Investigating the mechanisms of enzyme diffusion using mass photometry

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ABSTRACT: The existence of the phenomenon of enhanced enzyme diffusion (EED) has been a topic of debate in recent literature. The majority of experiments confirming the existence of this phenomenon have been conducted using fluorescence correlation spectroscopy (FCS), but an artefact has been found in some of these experiments. There are various proposed mechanisms to explain the origin of EED, such as conformational changes and oligomeric enzyme dissociation. In our study we use mass photometry (MP), a label-free single-molecule interferometric light scattering technique, to investigate the dependence of the oligomeric states of several enzymes on the presence or absence of substrate. The enzymes of interest in this study are catalase, aldolase, alkaline phosphatase and an alcohol oxidase (VAO) the first three of which were previously studied in the context of enhanced enzyme diffusion. We compared the ratios of oligomeric states in the presence and absence of substrate as well as different substrate and inhibitor concentrations. Catalase and aldolase were found to dissociate into smaller oligomers with substrate, while for alkaline phosphatase and VAO, different behaviors were observed. Intriguingly, the changes in oligomeric states in aldolase and catalase are independent of catalysis. Thus, we have identified a possible mechanism which explains the previously observed enhanced diffusion of enzymes by oligomer dissociation through ligand binding.

The idea that some enzymes diffuse faster than expected from Brownian motion in the presence of their substrates has been a topic of debate in recent literature. It has been observed in some studies [1–3] and denied by others [4,5]. Most experiments in which this phenomenon, termed enhanced enzyme diffusion (EED), has been observed, were conducted using fluorescence correlation spectroscopy (FCS) – an ensemble averaging technique requiring fluorescent labelling. Chen, et al.[5] discuss an artefact that arises in FCS: the substrate of alkaline phosphatase, *p*-nitrophenyl phosphate (pNPP), was found to interact with the fluorescent dye, which was wrongly interpreted as EED. The authors suggested that single-molecule methods should be employed in order to further study the phenomenon. It is important to note that this artefact has only been observed in the case of alkaline phosphatase and its substrate pNPP.

EED may be biochemically relevant on various scales. While its existence poses fundamental questions relating to the structure and dynamics of individual enzymes, it may also be significant on the cellular level, in relation to recently described observations of the upper limit on Gibbs free energy dissipation in cells [6,7]. However, its origin and mechanism are still unclear[8]. Current EED models can be categorized as being catalysis-independent, such as the conformational changes an enzyme undergoes during ligand binding [9,10] and those where catalysis is essential for diffusion enhancement [1–3]. One possible explanation for EED is based on a study by Jee, et al. [11] which suggests that oligomeric enzymes dissociate into their subunits in the presence of their substrate during catalysis. According to the Stokes-Einstein equation, the diffusion coefficient of a spherical particle is inversely proportional to its radius and therefore the subunits of an enzyme that has dissociated into smaller oligomers will diffuse faster than the enzyme in its larger, associated substrate-free state. Here, this mechanism is explored using mass photometry (MP). MP is a single molecule interferometric light scattering technique which can be used to determine the heterogeneity of a sample by measuring the masses of species present [12] (Figure 1). This technique can therefore be used to measure different oligomeric states of proteins. An advantage of MP is that measurements are performed in solution without fluorescent labelling. Furthermore, the concentrations used are in the nanomolar range, similar to those used in FCS. This makes MP a suitable technique to investigate EED under conditions close to those used in previous FCS measurements, but on the single-molecule level and under native conditions.

Catalase

Catalase is a fast exothermic enzyme that breaks down H_2O_2 to H_2O and O_2 and has been reported to exhibit EED up to 30 – 45% [1,13,14].

Catalase in the presence of substrate and inhibitor

The dissociation of oligomeric states of catalase was investigated with between 0 and 50 mM of substrate. Upon addition of substrate, the enzyme dissociates to its subunits with a sharp change in states between 0 and 5 mM of substrate. The states remain constant from 5 to 50 mM H_2O_2, suggesting that the enzyme reaches an equilibrium state in terms of dissociation (Fig. 2b).

To investigate the effect of catalysis on oligomeric state, measurements were performed in the presence of H_2O_2 and the non-competitive inhibitor NaN_3. To ensure that the inhibitor alone does not influence the oligomerization of the enzyme, a control experiment was performed (first two bars in the bar chart in Fig. 2e). In the middle two bars, the data presented is for catalase with inhibitor and substrate, using different substrate concentrations while keeping the inhibitor concentration constant, and the last two bars represent catalase with just substrate. It can be seen that the amount of tetramer decreases upon addition of substrate, regardless of the presence of inhibitor, suggesting that catalysis is not required for the dissociation of the enzyme. To further confirm this, the activity

of catalase was investigated in a bulk assay under the same conditions used in the MP experiments (Figures S3 and S5). This was further confirmed by the observation of bubbles, caused by the formation of dioxygen, only under turnover conditions (Figure S6).

