolecular constant (k_1) will decrease with ~~the~~ crowding[45-47]. In ~~the~~ literature, there are few works that report this situation[21, 37, 45-47]. In these reported cases, a high diffusion resistance in the sample is responsible of a K_m increase with Dextran concentration. However, most of the

enzymatic reaction studies in crowding situations showed a slight decrease in K_m compared to that of the dilute solution. These studies excluded a diffusion control of the enzymatic process and the modification of k_1 by the presence of crowding agents (e.g. reference 31). A variation in k_{cat} with crowding is usually found in the cases where a K_m decrease is reported. The K_m constant is sensitive to the sample composition since it depends on the substrate activity coefficient. Therefore, a decrease in K_m with crowding is usually attributed to several factors. On the one hand, due to non-ideal conditions of the crowded solution, chemical activity of the substrate is modified. On the other hand, there is an increase of the activity coefficients relation between the free enzyme and the complex formed by enzyme and substrate [27-28,30]. Moreover, substrate binding event is depending on water activity, because both the substrate and the active site must be dehydrated for the binding to occur. Therefore, k_2 value may be affected. In our opinion, as it was explained in detail in our previous work [37], both hindered diffusion and alterations in the active site must be considered to better understand the results. In fact, the behavior showed in this study by K_m at high concentration of large Dextrans is in agreement with these previous studies. In this case, at high concentration of large Dextrans, the decrease of both constants, v_{max} and K_m, are in agreement with a decrease in the k_2 value, which depends on of the amount of encounters between enzyme and substrates. The existence of a high quantity of large Dextrans reduces the number of these encounters and consequently the constant value.

In addition, if we compute the v_{max}/K_m ratio (Table 1), we observe a decrease in its value at high concentration of large Dextrans, which could indicate some possible effect of inhibition. Excess-substrate inhibition of rabbit muscle LDH is well documented for the case of pyruvate[48-51]. However, we observe a slight and constant

decrease of the value of the $^{v_{\max}}/_{K_{\mathrm{m}}}$ ratio with respect to the case of diluted solution until the excluded volume achieves some limit value. This behavior suggests that the possible substrate inhibition reported in the earlier literature does not explain the obtained values. Recent simulation and experimental studies reported on diffusion processes of enzymes in crowded media[47, 52-55] show that the effective diffusion coefficient is higher in dilute samples than in crowding situations. A decrease in the diffusion coefficient may imply a decrease in the bimolecular constant (k_1) with crowding[45-46]. In a situation where the enzymatic reaction depends on the diffusion, the K_{m} value should increase because of the decrease of k_1. However we find that K_{m} remains constant until high concentration of large Dextrans. On the other hand, our results show that k_2 decreases with crowding, which is in agreement with an activation control. Thus, a mixed activation-diffusion control could explain the rough decreases of the experimental fitted values of $v_{\max}$ and K_{m} for high Dextran sizes and concentrations In particular, the decrease $v_{\max}$ is produced by the decrease of k_2 with size and concentration of Dextrans. The quasi-constant value of K_{m} can be seen as a result of the combined effect of the decreasing of the diffusion-controlled constant k_1, with size and concentration of Dextrans and the decreasing of k_2. This concordance breaks for high concentration of large Dextrans when K_{m} is not constant anymore.

In conclusion, our results reveal, on the one hand, that the initial velocity of the reaction depends on both the size and amount of Dextran present in the media. In addition, and in contrast with previous reported works, we have found that the enzyme relative size respect to the crowding molecules represents another important factor influencing the velocity of the reactions occurring in crowded media. When enzymes are small the reaction rate mainly relies on the excluded volume of the solution.

