The effect of macromolecular crowding on oligomeric enzyme kinetics:
Negative cooperativity on GDH

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ABSTRACT

A study on the enzyme kinetics of glutamate dehydrogenase (GDH) is presented. Glutamate dehydrogenase is a NAD-dependent oxidoreductase that catalyses the deaminative oxidation of glutamate into α-oxoglutarate. It is an hexameric enzyme that presents negative cooperativity: the affinity of each catalytic centre decreases with the binding of substrates to the rest of the centres.

Macromolecular crowding is the alteration of the properties of a molecule due to the presence of high concentrations of unrelated macromolecules. It can be studied by mimicking these high concentrations of macromolecules with inert polymers. In the case of glutamate dehydrogenase, studies under crowded media are interesting because it is localized in the mitochondria of eukaryotes, which is a highly crowded environment.

In this work, experimental data, in dilute solution conditions and with increasing concentrations of a crowding agent, has been obtained by spectrophotometric measurements of the increase in absorbance caused by the formation of NADH.

Different negative cooperativity models, based on Adair’s equation, have been proposed and fitted to the experimental data. One of these models is capable to fit data obtained working in dilute solution and in crowded media. The model suggests a decrease in the affinity of the four first catalytic centres and a recovery of the affinity in the last two centres. The affinity decrease confirms the negative cooperative behaviour expected but experimental evidence is needed to confirm the affinity recovery.

Experimental data obtained under crowding conditions reveals a decrease in the maximum velocity of the reaction, suggesting a mixed-inhibition by product mechanism enhanced in crowded media. A dependence on crowder size has been found for high concentrations of crowding agent, which is in accordance with reports on other enzymes such as alkaline phosphatase and malate dehydrogenase.

Finally, the experimental data obtained in crowded media has been fitted to the proposed model. The results reveal that the cooperative behaviour on GDH does not seem to vary in crowded conditions.
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1. INTRODUCTION

Glutamate dehydrogenase (GDH) is a nicotine adenine dinucleotide (NAD) dependent oxidoreductase that catalyses the deaminative oxidation of \( L \)-Glutamate into \( \alpha \)-oxoglutarate and ammonia. It is an hexameric enzyme, with six catalytic centres, that has been reported to present negative cooperativity with its coenzyme\[^1\], which means that the affinity for each centre decreases as substrates bind to the other centres.

Macromolecular crowding is the alteration of the properties of molecules in a solution due to the presence of high concentrations of different macromolecules unrelated with the process under study. This phenomenon occurs typically in the cellular media and, therefore, performing experiments in dilute solution conditions (in absence of high concentrations of macromolecules) does not properly reflect cell environments.

Glutamate dehydrogenase is present in the mitochondria of eukaryotes, which consists of a highly crowded media. For this reason, the study of its kinetics in crowded media, using an \textit{in-vivo-like} strategy, is of great interest.

1.1 Cooperativity

Cooperativity is a phenomenon of universal importance in biological systems and involves identical or similar species that act in an interdependent way. The behaviour of a receptor molecule is considered as cooperative if its binding to a ligand depends with a nonlinear function on ligand concentration\[^2\]. It only applies to species with more than one binding site.

Cooperativity can be positive: if the binding of a ligand increases the apparent affinity of the receptor, facilitating the binding of the following ligand; or negative: if the binding of a ligand decreases the apparent affinity of the receptor, hindering the binding of the following ligand. If ligand binding to a site does not affect the others, the receptor can be considered non-cooperative.\[^2, 3\]

Cooperativity was originally found by C. Bohr in 1904 when he observed a sigmoid binding curve of oxygen to haemoglobin\[^4\]. This phenomenon was explained by stating that the binding of the first oxygen molecule caused an easier binding of the next oxygen molecule and hence was named cooperativity. Subsequent to this discovery, two different cooperativity theories were postulated\[^2\]: the Monod, Wyman and Changeux...
(MWC model) and the Koshland, Nemethy and Filmer (KNF model). The MWC model explains cooperativity as a change in the shape of the subunits in a manner that conserves the symmetry of the entire molecule, under the influence of a ligand. The KNF model, in contrast, explains cooperativity as an independent change in the conformation of the subunits that favours the change in the rest of subunits. Both theories were able to explain positive cooperativity cases but the MWC model cannot fit negatively cooperative enzymes. [4]

An easy way to identify cooperative behaviours is by its Scatchard plot (Figure 1), which is the representation of the variation the average number of occupied sites ($v$) divided by substrate concentration with the average number of occupied sites. The average number of occupied sites is related to the fractional occupancy of the sites ($\theta$) as:

$$v = s \cdot \theta \quad \text{(Eq.1)}$$

In which $s$ is the total number of sites. The fractional occupancy can be experimentally approximated as a ratio between the initial and maximum velocities (Eq. 2) and, therefore, the average number of occupied sites also depends on that ratio:

$$\theta \equiv \frac{v_0}{v_{max}} ; \quad v \equiv s \cdot \frac{v_0}{v_{max}} \quad \text{(Eq. 2)}$$

Where $v_0$ is the initial rate, $v_{max}$ is the maximum velocity.

### 1.1.1 Cooperative behaviour

The cooperative behaviour of an enzyme can be described using Adair’s equation[5]. First, it is necessary to define the reaction scheme:

$$E^- + S \rightleftharpoons E-S$$

Where $E$ represents the free binding sites, $S$ is the substrate and $E-S$ are the occupied sites. $K_c$ is the effective association constant of the substrate and can be expressed as:

$$K_c = \frac{[E-S]}{[E^-][S]} = \frac{1}{[S]} \cdot \left( \frac{\theta}{1-\theta} \right) = \frac{1}{[S]} \cdot \left( \frac{v}{v_{max}} \right) \quad \text{(Eq. 3)}$$

![Figure 1. Scatchard plot for different types of cooperativity. The values in the axis are arbitrary.](image)
In terms of the macroscopic or stoichiometric association constants \( (K_i) \), the average number of occupied sites can be written down as Eq. 4, which is known as Adair’s equation.

\[
\nu([S]) = \frac{\sum_{j=1}^{s} j b_j [S]^j}{\sum_{i=0}^{s} b_i [S]^i} \quad ; \quad b_j \equiv \prod_{i=1}^{j} K_i
\]  

(Eq. 4)

Where \( j \) represents the number of catalytic centres \((j=1,\ldots,s)\) and \( b_i \) parameters are the products of the macroscopic association constants \((K_i)\).

It is important to define intrinsic constants and its relation with the macroscopic ones. An intrinsic constant is an effective association constant for a certain site and it is independent of the other sites. It describes the equilibrium between the free substrate, the free site and the site-substrate complex. In contrast, a macroscopic association constant describes the equilibrium between the free substrate, the available enzyme and the enzyme-substrate complex (taking into account all the sites). To convert macroscopic constant into intrinsic constants an entropic term including the different distribution ways of the substrate in the free sites need to be included (for the conversion of macroscopic association constants, \( K_i \), into intrinsic association constants, \( K_i^{int} \), see Eq. 5).

\[
K_i = \left( \frac{s-j+1}{j} \right) \cdot K_i^{int}
\]  

(Eq. 5)

1.1.2 Non-cooperative behaviour

The non-cooperative or ideal behaviour corresponds to the Michaelis-Menten\(^6\) model. It is important to describe this model because it is commonly used to characterise kinetic behaviours, even when they are cooperative.