Aldolase

Aldolase is a slow endothermic enzyme with a K_M value of 0.013 mM for fructose-1,6-bisphosphate (FBP)[15]. The enzyme has been reported to exhibit EED [9]. While before this study it was hypothesized that exothermicity is necessary for EED and that a high turnover rate is required [1], the reports of aldolase exhibiting EED did not fit that hypothesis. Illien *et al.* [9] additionally report that the diffusion coefficient of aldolase increases in the presence of its competitive inhibitor pyrophosphate (PPi). It is important to note that as pointed out in [14], reports of EED of aldolase have been controversial across studies [16]. In a study by Zhang *et al.* [4] using dynamic light scattering, no diffusion enhancement was observed in the presence of the substrate, FBP.

Aldolase in the presence of substrate and inhibitor

To investigate the mechanisms behind the observed EED for aldolase, the same experiment was conducted as for catalase. As can be seen in Figure 3, the tetrameric aldolase dissociates in a similar manner to catalase upon the addition of substrate, but plateauing at around 1 mM. In [9] it is discussed that dissociation of enzymes was not the reason for the observed EED since the diffusion goes back to base values after all substrate has been consumed. We hypothesize that the two observations are not mutually exclusive as the dissociated subunits should be able to reassociate over time.

To investigate the role of catalysis on the dissociation of aldolase, the competitive inhibitor PPi, which binds in the active site, was used. Interestingly, the presence of an inhibitor triggers partial dissociation of the tetramers into dimers and monomers (Fig. 3e).

Alkaline phosphatase

In earlier research in enzyme diffusion, alkaline phosphatase (Fig. 4a) was reported to exhibit EED [1] but later papers show that what was interpreted as diffusion enhancement was an artefact related to the substrate interacting with the fluorescent labelling in FCS [5]. While these findings were confirmed by Zhang et al. [4], the following measurements can be regarded as yet another confirmation of the artefact. In Fig. 4b it can be seen that there is no statistically significant change in the oligomeric states of alkaline phosphatase when substrate is added, in the range 0 to 25 mM.

Vanillyl alcohol oxidase (VAO)

VAO is an enzyme that has not been studied previously in the context of EED. It is a fungal enzyme involved in the degradation of aromatic compounds and typically exhibits an octameric structure consisting of eight identical subunits of 64 kDa (Fig. 5a), each harboring a covalently bound FAD cofactor [17]. FAD binding stimulates the oligomerization of VAO into dimers and octamers, whereas the apo form of VAO consists of monomers and dimers [18]. The most stable form of VAO at neutral pH is its octameric state, which can be regarded as a tetramer of dimers. It has been shown that also the dimers show activity [19,20]. These features make VAO a good candidate for studying dissociation and oligomerization in solution. As can be seen in figure 5b, VAO is mainly present as dimers and octamers. In the presence of substrate (vanillyl alcohol) the number of octamers increases at the expense of dimeric VAO, while the monomeric population remains constant (Figs. 5c and 5d). It has been observed before that the dimer-dimer interface is relatively small [21]and that changes such as the addition of chaotropic salt or cysteine oxidation can induce dissociation of octamers into dimers. Here, we show that interaction with substrate triggers association of dimers into octamers.

In this study we show that some of the enzymes that have previously been reported to exhibit EED, namely catalase and aldolase, dissociate in the presence of their substrates, while alkaline phosphatase does not dissociate in the presence of its substrate. Additionally, the oligomeric states of VAO were observed to shift towards an octameric state in the presence of its substrate, showing opposite oligomeric states changes to catalase and aldolase. The behaviour of catalase and aldolase was also studied in the presence of their respective inhibitors and it was observed that the dissociation of their tetramers does still occur albeit to a lesser extent. It should be noted that since the measurements need to be performed in the nanomolar range, the equilibrium ratios of the oligomeric states may differ compared to what would be observed at higher concentrations, for example in the micromolar range which is more relevant to biological processes [22].

While oligomerization is essential for protein function, structural factors driving this oligomerization remain unclear for many proteins [23]. Previously it was reported that substrate binding influences oligomerization of an enzyme [24] and oligomerization can play a crucial role in enzymatic activity [25], yet we observe oligomer dissociation of catalase and aldolase upon ligand binding. This observation poses questions regarding the oligomerization in the first place and the need for its reversibility. We hypothesize that this dissociation may have a regulatory role.

We conclude that enzyme dissociation is a likely mechanism for previously reported EED. The dissociation is not catalysis driven and should be studied in context of the enzymes' structures and their oligomerization behavior. Thus, ligand binding may play a dominant role in the hydrodynamic properties of enzymes, while catalysis may be less relevant for EED.