However, for large enzymes (in our case LDH) the reaction rate is also influenced by the size of obstacles present in the reaction environment. On the other hand, we obtained a decrease in both Michaelis-Menten constants, $v_{\max}$ and K_{m}, with the amount of crowding agent in the sample, but this decrease is only significant in the case of samples with high concentrations of large Dextrans. The decrease in $v_{\max}$ also depends on the size of Dextran present in the media. This result is attributable to the activation control of the enzymatic process, but it must be taken into account the fact that as a result of the relative large size of LDH, large obstacles reduce the encounters among enzymes and substrates. However, an activation control of the LDH reaction is against the decay found in K_{m} only in cases with high concentration of large Dextrans. This decrease can be explained by a mixed activation-diffusion control of this enzymatic process in crowding media produced by Dextrans. Only a mixed control can explain the behavior found in both Michaelis-Menten constants, and probably it is due to the relative large size of the LDH and the difference between the enzyme and the crowding agent size. In our opinion, the enzyme relative size with respect to the crowding agent represents a significant factor to be considered in enzymatic reaction studies carried out in macromolecular crowded media.

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REFERENCES

1. Ellis R. J. Macromolecular Crowding: Obvious but Underappreciated. *Trends Biochem. Sci.* **2001**, *26*, 577-604.

2. Muramatsu, N.; Minton, A. P. Tracer Diffusion of Globular Proteins in Concentrated Protein Solutions. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 2984-2988.

3. Wachsmuth, M.; Waldeck, W.; Langowski, J. Anomalous Diffusion of Fluorescent Probes inside Living Cell Nuclei Investigated by Spatially-Resolved Fluorescence Correlation Spectroscopy. *J. Mol. Biol*. **2000**, *298*, 677-689.

4. Arrio-Dupont, M.; Foucault, G.; Vacher, M.; Devaux, P. F.; Cribier, S. Translational Diffusion of Globular Proteins in the Cytoplasm of Cultured Muscle Cells. *Biophys. J.* **2001**, *78*, 901-907.

5. Verkman, A. S. Solute and Macromolecular Diffusion in Cellular Aqueous Compartments. *Trends Biochem. Sci.* **2002**, *27*, 27-32.

6. Weiss, M.; Elsner, M.; Kartberg, F.; Nilsson, T. Anomalous Subdiffusion is a Measure for Cytoplasmic Crowding in Living Cells. *Biophys. J.* **2004**, *87*, 3518-3524.

7. Dix, J. A.; Verkman, A. S. Crowding Effects on Diffusion in Solutions. *Annu. Rev. Biophys.* **2008**, *37*, 247-263.

8. Zhou, H. X.; Rivas, G.; Minton, A. P. Macromolecular Crowding and Confinement: Biochemical, Biophysical and Potential Physiological Consequences. *Annu. Rev. Biophys.* **2008**, *37*, 375-397.

9. Minton, A. P.; Wilf, J. Effect of Macromolecular Crowding upon the Structure and Function of an Enzyme: Glyceraldehyde-3-phosphate dehydrogenase. *Biochemistry*. **1981**, *20*, 4821-4826.

10. Minton, A. P. Excluded Volume as a Determinant of Macromolecular Structure and Reactivity. *Biopolymers*. **1981**, *20*, 2093-2120.

11. Zimmerman, S. B.; Minton, A. P. Macromolecular Crowding: Biophysical, Biochemical, and Physiological Consequences. *Annu. Rev. Biophys. Biomol. Struct*. **1993**, *22*, 27-65.

12. Rivas, G.; Fernandez, J. A.; Minton, A. P. Direct Observation of the Self-Association of Dilute Proteins in the Presence of Inert Macromolecules at High Concentration Via Tracer Sedimentation Equilibrium: Theory, Experiment, and Biological Significance. *Biochemistry*. **1999**, *38*, 9379-9388.

13. Van den Berg, B.; Ellis, R. J.; Dobson, C. M. Effects of Macromolecular Crowding on Protein Folding and Aggregation. *EMBO J.* **1999**, *18*, 6927-6933.

14. Minton, A. P. Implications of Macromolecular Crowding for Protein Assembly. *Curr. Opin. Struct. Biol.* **2000**, *10*, 34-39.

15. Rivas, G.; Fernandez, J. A.; Minton, A. P. Direct Observation of the Enhancement of Non-Cooperative Protein Assembly by Macromolecular Crowding: Indefinite Self-Association of the Bacterial Cell Division Protein FtsZ. *Roc. Natl. Acad. Sci. USA* **2001**, *98*, 3150-3155.

16. Kozer, N.; Schreiber, G. Effect of Crowding on Protein–Protein Association Rates: Fundamental Differences between Low and High Mass Crowding Agents *J. Mol. Biol.* **2004**, *336*, 763-774.