First, the model involves an enzyme (E) which is combined with the substrate or ligand (L) in a first and reversible step to form the enzyme-substrate complex (EL). The dissociation of this complex yields to the product (P) and the recovery of the enzyme. This can be schematised as:

\[
E + S \rightleftharpoons \frac{k_f}{k_b} ES \rightarrow \frac{k_{cat}}{E + P}
\]

Where \( k_f \), \( k_b \) are the rate constants of formation (forward) and decomposition (backward) of the complex, respectively, and \( k_{cat} \) is the rate constant for product formation (catalytic). By assuming that product formation is an irreversible step and that
the complex and substrate concentrations are constants over time (stationary state), the Michaelis-Menten equation for the initial rate \( (v_0) \) can be written down as Eq. 6.

\[
v_0 = \frac{v_{\text{max}}[S]}{K_m+[S]} \quad \text{(Eq. 6)}
\]

where \( v_{\text{max}} \) is the maximum velocity and can be expressed as Eq. 7:

\[
v_{\text{max}} = \lim_{[S] \to \infty} v_0 = k_{\text{cat}} \cdot [E] \quad \text{(Eq. 7)}
\]

and \( K_m \) is the pseudo-dissociation constant for the complex (Eq. 8), which is named as Michaelis constant and can be related to the enzyme affinity for the substrate.

\[
K_m \equiv \frac{[E][S]}{[ES]} = \frac{k_{\text{cat}}+k_b}{k_f} \quad \text{(Eq. 8)}
\]

Michaelis-Menten equation can be represented as Figure 2.

The parameters \( v_{\text{max}} \) and \( K_m \) are used to describe kinetic behaviours even when they are cooperative; in that case they are used as apparent parameters \( (v_{\text{max}}^{\text{app}}, K_m^{\text{app}}) \).

\[
v_0 = \frac{v_{\text{max}}^{\text{app}}[S]}{K_m^{\text{app}}+[S]} \quad \text{(Eq. 9)}
\]

The non-cooperative case can be also described by Adair’s equation (see section 1.1.1) in which the association intrinsic constants of all the sites will be equal.

\[
\psi^i d ([S]) = \frac{sK_c^i d [S]}{1+K_c^i d [S]} \quad \text{(Eq. 10)}
\]

\[
K_c^i d = \lim_{[S] \to 0} K_c = \frac{K_1}{s} \quad \text{(Eq. 11)}
\]

If the expression is rearranged in terms of the Michaelis constant, which is the inverse of the association constant, the Michaelis-Menten equation is obtained.

\[
\psi^i d ([S]) = \frac{s [S]}{K_m^{\text{app}}+[S]} : K_c^i d = K_m^{-1} \quad \text{(Eq. 11)}
\]

**1.1.3 Negative cooperativity role**

As previously mentioned, negative cooperativity involves a decrease in the binding affinity of the protein as ligands bind to it. This type of cooperative behaviour was first reported for rabbit-muscle Glyceraldehyde 3-phosphate dehydrogenase in 1968 but,
despite the years that have passed, its physiological role remains unclear because the majority of negative cooperativity studies have been focusing only on explaining the cooperative mechanisms. [7]

The first interpretation of the role of negative cooperativity is in terms of response to substrate concentration. Positive cooperativity amplifies the sensitivity for a signal than in a non-cooperative system. However, negative cooperativity has less sensitivity than non-cooperative systems but extends the range over which some response is generated[8] (lower substrate concentrations) as can be seen in Figure 3.

Negative cooperativity could be explained as a link between mechanical energy with catalysis, by transferring the energy associated with the binding of a substrate in one subunit to facilitate product release from other subunits. [9]

Another interpretation of the presence of negative cooperativity in several enzymes is focused on enzymes maintaining a certain rate of reaction. Experimentally, substrate concentrations are chosen by the investigator and the enzyme responds with the appropriate rates. In the physiological conditions, however, enzymes do not alter their velocities but adjust or vary substrate and product concentrations to maintain a certain rate[7]. Consequently, negative cooperativity could be important to maintain a certain level of enzymatic activity over a wide range of substrate or coenzyme concentration[8]. With low substrate concentrations, enzyme would have a higher response than a non-cooperative enzyme; and with higher substrate concentrations, the enzyme would have a minor response than the non-cooperative but very similar to its own response at low substrate concentrations (see Figure 3).

A more general conclusion is that cooperativity can only be understood when considering it as a contributor of an entire pathway and not as a mechanism for regulating fluxes but to maintain homeostasis responding to changes on demand. [10]
1.2 Macromolecular Crowding

One important feature of the intracellular media is that consists of large amounts of different macromolecules like proteins, nucleic acids, polysaccharides, etc. Such environment is termed crowded rather than concentrated\cite{11} due to the fact that not an individual macromolecule species is present at high concentration but all the different molecules taken together occupy a significant fraction of the total volume (as can be seen in Figure 4).

The total concentration of the macromolecules present inside cells is up to 400 g/L, which means that between 5% and 40% of the total volume is occupied and unavailable to other molecules\cite{12}. For example, the total concentration of proteins and nucleic acids in an E. Coli cell is about 300-400 g/L\cite{13}. These high concentrations of macromolecules are not only present in cellular interiors, but also occur in the extracellular matrix of tissues and even in blood plasma where the protein concentration is about 80 g/L\cite{14} (and it is sufficient to cause crowding effects).

As previously mentioned, macromolecular crowding effect is the alteration of the properties of molecules in solution as a result of the presence of high concentrations of unrelated macromolecules\cite{15}. This effect is also named the excluded volume effect because molecules cannot occupy the same space in solution and, as a result, they exclude volume to each other increasing the effective concentration of solutes.

The importance of the excluded volume effect lies in that the non-specific steric interactions that occur are universal, regardless of any other attractive or repulsive interactions between solute molecules, and have the potential to modulate the kinetics and equilibria of reactions in physiological fluid media.\cite{14}

Despite macromolecular crowding was discovered four decades ago\cite{16} and started to be commonly known since the 80s\cite{17}, \textit{in-vitro} experiments focused on studying the properties of macromolecules have usually been carried out with a total macromolecular concentration of 1 to 10 g/L.
These uncrowded media do not properly represent the cellular environment (as can be seen in Figure 5) and kinetic data obtained in these conditions can differ by orders of magnitude from those obtained performing experiments under crowded conditions within cells\cite{18}. Furthermore, reactions and processes that depend on available volume are usually affected by macromolecular crowding.

The available volume, defined as the fraction that can be occupied by a molecule, for a macromolecule is lesser than for a small molecule, as shown in Figure 6. The degree of unavailability of the total volume depends on the concentration, the shape and the size of all the molecules present.