17. Munishkina, L. A.; Cooper, E. M.; Uversky, V.; Fink, A. The Effect of Macromolecular Crowding on Protein Aggregation and Amyloid Fibril Formation *J. Mol. Recognit.* **2004**, *17*, 456-464.

18. Chebotareva, N. A.; Kurganov, B. I.; Livanova, N. B. Biochemical Effects of Molecular Crowding. *Biochemistry (Mosc).* **2004**, *69*, 1239-1251.

19. Zhou, H-X. Protein Folding and Binding in Confined Spaces and in Crowded Solutions. *J. Mol. Recognit.* **2004**, *17*, 368-375.

20. Minton, A. P. Influence of Macromolecular Crowding upon the Stability and State of Association of Proteins: Predictions and Observations. *J. Pharm. Sci.* **2005**, *94*, 1668-1675.

21. Schreiber, G.; Haran, G.; Zhou, H-X. Fundamental Aspects of Protein-Protein Association Kinetics *Chem. Rev.* **2009**, *109*, 839-860.

22. Dong, H.; Qin S.; Zhou, H-X. Effects of Macromolecular Crowding on Protein Conformational Changes. *PLoS Comput. Biol.* **2010**, *6*, e1000833.

23. Laurent, T. C. Enzyme Reactions in Polymer Media. *Eur. J. Biochem.* **1971**, *21*, 498-506.

24. Wenner, J. R.; Bloomfield, V. A. Crowding Effects on EcoRV Kinetics and Binding. *Biophys. J.* **1999**, *77*, 3234-3241.

25. Asaad, N.; Engberts, J. B. F. N. Cytosol-Mimetic Chemistry: Kinetics of the Trypsin-Catalyzed Hydrolysis of p-Nitrophenyl Acetate upon Addition of Polyethylene Glycol and N-tert-Butyl Acetoacetamide. *J. Am. Chem. Soc.* **2003**, *125*, 6874-6875.

26. Derham, B. K.; Harding, J. J. The Effect of the Presence of Globular Proteins and Elongated Polymers on Enzyme Activity. *Biochim. Biophys. Acta* **2006**, *1764*, 1000-1006.

27. Olsen, S. N. Applications of Isothermal Titration Calorimetry to Measure Enzyme Kinetics and Activity in Complex Solutions *Thermochim. Acta* **2006**, *448*, 12-18.

28. Sasaki, Y.; Miyoshi, D.; Sugimoto, N. Effect of Molecular Crowding on DNA Polymerase Activity. *Biotechnol. J.* **2006**, *1*, 440-446.

29. Homchaudhuri, L.; Sarma, N.; Swaminathan, R. Effect of Crowding by Dextrans and Ficolls on the Rate of Alkaline Phosphatase-Catalyzed Hydrolysis: a Size-Dependent Investigation. *Biopolymers* **2006**, *83*, 477-485.

30. Olsen, S. N.; Ramlɸv, H.; Westh, P. Effects of Osmolytes on Hexokinase Kinetics Combined with Macromolecular Crowding Test of the Osmolyte Compatibility Hypothesis Towards Crowded Systems. *Comp. Biochem. Physiol. A*. **2007**, *148*, 339-345.

31. Moran-Zorzano, M. T.; Viale, A.; Muñoz, F.; Alonso-Casajas, N.; Eydaltm, G. et al. Escherichia coli AspP Activity is Enhanced by Macromolecular Crowding and by Both Glucose-1,6-bisphosphate and Nucleotide-Sugars *FEBS Lett.* **2007**, *581*, 1035-1040.

32. Jiang, M.; Gou, Z. H. Effects of Macromolecular Crowding on the Intrinsic Catalytic Efficiency and Structure of Enterobactin-Specific Isochorismate Synthase. *J. Am. Chem. Soc.* **2007**, *129*, 730-731.

33. Sasaki, Y.; Miyoshi, D.; Sungimoto, N. Regulation of DNA Nucleases by Molecular Crowding. *Nucleic. Acids. Res.* **2007**, *35*, 4086-4093.