The principal consequence of the excluded volume effect is an entropic decrease owing to the reduction of free space that causes a decrease in the randomness of particle distribution. The reduction of entropy increases the free energy of the solute and consequently produces an increase in its thermodynamic activity. As a result, crowding is expected to affect processes determined by activity. It can alter protein structure, folding, shape, conformational stability, binding of small molecules, enzymatic activity, protein-protein interactions, protein-nucleic acid interactions and pathological aggregation.\cite{11}

Another phenomenon related to the excluded volume is macromolecular confinement, which can be defined as the effects on the free energy and reactivity attributed to volume excluded by a fixed boundary to a macromolecule.\cite{15}

1.2.1 In-vivo-like approaches

In order to study the effects of macromolecular crowding it would be reasonable to think of performing experiments with living cells, in-vivo. However, working in in-vivo conditions or even with cell extracts would be arduous due to the complexity and the high heterogeneity in geometrical and physical properties of such media\cite{11}. Furthermore, the interpretation of the results would be challenging because of the great
the number of processes taking place and sharing common features and reaction species at the same time. As an example, to study a reaction involving nicotine adenine dinucleotide coenzymes (NAD oxidised or reduced), following the change of absorbance would be nearly impossible as these coenzymes participate in many other reactions throughout metabolism. Therefore, the effects of excluded volume need to be studied in model systems that ease the control of the reactions and experimental conditions.

To mimic the high concentration of macromolecules present in cell media it is possible to add known amounts of inert polymers that act as crowding agents. This approach, named *in-vivo-like*, facilitates the study of macromolecular crowding avoiding the intricacy of *in-vivo* conditions. Notwithstanding, this strategy simplifies the complexity of background species in cellular environments since they are represented by particles with identical size and shape, which differs greatly from the cell environment.

The ideal crowding agent should have a molecular weight between 50-200 kDa and a high solubility in water, it should not be prone to self-aggregation, it should be globular (in order to prevent solutions to become too viscous to handle) and available with high purity (to avoid any contamination) and it should only interact with the system via steric repulsion.\[^{14}\]

There are many types of available crowding agents but the most used are synthetic polymers like Dextran, Ficoll and Polyethylene glycol (PEG), which are neutral crowders, and proteins such as Bovine Serum Albumin (BSA), lysozyme or haemoglobin, which are in general negatively charged. When using neutral crowders, the interaction of the crowders with the reactant species would be only via steric repulsion. In contrast, with charged crowders there are steric and also electrostatic interactions. The use of charged crowders adds intricacy to the system and complicates the interpretation of results\[^{19}\]. However, this strategy represents more accurately the cytosol composition as it is formed by proteins majorly charged.

There is also a strategy to mimic macromolecular confinement by encapsulating the protein of interest in different polymeric matrixes such as silica glass using sol-gel techniques. The non-covalent binding of the proteins to the silica matrix impedes the rotational freedom of the protein and the highly porous character of the glass allows the exchange of solvent while proteins remain attached to the matrix. Since the glass is
transparent structures can be monitored and reactions can be followed by spectroscopic techniques including fluorescence and circular dichroism. \(^{[11]}\)

It is important to remark that both of these strategies to mimic macromolecular crowding and confinement are still far from accurately represent the cellular environment, which is made of high concentrations of different macromolecules, filamentous structures, confined spaces, etc. But these recreations can be useful to obtain activity values closer to the physiological ones than results obtained in dilute solution\(^{[20]}\). For example, it could be used in enzymatic activity assays for drug development avoiding the cost and difficulties of cell cultures and animal manipulations in pre-clinical stages.

### 1.2.2 Experimental crowding effects

Macromolecular crowding can affect some variables contributing to the enzymatic rate as: macromolecule diffusion, interactions between macromolecules, conformational equilibria, protein stability and enzyme kinetics.

Different effects caused by the excluded volume on enzyme kinetics have been reported over the last years. The effects of macromolecular crowding seem to differ between diffusion-controlled reactions and activation-controlled reactions. These effects can be explained by alterations in protein diffusion and modifications of the active site caused by crowding. \(^{[21]}\)

In terms of Michaelis-Menten kinetic parameters: \(v_{\text{max}}\) decreases in the majority of cases but the variation of \(K_m\) cannot be predicted since it has been reported to increase, decrease or remain constant\(^{[20]}\). Some examples of the effect of the excluded volume on enzyme kinetics are summarized below.

First, a study on the enzyme α-chymotrypsin\(^{[22]}\) (\(M_w=25\) kDa) revealed a decrease in \(v_{\text{max}}\) as a result of increased mixed inhibition by product in crowded media and an increase in \(K_m\) due to a decrease in protein diffusion caused by the presence of crowder. In the case of horseradish peroxidase (HRP)\(^{[23]}\) (\(M_w=42\) kDa) showed a decrease in both \(v_{\text{max}}\) and \(K_m\) parameters suggesting an activation control mechanism, which means that the catalytic constant \(k_{\text{cat}}\) brings a significant contribution and it is affected by crowding. Both of these enzymes showed dependence on the crowder concentration but not on its size.
However, a macromolecular crowding study on lactate dehydrogenase (LDH)\textsuperscript{[24]} (M\textsubscript{w}=140 kDa), which is a much bigger enzyme, exhibited a decrease in $K_m$ due to a modification on chemical activity of the substrate and an increase of the activity coefficient relation between the free enzyme and the complex. It also displayed a decrease in $v_{\text{max}}$ that depends on both crowder concentration and size, being more affected by the larger crowders. Other enzymes such as malate dehydrogenase (MDH)\textsuperscript{[19]} (M\textsubscript{w}=140 kDa) and alkaline phosphatase (ALKP)\textsuperscript{[25]} (M\textsubscript{w}=104 kDa) also showed a similar behaviour but they were more affected by crowder sizes similar to the enzyme size. This size-dependence effect can be explained by the rise of depletion forces, which are entropic forces caused by the difference between enzyme and crowder sizes\textsuperscript{[20]}. The sizes of the studied enzymes and the crowders used are schematized in figure 7.

![Figure 7. Schematic representation of the reviewed enzymes with their corresponding sizes and the sizes of the crowders used (Dextrans).](image)

The effects of macromolecular crowding on enzyme kinetics can also be studied by computational simulations performing either Monte Carlo simulations in a three dimensional lattice\textsuperscript{[26]}, or Brownian Dynamics simulations for the diffusion process coupled to Monte Carlo simulation of the reaction process. This last simulation can be approached using the ReaDDy software package\textsuperscript{[27]}. The evolution of the system over time in different excluded volume conditions allows studying reaction and diffusion processes.

### 1.3 Glutamate dehydrogenase

Glutamate dehydrogenase (GDH) in an homohexameric enzyme found in all living organisms that catalyses the reversible oxidative deamination of L-glutamate to α-oxoglutarate by converting nicotine adenine dinucleotide and nicotine adenine dinucleotide phosphate in its oxidised form (NAD\textsuperscript{+} and NADP\textsuperscript{+}, respectively) to their
corresponding reduced forms (NADH and NADPH), as can be seen in Figure 8. Mammalian GDH exhibits the unusual ability to use both forms of coenzyme with nearly equal efficacy, in contrast to GDH from primitive organisms.\textsuperscript{[28]}

Glutamate dehydrogenase is located within the inner mitochondrial matrix and it feeds α-oxoglutarate to the Krebs cycle whereas supplies nitrogen for several biosynthetic pathways.\textsuperscript{[8]}

The study of the kinetics of GDH in crowded media is interesting due to the fact that the mitochondrial matrix is a highly crowded environment. Mimicking this media can give more realistic results of GDH’s kinetics.

In mammals, the reaction is expected to happen only in the oxidative deamination sense under normal conditions since the reverse reaction has a high Michaelis constant (\(K_m\)), enzyme has very low affinity for ammonia. Therefore, the conversion of α-oxoglutarate into \(\text{L-glutamate}\) only occurs when very high levels of ammonia are present. Moreover, it has been reported that some bacteria use GDH instead of the normal glutamine synthase pathway to fix nitrogen under high ammonia conditions.\textsuperscript{[29]}

Glutamate dehydrogenase from animal sources is allosterically regulated by a wide range of metabolites. A summary of GDH regulation is depicted in Table 1. It is important to highlight that there is product inhibition by NADH.

<table>
<thead>
<tr>
<th>Activators</th>
<th>Adenosine diphosphate (ADP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leucine</td>
</tr>
<tr>
<td></td>
<td>Monocarboxilic acids</td>
</tr>
<tr>
<td></td>
<td>Guanosine-5’-triphosphate (GTP)</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
</tr>
<tr>
<td></td>
<td>Adenosine triphosphate (ATP)</td>
</tr>
<tr>
<td></td>
<td>Steroid hormones such as diethylbestrol (DES)</td>
</tr>
<tr>
<td></td>
<td>Hydrophobic compounds such as Palmitoyl CoA</td>
</tr>
<tr>
<td></td>
<td>(\text{Zn}^{2+})</td>
</tr>
<tr>
<td></td>
<td>Coenzyme (NAD(P)(^{+}))</td>
</tr>
</tbody>
</table>


In contrast to this extensive regulation that mammalian GDH presents, bacterial forms of this enzyme are relatively unregulated, they are mainly regulated at the transcriptional level. This difference has been attributed to an additional amino acid
sequence in mammalian GDH that expresses an antenna that intercommunicates the different subunits. [8]

The possible roles for this intricate allosteric regulation are only starting to emerge. One of the most interesting is the inappropriate insulin secretion (hyperinsulinism) related to a loss on GDH inhibition. The lack of inhibition leads to an uncontrolled catabolism of amino acids yielding high ATP levels that stimulate insulin secretion. This GDH-mediated hyperinsulinism is related to: hyperinsulinism/hyperammonemia (HHS), mutations in NAD-dependent deacetylase sirtuin 4 (SIRT4) (implicated in aging, stress response and apoptosis) and deficiencies in the short-chain 3-hydroxyacyl-CoA dehydrogenase that cause protein-induced hypoglutecemia. [30]

1.3.1 Structure

As previously mentioned, Glutamate dehydrogenase is an hexamer and it is structured as two trimmers of subunits stacked directly on top of each other (Figure 9). Each subunit is composed of at least three domains: the bottom domain that makes extensive contacts with a subunit from the other trimer, the NAD-binding domain which is on top of the bottom domain and the antenna which rises above the NAD-binding domain and lies immediately behind the adjacent, counter-clockwise neighbour. [8]

Substrate binds in the depths of the cleft between the NAD-binding domain and the bottom domain. Coenzyme binds on the NAD-binding domain and it closes down upon the substrate and coenzyme. As the catalytic site closes, the base of each antenna rotates out in a counter-clockwise manner, pushing away the pivot helix of the adjacent subunit. The three pairs of subunits move rigid towards each other, compressing the cavity at the core of the hexamer. [30]

The antenna is only found in the allosteric regulated forms of GDH hence it is thought to be strongly related to regulation.

Figure 9. Glutamate dehydrogenase structure. Each of the six identical subunits is shown in different colours. Extracted image from Smith, T.J. and Stanley, C.A. Trends. Biochem. Sci. 2008 33: 557-564.
1.3.2 Negative cooperativity

Glutamate dehydrogenase exhibits an unusual kinetic behaviour with an unclear physiological role, negative cooperativity. This phenomenon was first reported for this enzyme in 1969\cite{1}, in a study in which deviation from Michaelis-Menten behaviour was detected when varying NAD$^+$ and NADP$^+$ concentrations.

Negative cooperativity in GDH is caused by coenzyme-induced conformational changes and requires a dicarboxylic acid substrate or an analog with a 2-position substituent\cite{28}. With alternative amino acidic substrates, such as Norvaline, cooperative interactions are absent\cite{1}.

Over the years some studies have explained negative cooperativity in GDH with a compulsory order mechanism, two different mechanisms depending on pH. However, some mathematical inconsistencies detected in 1970\cite{31} provided clear proof that the enzyme does not follow a compulsory mechanism. Independent evidence\cite{32} for a random-order mechanism obtained by studying isotope exchange at equilibrium was also found. Although alternative models have been proposed\cite{33}, a model to explain the negative cooperativity in GDH is still not found.

The unusual kinetic behaviour exhibited by Glutamate dehydrogenase has been attributed to the interactions between its six sites (or subunits) and also by different conformational transitions. The presence of cooperativity caused by interactions within the sites was demonstrated by the ideal kinetic behaviour presented by a bacterial form of the enzyme when performing experiments deactivating five of the six subunits\cite{34}. By mutating tryptophan and phenylalanine residues at the trimer-trimer interface results suggest that conformational transitions between different active sites mediate the cooperative behaviour\cite{35}.
2. OBJECTIVES

The aim of this work is to study the kinetics of the oxidative deamination of \(L\)-glutamate catalysed by glutamate dehydrogenase. The study is focused on characterising the cooperative behaviour that presents the enzyme and analysing the effect of macromolecular crowding on its kinetics.

All the experiments will consist on measuring the initial rate of the reaction varying coenzyme (NAD\(^+\)) concentration and following the reaction by spectrophotometric measurements of NADH absorbance as it is formed. The experiments will be carried out first in dilute solution conditions and, to analyse the effect of macromolecular crowding, experiments in crowded media will be performed by adding a crowding agent to the solutions. Increasing amounts of the crowding agent, dextran, and different sizes of it (60, 250 and 500 kDa) will be used to simulate the crowding effect.

In order to properly define negative cooperativity, some cooperative models are going to be proposed. These models are based on Adair’s equation and take into account the different interactions between the substrate and the catalytic centres of the different subunits that form the enzyme. Experimental data will be fitted to these models and its suitability is going to be evaluated in terms of error and accuracy. Other reaction systems with similar features will be used to validate the chosen model.

Experimental data obtained working in crowded conditions will be compared to the measurements acquired working in dilute solution conditions. Variations in kinetic parameters will be evaluated and compared with the ones obtained previously in other reaction systems.

Finally, crowded media data will be fitted to the chosen model and the interpretation of negative cooperativity in crowding conditions will be discussed.
3. MATERIALS AND METHODS

The studied enzyme, Glutamate dehydrogenase, was chosen mainly for its cooperative behaviour, with the purpose of proposing a general model for negative cooperativity in hexameric enzymes. Other reasons to choose GDH were: that, due to a change in absorbance, reaction can be followed by spectrophotometric measurements; that the enzyme size is in between the crowder sizes available and it is higher than the other reviewed enzymes; and that it can be studied under crowding conditions.