34. Norris, M.G.S.; Malys N. What is the True Enzyme Kinetics in the Biological System? An Investigation of Macromolecular Crowding Effect upon Enzyme Kinetics of glucose-6-phosphate dehydrogenase. *Biochem. Biophys. Res. Commun.* **2011**, *405*, 388-392.

35. Verma, P. K.; Rakshit, S.; Mitra, R. K.; Pal, S. K. Role of Hydration on the Functionality of a Proteolytic Enzyme α-Chymotrypsin under Crowded Environment. *Biochimie* **2011,** *93,* 1424-1433.

36. Hou, S.; Ziebacz, N.; Kalwarczyk, T.; Kaminski, T. S.; Wieczoreka, S. A.; Holyst, R. Influence of Nano-Viscosity and Depletion Interactions on Cleavage of DNA

by Enzymes in Glycerol and Poly(Ethylene Glycol) Solutions: Qualitative Analysis. *Soft Matter* **2011,** *7,* 3092-3099.

37. Pastor I, Vilaseca E, Madurga S, Garces JL, Cascante M, Mas F. Effect of Crowding by Dextrans on the Hydrolysis of N-Succinyl-l-phenyl-Ala-p-nitroanilide Catalyzed by α-Chymotrypsin. *J. Phys. Chem. B* **2011**, *115*, 1115-1121.

38. Akabayov, S. R.; Akabayov, B.; Richardson, C. C.; Wagner, G. Molecular Crowding Enhanced ATPase Activity of the RNA Helicase elF4A Correlates with Compaction of Its Quaternary Structure and Association with elF4G. *JACS* **2013,** *135,* 10040-10047.

39. Akabayov, B.; Akabayov, S. R.; Lee, S. J.; Wagner, G.; Richardson, C. C. Impact of Macromolecular Crowding on DNA Replication. *Nat. Commun.* **2013,** *4,* 1615:1-10.

40. Liu, J.; Peng, J.; Shen, S.; Jin, Q.; Li, C.; Yang, Q. Enzyme Entrapped in Polymer-Modified Nanopores: the Effects of Macromolecular Crowding and Surface Hydrophobicity. *Chem. Eur. J.* **2013,** *19,* 2711-2719.

41. Pitulice, L.; Pastor, I.; Vilaseca, E.; Madurga, S.; Isvoran, A.; Cascante, M.; Mas, F. Influence of Macromolecular Crowding on the Oxidation of ABTS by Hydrogen Peroxide Catalyzed by HRP. *J Biocatal Biotransformation*, **2013**, *2*, 1:1-5.

42. Cornish-Bowden, A. *Fundamentals of Enzyme Kinetics*. 3rd edition. Published by Portland Press Ltd. London (U.K.) 2004.

43. Lonhienne, T. G. A.; Winzor, D. A Potential Role for Isothermal Calorimetry in Studies of the Effects of Thermodynamic Non-Ideality in Enzyme-Catalyzed Reactions. *J. Mol. Recognit*. **2004**, *17*, 351-361.

44. Minh, D. D.; Chang, C.; Trylska, J.; Tozzini, V.; McCammon, J. A. The Influence of Macromolecular Crowding on HIV-1 Protease Molecular Dynamics. *J. Am. Chem. Soc.* **2006**, *128*, 6006-6007.

45. Berry, H. Monte-carlo Simulations of Enzyme Kinetics in Two Dimensions: Fractal Kinetics and Spatial Segregation. *Biophys. J.* **2002**, *83*, 1891-1901.

46. Schnell, S.; Turner, T. E. Reaction Kinetics in Intracellular Environments with Macromolecular Crowding: Simulations and Rate Laws. *Progr. Biophys. Mol. Biol.* **2004**, *85*, 235-260.

47. Pitulice, L. *Aspecte fractale ale structurii şi dinamicii biomoleculelor*. PhD thesis. Vest Timişoara University, Timişoara (Romania), 2009; Pitulice, L.; Vilaseca, E.; Pastor, I.; Madurga, S.; Garcés, J. L.; Isvoran, A.; Mas, F. Monte Carlo Simulations of Enzymatic Reactions in Crowded Media. Effect of the Enzyme-Obstacle Relative Size. *Submitted to Math. Biosci.,* **2014**, *in revised form.*

48. Zewe, V; Fromm, H. J. Kinetics Studies of Rabbit Muscle Lactate Dehydrogenase. *J. Biol. Chem.* **1962**, *237*, 1668-1675.

49. Zewe, V; Fromm, H. J. Kinetics Studies of Rabbit Muscle Lactate Dehydrogenase II. Mechanism of Reaction. *Biochemistry.* **1965**, *4*, 782-792.

50. Stambaugh, R.; Post, D. Substrate and Product Inhibition of Rabbit Muscle Lactic Dehydrogenase Heart (H_4) and Muscle (M_4) Isozyme. *J. Biol. Chem.* **1966**, *241*, 1462-1967.

51. Griffin J. H. and Criddle R.S. Substrate-Inhibited Lactate Dehydrogenase. Reaction Mechanism and Essential Role of Dissociated Subunits. *Biochemistry.* **1970**, *9*, 1195-1205.

52. Saxton, M. J. Anomalous Diffusion Due to Obstacles: a Monte Carlo Study *Biophys. J.* **1994**, *66*, 394-401

53. Pastor, I.; Vilaseca, E.; Madurga, S.; Garcés, J. L.; Cascante, M.; Mas, F. Diffusion of Alpha-Chymotrypsin in Solution-Crowded Media. A Fluorescence Recovery After Photobleaching Study. *J. Phys. Chem. B* **2010**, *114*, 4028-4034. *Erratum J. Phys. Chem. B* **2010**, *114*, 12182.

54. Vilaseca, E.; Isvoran, A.; Madurga, S.; Garcés, J. L.; Pastor, I.; Mas, F. New Insights into Diffusion in 3D Crowded Media by Monte Carlo Simulations: Effect of Size, Mobility and Spatial Distribution of Obstacles *Phys. Chem. Chem. Phys.,* **2011***, 13,* 7396–7407.

55. Vilaseca, E., Pastor, I., Isvoran, A., Madurga, S.,Garcés, JL., Mas, F. Diffusion in Macromolecular Crowded Media. Monte Carlo Simulation of Obstructed Diffusion vs. FRAP Experiments. *Theoretical Chemistry Accounts* **2011**, *128,* 795-805.

Table 1

	Concentration (mg/mL)	v_{max} (mM/s)	K_m (mM)	v_{max}/K_m (s^{-1})	r^2
Diluted Solution	-	0.81 ±0.01	0.47 ± 0.01	1.7 ± 0.1	0.9825
D50	25	0.63± 0.02	0.45 ± 0.01	1.4 ± 0.1	0.9893
	50	0.57 ± 0.02	0.46 ± 0.01	1.2 ± 0.1	0.9758
	100	0.54 ± 0.05	0.46 ± 0.01	1.2 ± 0.1	0.9733
D150	25	0.53 ± 0.03	0.43 ± 0.01	1.2 ± 0.1	0.9879
	50	0.53 ± 0.03	0.43 ± 0.01	1.2 ± 0.1	0.9820
	100	0.21 ± 0.01	0.34 ± 0.01	0.6 ± 0.1	0.9918
D275	25	0.58 ± 0.03	0.41 ± 0.01	1.4 ± 0.1	0.9865
	50	0.59 ± 0.02	0.41 ± 0.01	1.4 ± 0.1	0.9846
	100	0.21 ± 0.01	0.35 ± 0.01	0.6 ± 0.1	0.9965
D410	25	0.59 ± 0.02	0.43 ± 0.01	1.4 ± 0.1	0.9925
	50	0.39 ± 0.03	0.43 ± 0.01	0.9 ± 0.1	0.9855
	100	0.11 ± 0.07	0.37 ± 0.01	0.3 ± 0.1	0.9970