In order to properly study the effect of macromolecular crowding the reaction must satisfy certain requirements: it must be accompanied by a minimum change in the excluded volume due to the conversion of reagents into products, any species involved cannot present specific interactions with the crowding agent and the enzyme size must be intermediate between the available crowder agent sizes. Glutamate dehydrogenase fulfils all these requirements.

3.1 Macromolecular crowder

As previously mentioned, macromolecular crowding can be simulated by adding known amounts of an inert polymer. In this case DextranS (Figure 10) were used to that purpose.

DextranS are neutral, inert, random-coil synthetic polymers of glucose polysaccharides with high water solubility. They are available in a wide range of sizes and in large quantities. Due to these properties, dextranS are the most commonly used crowding agents and that makes them particularly suitable for comparing experimental results between different studies.

3.2 Chemicals

Bovine liver Glutamate dehydrogenase, type II (EC 1.4.1.3, $M_w=\text{310}-350$ kDa in hexameric form) was received already purified and in an aqueous glycerol solution form. The enzyme, L-Glutamic acid and $\beta$-Nicotinamide adenine dinucleotide in its oxidised form ($\text{NAD}^+$) were purchased from Sigma-Aldrich Chemical (Milwaukee, WI, USA). DextranS with 60, 250 and 500 kDa were acquired from Pharmacosmos (Hoelbrak, Denmark) and used without any further purification. The buffer solution was prepared by diluting a phosphate buffer solution 1M purchased from Sigma-Aldrich.
Chemical (Milwaukee, WI, USA). All chemicals were of analytical or spectroscopic reagent grade.

3.3 Oxidation of L-Glutamate

Spectrophotometric measurements were carried out at 25°C in a 0.01 M phosphate buffer adjusted to pH=7 to enhance enzyme stability (Engel 2011). Each sample contained a constant concentration of L-Glutamate (5 mM), which is in excess to consider the reaction as monosubstrate, and enzyme (0.18 µM; 1.3 µg/ml). At protein concentrations below 0.1 mg/ml, the enzyme only exists in the hexameric form. Different NAD⁺ concentrations in a range between 0.1 and 2 mM were used.

Experiments were performed in dilute solution conditions and in crowded conditions by adding increasing concentrations (25-150 g·L⁻¹) of Dextran with 60, 250 and 500 kDa sizes. An increase in absorbance caused by the formation of NADH was measured at 340 nm with a Shimadzu UV-1800 spectrophotometer. The initial reaction rate, \( v_0 \), was obtained by linear fitting of first data points in the absorbance-time plot (5-25 seconds). This data was fitted to the proposed models.

Although the oxidative deamination of L-glutamate is a fast reaction, experiments were performed by manual mixing of the reactants. However, some experiments were performed with a stopped-flow system\[36\] coupled to the spectrophotometer, which is a rapid mixing device to study fast reactions in solution. Both methods yield to identical results and therefore manual mixing was used to reduce wasting reagents.

Each measurement was repeated at least three times to minimize error and ensure reproducibility. The enzyme activity was controlled by absorbance at 270 nm every day and by measuring a control mixture twice a week.

3.4 Data treatment

In order to obtain the initial velocities, the linear section in the absorbance-time plot was selected (5-25 seconds) using Origin ver. 7.0. The slope of that section, dividing among the molar absorptivity (\( \epsilon \)) of NADH (6220 M⁻¹·cm⁻¹) and multiplying by the optical path (1 cm), is the initial velocity of the reaction. This data was fitted to the different proposed models using a least square adjustment with GNU Octave.
4. RESULTS
4.1 Negative cooperativity model for hexameric enzymes

One of the objectives of this study was to properly define the cooperative behaviour of GDH by proposing a model that fits the experimental data and allow some interpretation of its functioning. To this end, and owing to the background of the group, some models based on Adair’s equation (see section 1.1.1) were proposed and are outlined.

The \( \{b_j\} \) parameters of Adair’s equation in the proposed models depend on other parameters, in which are included microscopic association constants and the interaction between trimmers. Four types of models have been proposed varying the number and type of these parameters, they can be schematized as:

- **Two parameters \( (k_1, k_2) \)**

- **Three parameters \( (k_1, k_2, k_3) \)**

- **Four parameters \( (k_1, k_2, k_3, w) \), with an alternated conformation of the two trimmers**

- **Four parameters \( (k_1, k_2, k_3, w') \) with an eclipsed conformation of the two trimmers**
The terms of the interaction between trimmers (w and w') are Boltzmann’s factors associated to the free energy of interactions:

$$w = e^{-E_{\text{int}}/k_B T}$$  \hfill (Eq. 12)

Where $E_{\text{int}}$ is the energy associated with the interaction, $k_B$ is Boltzmann constant and $T$ is temperature.

In table 2, the relationship between $b$ parameters and the microscopic association constants for the four different models are depicted.

<table>
<thead>
<tr>
<th>$b_i$</th>
<th>Config.</th>
<th>Statistical weight</th>
<th>Relationship of microscopic parameters</th>
</tr>
</thead>
</table>
|       |         |                    |\begin{align*}
&2 \text{ parameters} \\
&(k_1, k_2) & 3 \text{ parameters} \\
&(k_1, k_2, k_3) & 4 \text{ parameters} \\
&(k_1, k_2, k_3, w) & 4 \text{ parameters} \\
&(k_1, k_2, k_3, w')
\end{align*}|
| 0     |         | 1                  | 1 1 1 1 |
| 1     |         | 6                  | $6k_1$ $6k_1$ $6k_1$ $6k_1$ |
| 2     |         | 9                  | $9k_1^2 + 6k_1k_2$ $9k_1^2 + 6k_1k_2$ $3k_1^2 + 6k_1^2w + 6k_1k_2$ $6k_1^2 + 3k_1w' + 6k_1k_2$ |
|       |         | 18                 | $18k_1^2k_2 + 2k_1k_2^2$ $18k_1^2k_2 + 2k_1k_2^2$ $12k_1^2k_2w + 6k_1^2k_2w^2 + 2k_1^2k_3$ $12k_1^2k_2w' + 6k_1^2k_2$ |
| 4     |         | 9                  | $9k_1^2k_2^2 + 6k_1k_2^3$ $9k_1^2k_2^2 + 6k_1k_2^3$ $3k_1^2k_2^2w + 6k_1^2k_2^3w^2 + 6k_1^2k_2k_3w^2$ $3k_1^2k_2^2w' + 6k_1^2k_2^3w + 6k_1^2k_2k_3w'$ |
| 5     |         | 6                  | $6k_1k_2^4$ $6k_1k_2^4$ $6k_1k_2^4$ $6k_1k_2^4w^2$ |
| 6     |         | 1                  | $k_2^6$ $k_1^2k_2^2k_3^2$ $k_1^2k_2^2k_3^2w^6$ $k_1^2k_2^2k_3^2w'^3$ |

Table 2. Relationship of microscopic parameters for each $b_i$ parameter and schematic representation of substrate binding on the possible configurations.

It should be noticed that for each configuration there is a product of microscopic association parameters, and the resulting $b_i$ parameter is the sum of the products for all the possible configurations.