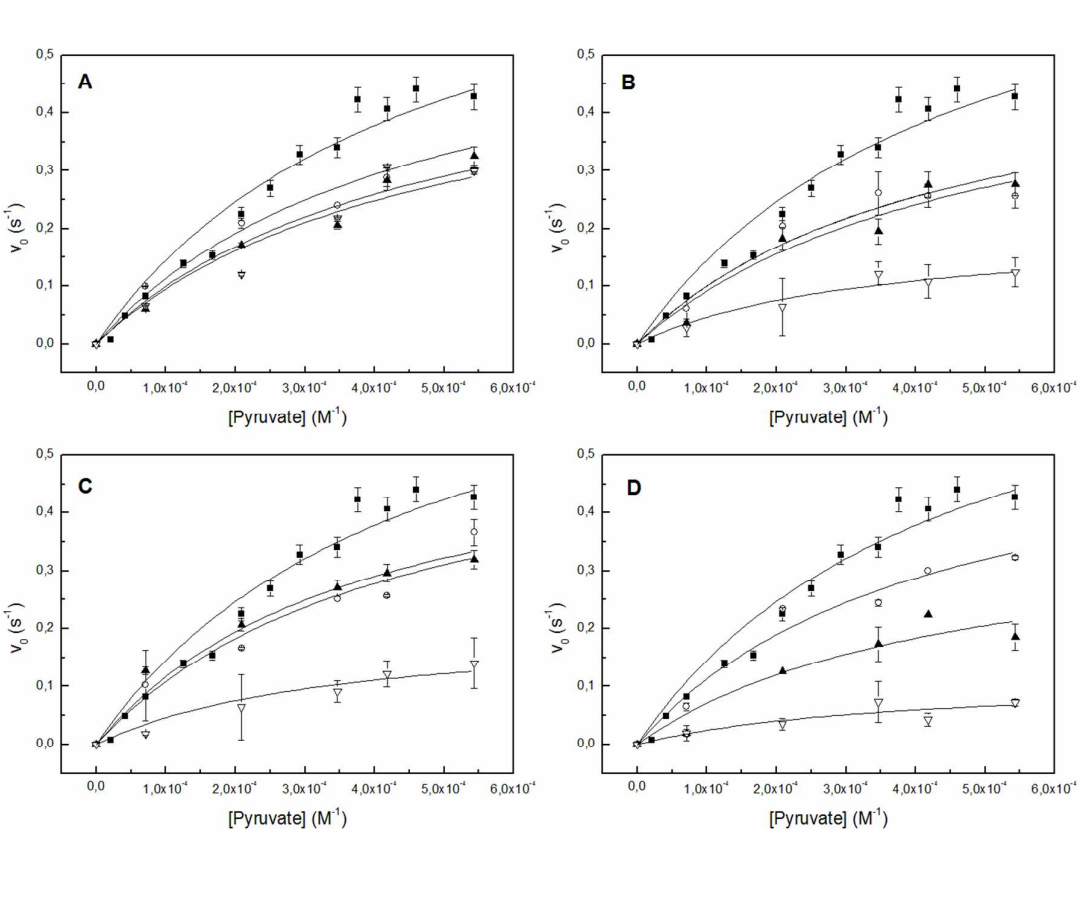
FIGURE 1. v_0 versus substrate concentration plot for the oxidation of NADH in presence of pyruvate catalyzed by LDH in Dextran crowded media with different Dextran sizes: (**A**) M_w = 50 kDa; (**B**) M_w = 150 kDa; (**C**) M_w = 275 kDa and (**D**) M_w = 410 kDa. In each figure, the curves corresponding to four Dextran concentrations are plotted: 0 mg/mL (solid square), 25 mg/mL (open circle), 50 mg/mL (solid up-triangle) and 100 mg/mL (open down-triangle).

FIGURE 2. Example of v_0 variation with Dextran size and concentration for the oxidation of NADH in presence of pyruvate catalyzed by LDH for three fixed substrate concentrations: 0.22 mM (**A**), 0.34 mM (**B**) and 0.40 mM (**C**). In each figure, three Dextran concentrations are plotted: 25 mg/mL (open circle), 50 mg/mL (solid up-triangle) and 100 mg/mL (open down-triangle).

FIGURE 3. Plot of the variation of v_{max} values with Dextran size and concentration for the oxidation of NADH in presence of pyruvate catalyzed by LDH. The four Dextran sizes used are plotted: D50 (solid square), D150 (open circle), D275 (solid up-triangle) and D410 (open down-triangle).

FIGURE 4. Plot of the variation of K_m values with Dextran size and concentration for the oxidation of NADH in presence of pyruvate catalyzed by LDH. The four Dextran sizes used are plotted: D50 (solid square), D150 (open circle), D275 (solid up-triangle) and D410 (open down-triangle).

Figure 1


A
B
C
D
v_0 (s^{-1})
[Pyruvate] (M^{-1})
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0,1
0,2
0,3
0,4
0,5
1,0x10⁻⁴
2,0x10⁻⁴
3,0x10⁻⁴
4,0x10⁻⁴
5,0x10⁻⁴
6,0x10⁻⁴

Figure 2

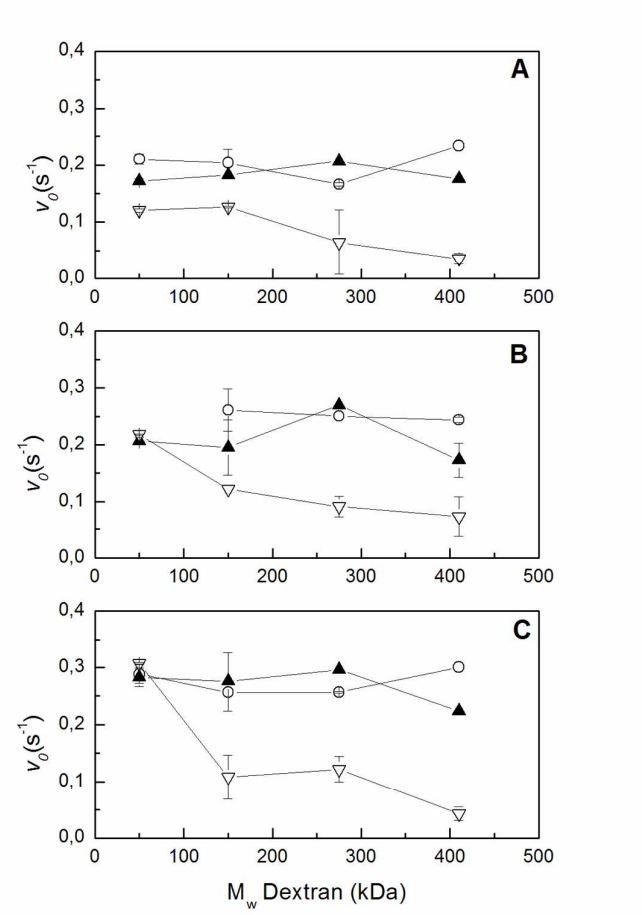


Figure 3

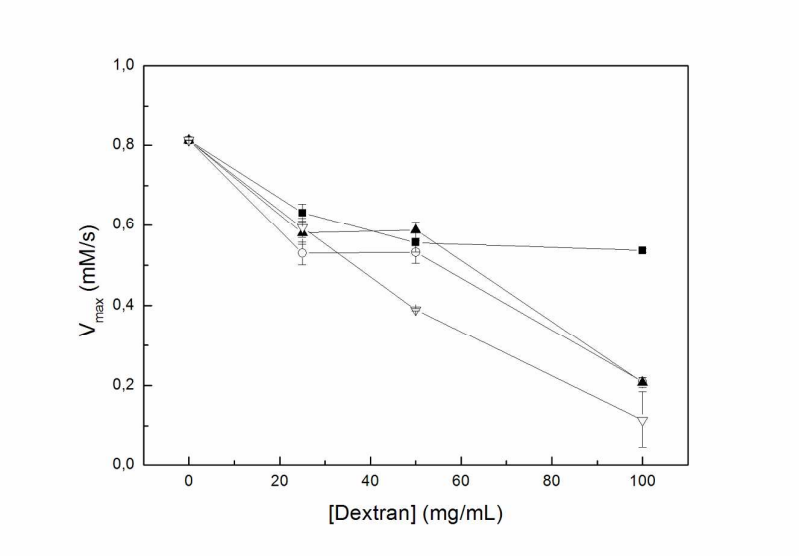
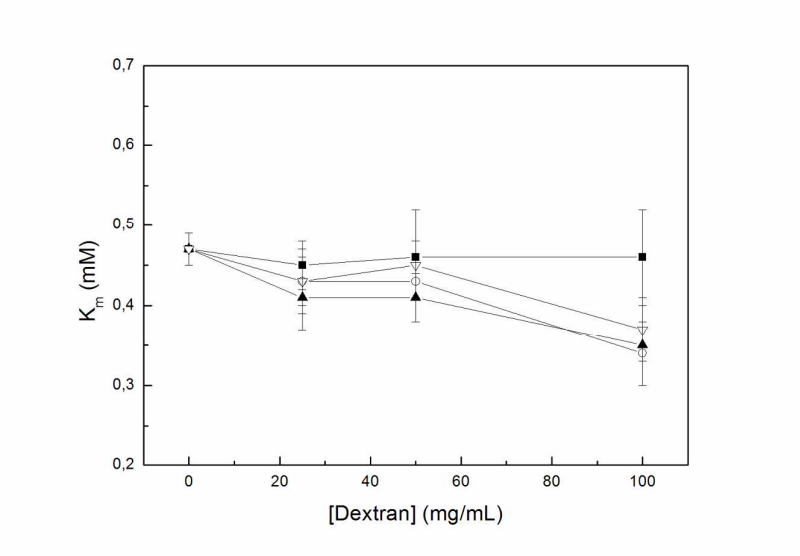