These $b_i$ parameters are introduced in Adair’s equation to obtain the final fitting equation for each model. The data was fitted by a least square adjustment with GNU Octave, varying the microscopic parameters.
4.2 Fitting results

First, the experimental data used to perform the fitting to the different models was obtained in dilute solution conditions and it is shown in Figure 11.

As can be seen in Figure 11-B, the Scatchard plot presents the typical curvature expected in negative cooperativity and confirms that the enzyme presents a negative cooperative behaviour.

In order to fit the data to the proposed models, it is necessary to obtain the $v_{\text{max}}$ value. A simple manner to calculate it is by fitting the experimental data to the Michaelis-Menten equation (it was performed using Origin). The $v_{\text{max}}$ value obtained was: $(1.39 \pm 0.03) \times 10^{-6} \text{ M} \cdot \text{s}^{-1}$.

The fittings were performed using GNU Octave. Two different plots of the experimental data and the four different fittings to the models proposed are shown in Figure 12.
The first plot shows a good fitting for all the models, although the 3 parameters and 4 parameters (\(w'\)) models seem to fit better the data.

In the Scatchard plot (Figure 12-B), it can be seen that only the 3 parameters and the 4 parameters (\(w'\)) models fit properly the data. The 2 parameters model seems to represent a non-cooperative behaviour and the 4 parameters (\(w\)) clearly does not fit the data.

In order to numerically compare the models, the values and errors of the microscopic parameters and also the reduced \(\chi^2\) for every model are depicted (in Table 3).

<table>
<thead>
<tr>
<th>Model</th>
<th>Fitted parameters</th>
<th>Reduced (\chi^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 parameters</strong></td>
<td>(k_1) 1.91 ± 0.10 (mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(k_2) 1.72 ± 0.02 (mM)</td>
<td>0.0618</td>
</tr>
<tr>
<td><strong>3 parameters</strong></td>
<td>(k_1) 2.8 ± 0.3 (mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(k_2) 1.0 ± 0.2 (mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(k_3) 2.6 ± 0.4 (mM)</td>
<td>0.0590</td>
</tr>
<tr>
<td><strong>4 parameters (w)</strong></td>
<td>(k_1) 3.6 ± 1.3 (mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(k_2) 1.2 ± 1.3 (mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(k_3) 14 ± 8 (mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(w) -(5 ± 2) \cdot 10^2</td>
<td></td>
</tr>
<tr>
<td><strong>4 parameters (w')</strong></td>
<td>(k_1) 2.3 ± 0.4 (mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(k_2) 1.4 ± 0.6 (mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(k_3) 8 ± 5 (mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(w') (3.9 ± 1.2) \cdot 10^2</td>
<td>0.0592</td>
</tr>
</tbody>
</table>

Table 3. Parameters values and reduced \(\chi^2\) obtained fitting the experimental data to four different models.

The first two models have reasonable microscopic parameters and their corresponding errors. The 4 parameters (\(w\)) model has greater relative errors and a negative constant, which means that it does not fit well the data. It is also in accordance with the graphical results shown in Figure 12. The 4 parameters (\(w'\)) model has also higher errors than the first ones.

In terms of \(\chi^2\) reduced, the 3 parameters model has the smallest \(\chi^2\) reduced. Therefore, the 3 parameters model is the one that fits better the data and, consequently, the one that defines better with the minimum number of parameters GDH’s negative cooperativity.

In Figure 13, the fitting of the data to the 3 parameters model can be seen in different plots. It should be noticed that the \(K_c\) plot (Figure 13-C) shows a decrease over the ideal value expected, which is another evidence of negative cooperativity.
Figure 13. Variation of the initial rate with substrate concentration plot (A), Scatchard plot (B) and variation of the effective association constant ($K_c$) with substrate concentration plot (C) of the experimental data and the fitting to the 3 parameters model. The ideal or non-cooperative behaviour is shown in B and C, with its deduction.

The $\{b_j\}$ parameters and the intrinsic association constants obtained by fitting the data are depicted in Table 4.

<table>
<thead>
<tr>
<th>$b_1$ (mM$^{-1}$)</th>
<th>$b_2$ (mM$^{-2}$)</th>
<th>$b_3$ (mM$^{-3}$)</th>
<th>$b_4$ (mM$^{-4}$)</th>
<th>$b_5$ (mM$^{-5}$)</th>
<th>$b_6$ (mM$^{-6}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17±2</td>
<td>(9±2)-10</td>
<td>(1.5±0.4)-10$^3$</td>
<td>(10±4)-10</td>
<td>(2.1±1.3)-10</td>
<td>1.4±1.5</td>
</tr>
</tbody>
</table>

### Intrinsic association constants (mM$^{-1}$)

<table>
<thead>
<tr>
<th>$K_{1}^{\text{int}}$</th>
<th>$K_{2}^{\text{int}}$</th>
<th>$K_{3}^{\text{int}}$</th>
<th>$K_{4}^{\text{int}}$</th>
<th>$K_{5}^{\text{int}}$</th>
<th>$K_{6}^{\text{int}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.8±0.3</td>
<td>2.1±0.2</td>
<td>1.6±0.2</td>
<td>1.4±0.2</td>
<td>1.6±0.2</td>
<td>2.6±0.4</td>
</tr>
</tbody>
</table>

Table 4. Values for the $\{b_j\}$ parameters and the intrinsic association constants obtained by fitting the experimental data to the proposed model.

The values of the intrinsic association constants allow us to predict the affinity of the enzyme for the substrate at every situation. As can be deduced by observing the values of the $K_{i}^{\text{int}}$ in Table 4, the enzyme seems to present negative cooperativity, the constants values decrease, until four substrates are bind. It also seems to recover its initial affinity in the last two binding events. This behaviour can be clearly seen when representing the intrinsic association constants and the variation of the effective association constant ($K_c$) with the average number of occupied sites (Figure 14).
Although it seems to recover its initial association affinity, there is not an experimental evidence about this because the last experimental point was obtained before $\nu=5$ (it can be clearly seen in the Scatchard plot in Figure 13-B). It was not possible to obtain values further than $\nu=5$ in the conditions of this work because then glutamate would not be in excess and the reaction could not be considered as monosubstrate. A further discussion can be found at section 5.

4.3 Crowded media results

4.3.1 Effect of the crowder concentration

To focus only in the effect of crowder concentration, the results shown in this section were all obtained working with the same size of Dextran, which is 250 kDa. This size was chosen due to its similarity to the enzyme size (310-350 kDa). Using crowders with similar size to the enzyme increases the crowding effect$^{[11]}$. 

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**Figure 14.** Plot of the intrinsic association constants ($K_{i\text{int}}$) values and the variation of the fitted effective association constant ($K_c$) with the average number of occupied sites ($\nu$).

**Figure 15.** Experimental data obtained for the variation of the initial rate with substrate concentrations in dilute solution conditions and working with different concentrations of 250 kDa Dextran (crowder).
In Figure 15, the experimental data obtained in dilute solution conditions and with increasing concentrations of Dextran is displayed.

First, it can be noticed that the initial rates generally decrease in crowded media and tend to decrease more with crowder concentration. It follows that $v_{\text{max}}$ seems to decrease in crowded media.