Figure 4



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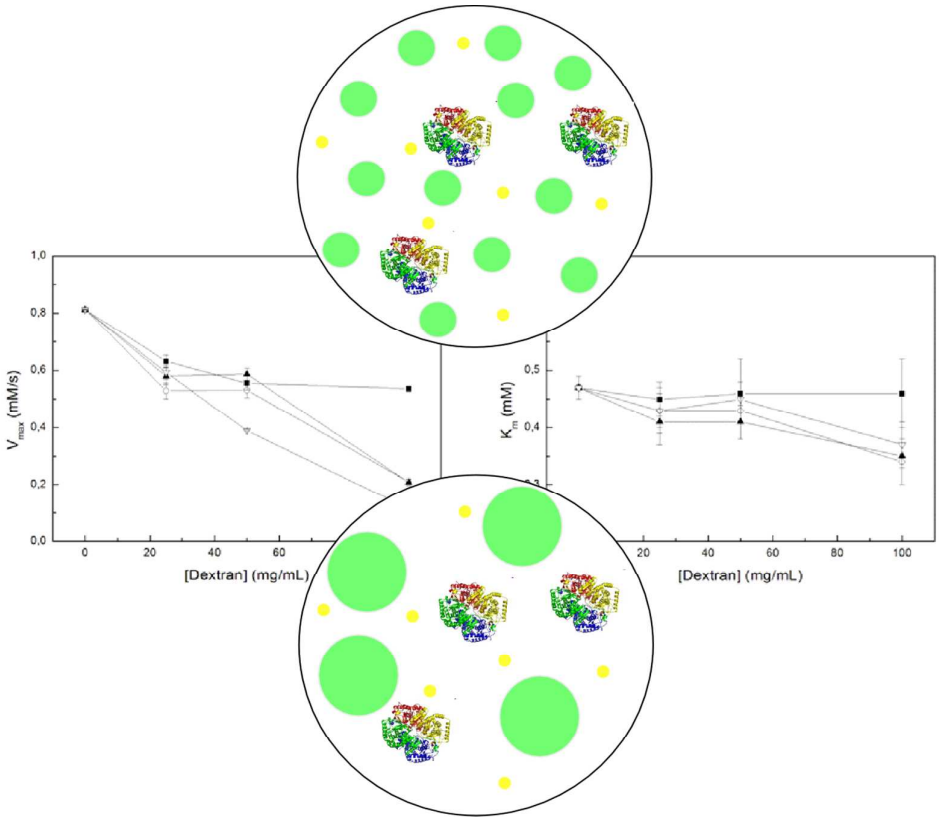
1,0
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0,6
0,4
0,2
0,0
V_{max} (mM/s)
0
20
40
60
[Dextran] (mg/mL)
0,5
0,4
K_m (mM)
20
40
60
80
100
[Dextran] (mg/mL)