Secondly, the greater decrease in the initial rate corresponds to 150 g·L$^{-1}$ concentration. This curve presents a different shape than the rest of curves.

Finally, fittings to Michaelis-Menten equation were performed to the data in order to quantify variations in the kinetic parameters. Although this enzyme does not behave ideally, this strategy is a simple way to compare the effect of crowding in terms of the kinetic parameters, which in this case, as our enzyme is cooperative, they are apparent.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Dilute solution</th>
<th>Crowder 25 g·L$^{-1}$</th>
<th>Crowder 50 g·L$^{-1}$</th>
<th>Crowder 100 g·L$^{-1}$</th>
<th>Crowder 150 g·L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{\text{m,app}}$ (mM)</td>
<td>0.58±0.03</td>
<td>0.73±0.06</td>
<td>0.60±0.06</td>
<td>0.58±0.08</td>
<td>~3</td>
</tr>
<tr>
<td>$v_{\text{max,app}}$ ($10^{-7}$ M·s$^{-1}$)</td>
<td>13.9±0.3</td>
<td>14.2±0.5</td>
<td>12.5±0.5</td>
<td>10.9±0.5</td>
<td>~10</td>
</tr>
</tbody>
</table>

Table 5. Michaelis constant and maximum velocity values obtained by fitting the experimental data in several crowded conditions to a Michaelis-Menten equation using Origin. The crowder size used was 250 kDa.

The results of the fitting to Michaelis-Menten equation are shown in Table 3. The 150 g·L$^{-1}$ data was not well fitted to Michaelis-Menten equation due to its shape, the results shown for this data are only orientative. The Michaelis constant remains practically equal for all the crowded conditions except for the highest one, in which $K_{\text{m}}$ seems to increase. Maximum velocity decreases with crowder concentration as predicted by observing the plot. For 25 g·L$^{-1}$ of crowder concentration $v_{\text{max}}$ is almost equal to the one obtained in dilute solution, but the initial rates in general are lower than in dilute solution (as seen in Figure 15). The maximum velocity of 150 g·L$^{-1}$ seems to be lower than the value obtained by the fitting.

In conclusion, the concentration of crowder diminishes initial rates and maximum velocity but does not affect $K_{\text{m}}$ it seems to increase at high concentrations of crowder.

### 4.3.2 Effect of the crowder size

It is also important to analyse the effect of crowder size. For this purpose, experimental data obtained with different concentrations and sizes of Dextran is depicted in this section.
For 25 and 100 g·L\(^{-1}\) crowder concentrations the initial rate remains essentially equal independently of the crowder size used as can be seen in Figure 16.

![Figure 16](image_url)

Figure 16. Experimental data obtained for the variation of the initial rate with substrate concentration working in dilute solution conditions and with two concentrations: 25 g·L\(^{-1}\) (A) and 100 g·L\(^{-1}\) (B). Both were obtained with different Dextran sizes: 60, 250 and 500 kDa.

Experimental data for 150 g·L\(^{-1}\) of crowder with different sizes is shown in Figure 17.

![Figure 17](image_url)

Figure 17. Experimental data obtained for the variation of the initial rate with substrate concentration working in dilute solution conditions and with a 150 g·L\(^{-1}\) concentration and different Dextran sizes (60, 250 and 500 kDa).

It can be noticed that the initial rate, for a 150 g·L\(^{-1}\) crowder concentration, depends on the crowder size and it depends in an unexpected manner. The decrease on the initial rate is higher with the 250 kDa Dextran, which is the intermediate crowder size used. The 500 kDa Dextran has less decreasing effect than the 250 kDa and the 60 kDa Dextran has the lower decreasing effect.

To properly analyse the effect of the different size in 150 g·L\(^{-1}\) crowder concentration, it would be useful to fit the data to a Michaelis-Menten equation to compare the kinetic constants but, due to the shape of the obtained curves, it was not possible to fit the data correctly to that model.
4.3.3 Negative cooperativity in crowded media

The last objective of this work was to analyse the cooperative behaviour of the enzyme in crowded media. With this purpose, some data series obtained in crowded conditions (Dextran 250 kDa) were fitted to the proposed model (3 parameters).

![Graphs showing initial rate variation with substrate concentration, Scatchard plots, and variation of $K_c$ with substrate concentration.](image)

Figure 18. Variation of the initial rate with substrate concentration (A), Scatchard (B) and variation of $K_c$ with substrate concentration (C) plots of the experimental data in dilute solution and in crowded conditions (250 kDa) and fittings to the model proposed (3p). To clarify the goodness of fitting, the Scatchard plot (B) does not include errors.

As shown in Figure 18, the fitting of the data was performed with great results. All the plots corroborate that the 3 parameters model proposed is capable to fit the data in crowded conditions. Now, to properly analyse the effect of crowding in the cooperative behaviour of GDH the values obtained for the fitted parameters are depicted in Table 6.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$k_1$ (mM$^{-1}$)</th>
<th>$k_2$ (mM$^{-1}$)</th>
<th>$k_3$ (mM$^{-1}$)</th>
<th>Reduced $\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilute solution</td>
<td>2.8 ± 0.3</td>
<td>1.0 ± 0.2</td>
<td>2.6 ± 0.4</td>
<td>0.059</td>
</tr>
<tr>
<td>25 g/L crowding agent</td>
<td>2.3 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>2.6 ± 0.6</td>
<td>0.031</td>
</tr>
<tr>
<td>50 g/L crowding agent</td>
<td>2.9 ± 0.4</td>
<td>0.9 ± 0.2</td>
<td>2.7 ± 0.6</td>
<td>0.032</td>
</tr>
<tr>
<td>100 g/L crowding agent</td>
<td>2.1 ± 0.5</td>
<td>1.2 ± 0.3</td>
<td>2.0 ± 0.5</td>
<td>0.093</td>
</tr>
</tbody>
</table>

Table 6. Parameters obtained by fitting the experimental data to the proposed model (3 parameters) and reduced $\chi^2$ of the fitting.
The first thing that should be noticed is that the reduced $\chi^2$ for the 25 and 50 g·L$^{-1}$ is lower than the obtained fitting dilute solution data, which means that the fitting is even better for this crowding conditions. For the 100 g·L$^{-1}$ the reduced $\chi^2$ is worse that for the dilute solution fitting, which is probably caused by some dispersion on the shape of the curve.

Now, focusing on the parameters values, it can be seen that they remain equal with increasing crowder concentrations. Even the 100 g·L$^{-1}$ parameters are practically equal to the rest of parameters for different conditions. The explanation for these results would be discussed in the section below.
5. DISCUSSION

First, four cooperative models were proposed to characterise the negative cooperativity observed in glutamate dehydrogenase. The fitting of the experimental data to the models revealed that the best model, with the minimum number of parameters, is the one with 3 parameters. Therefore, this was chosen to explain the cooperative behaviour and also to fit the data obtained in crowded conditions. The proposed model has only microscopic association constants (it does not include the interaction between trimmers term), which means that the enzyme works as two equal trimmers. In each trimmer, the union of a substrate to a site affects the rest of the sites.

Analysing the intrinsic constants obtained (Table 4, Figure 14), it can be seen that the enzyme decreases its affinity for the substrate until the first four sites are occupied and, in the last two, it seems to recover its initial affinity. This combination of two different behaviours could be explained by: the presence of negative cooperativity, until the fourth site is occupied; and positive cooperativity, in the last two sites.

But, considering that there is experimental evidence only before the fifth intrinsic association constant (or \( \nu=5 \)), this recovery cannot be confirmed. To obtain data with substrate concentration above the concentrations used in this study is necessary to rethink all the experiments, due to the fact that in higher substrate concentrations glutamate would not be in excess and the reaction could not be considered as monosubstrate. Besides, to increase the concentration of glutamate increments the possibilities of substrate inhibition\(^{[29]}\).

The proposed model was also used to explain the negative cooperativity on Aspartate carbamoyltransferase, which is also an hexameric enzyme that presents negative cooperativity. Experimental data from other studies\(^{[38]}\) was fitted to all the models and the one that fitted better the data was also the 3 parameters model\(^{[39]}\). It allows us to think that it is a promising model that could bring some light into the negative cooperative behaviour topic.

The next point is the effect of macromolecular crowding on the kinetics of our enzyme. From here we found that crowding, in general, decreases the apparent maximum velocity but does not alter the apparent Michaelis constant until high concentrations of crowder.
The alteration of \( v_{\text{max}}^{\text{app}} \) could be attributed to a decrease in \( k_{\text{cat}} \) (\( v_{\text{max}} = k_{\text{cat}} \cdot [E] \)), which is normally caused by conformational changes\[^{20}\]. The hypothesis of the conformational changes can be refused due to the results of negative cooperativity in crowded media, which are equal to the ones obtained working in solution. It is difficult to think that crowding changes the enzyme conformation without altering the binding sites and, therefore, the cooperativity mechanism. Instead of that and as no evidence of the decrease of the catalytic constant has been found, the decrease in \( v_{\text{max}}^{\text{app}} \) could be attributed to inhibition by product, which has previously been reported for this enzyme\[^{29}\].

The increase in \( K_m \) has only been seen in the highest crowder concentration but it could mean that in high crowding conditions the control of the reaction is by diffusion, as reported for other enzymes\[^{20}\].

Consequently, as the ratio \( v_{\text{max}}^{\text{app}}/K_m^{\text{app}} \) is decreasing, a mixed inhibition mechanism could explain the results\[^{22, 36}\]. The mixed inhibition mechanism can be schematized as:

\[
E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E' + P
\]

Where the product (P) acts as a mixed inhibitor, instead of a competitive inhibitor, due to the fact that it binds to a different form of the enzyme (E') than the one that substrate binds (E). The effect of this inhibition affects the \( v_{\text{max}}^{\text{app}}/K_m^{\text{app}} \) ratio as:

\[
\frac{v_{\text{max}}^{\text{app}}}{K_m^{\text{app}}} = \left( \frac{1}{1+\frac{[P]}{K_{1c}}} \right) \cdot \frac{v_{\text{max}}}{K_m} ; K_{1c} = \frac{(k_{-1}+k_2)k_3}{k_1k_{-2}} \tag{Eq. 13}
\]

As can be seen in Eq. 13, the term multiplying the \( v_{\text{max}}/K_m \) ratio is <1 and, therefore, it is able of diminish the value of \( v_{\text{max}}^{\text{app}}/K_m^{\text{app}} \) if it decreases with crowding. Crowding increases the effective concentrations of the species present in solution and, therefore, it increases the product concentration. If product concentration is higher, the term multiplying the \( v_{\text{max}}/K_m \) ratio decreases and the apparent maximum velocity decreases.

The following topic to discuss is the effect of crowder size. It has been found that, only in the highest crowding conditions, the initial rates depend on the size of the crowding agent. In fact, the size that highly affects the rates has a 250 kDa size. Taking into
account that our enzyme size is about 310-350 kDa, it seems that the crowder with similar size to the enzyme is the one exerting a highest effect. The effect of the crowder size in this case is similar than in other enzymes reported as ALKP\textsuperscript{[25]} and MDH\textsuperscript{[19]}. As previously mentioned, this size-dependence effect can be explained by the rise of depletion forces\textsuperscript{[20]}.

Finally, the effect of macromolecular crowding on negative cooperativity has been found to not affect the values of the parameters, which means that the cooperative behaviour of GDH is not affected by crowding.

These results suggest that crowding is not affecting the enzyme conformation, because it would probably alter the cooperativity exerted by GDH. Instead of that, it seems more likely to think that crowding is only diminishing the diffusion and hindering the encounters between the substrates and the enzymes. Binding experiments in crowded media would be a great alternative to confirm and corroborate the results presented in this study.
6. CONCLUSIONS

A study on glutamate dehydrogenase focused on its cooperative behaviour and the effect of macromolecular crowding on its kinetics has been presented. Experimental data in dilute solution conditions and in crowded media, obtained by spectrophotometric measurements of the increase in absorbance caused by the formation of NADH, has been depicted in this work.

Some models based on Adair’s equation have been proposed and one of them has successfully fitted the data in both dilute and crowded conditions. The model has three microscopic constants which imply that there is not an interaction between the two trimmers that form the enzyme. It has also shown a decrease in the affinity for the substrate in the first four binding events and an affinity recovery, that needs experimental corroboration, in the last two binding sites. The decrease in the affinity confirms the expected negative cooperativity.

Experimental data in crowded conditions reveals a decrease in the maximum velocity although the Michaelis constant remains equal and only seems to increase in the highest crowder concentration used. These results, and the fact that there is not an evidence of the decrease of the catalytic constant, suggest a mixed-inhibition by product mechanism of reaction.

The data obtained in crowded media also exhibits a dependence on crowder size in the highest crowder concentration used. These results are in agreement with other reported cases of enzymes such as alkaline phosphatase and malate dehydrogenase.

Finally, the experimental data obtained in crowding conditions was fitted to the proposed model. The fitting results showed that negative cooperativity does not vary with the presence of crowding and, therefore, crowding seems to not alter significantly the conformation of the protein.
ACRONYMS

- GDH: Glutamate dehydrogenase
- NAD: Nicotinamide adenine dinucleotide
- MWC: Monod, Wyman and Changeux
- KNF: Koshland, Nemethy and Filmer
- PEG: Polyethylene glycol
- BSA: Bovine serum albumin
- HRP: Horseradish peroxidase
- LDH: L-lactate dehydrogenase
- MDH: Malate dehydrogenase
- ALKP: Alkaline phosphatase
- NAD$: Nicotinamide adenine dinucleotide (oxidized form)
- NADP$: Nicotinamide adenine dinucleotide phosphate (oxidized form)
- NADH: Nicotinamide adenine dinucleotide (reduced form)
- NADPH: Nicotinamide adenine dinucleotide phosphate (reduced form)
- ADP: Adenosine diphosphate
- GTP: Guanosine-5’-triphosphate
- ATP: Adenosine triphosphate
- DES: Diethylbestrol
- HHS: Hyperinsulinism/hyperammonemia
- SIRT4: Sirtuin 4
REFERENCES