Figures

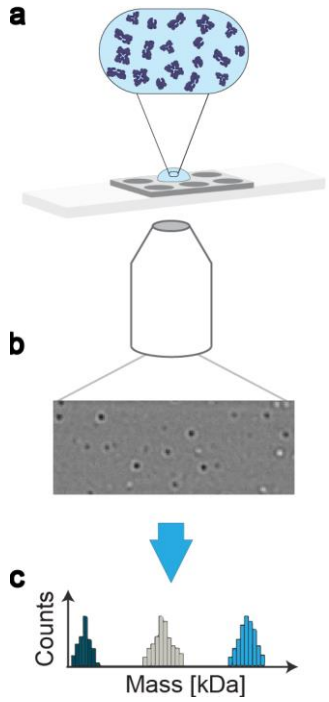

Figure 1. A mass photometry experiment. **a.** A glass slide with a gasket which contains six wells, each of which is used for a single measurement. For each measurement, 20 µL of diluted sample was used. **b.** The light goes through an interferometric microscope and the contrast change of each individual particle is obtained. **c.** The contrast change is directly proportional to the mass of a particle. Mass histograms of the populations present in the sample are obtained.

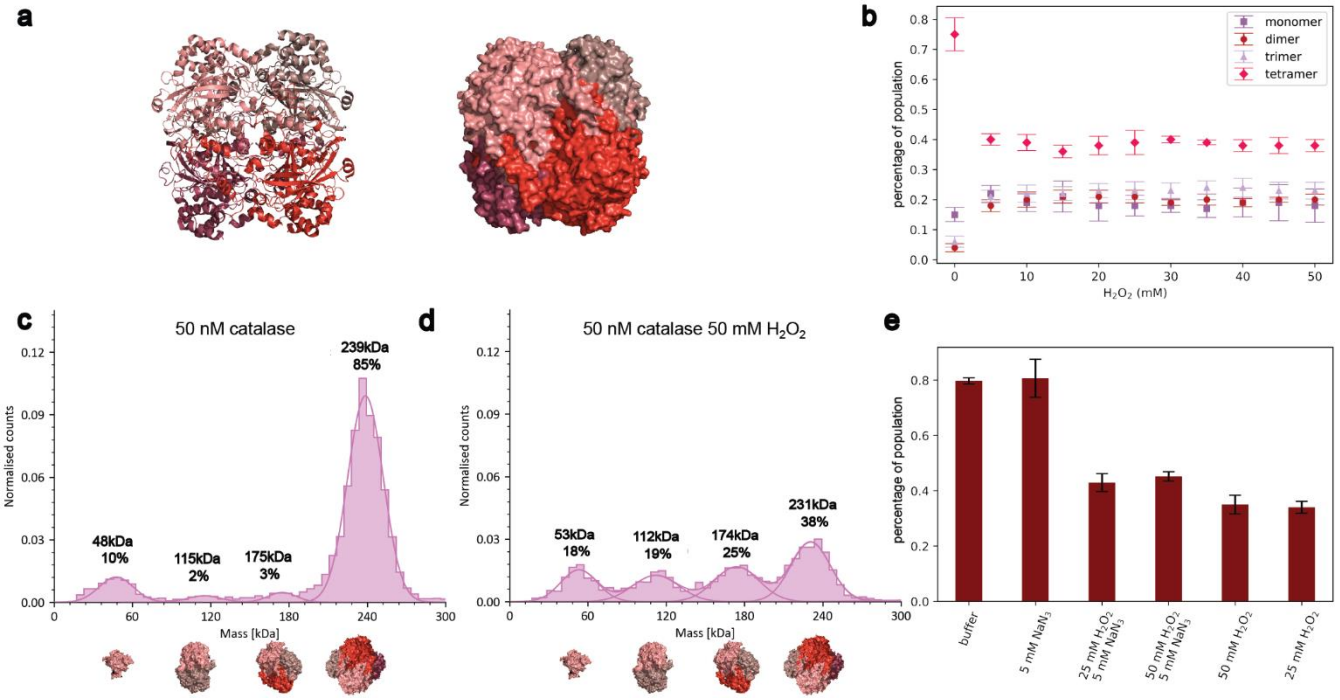

Figure 2. Oligomeric states of catalase (*Bos taurus*) in the presence of H_2O_2 (substrate) and NaN_3 (inhibitor). **a.** Crystal structure (PDB 1TGU) in cartoon representation (left) and surface representation (right) of catalase. The different shades of red represent the monomers. **b.** Oligomeric states of catalase as a function of substrate concentration, where normalized oligomeric states as percentage of all oligomeric states are shown. **c.** Histogram of catalase in potassium phosphate buffer (pH 7.5) and **d.** in the presence of substrate. **e.** Catalase tetramers in the presence and absence of substrate and inhibitor. On the y-axis the percentage of tetramer population is shown.

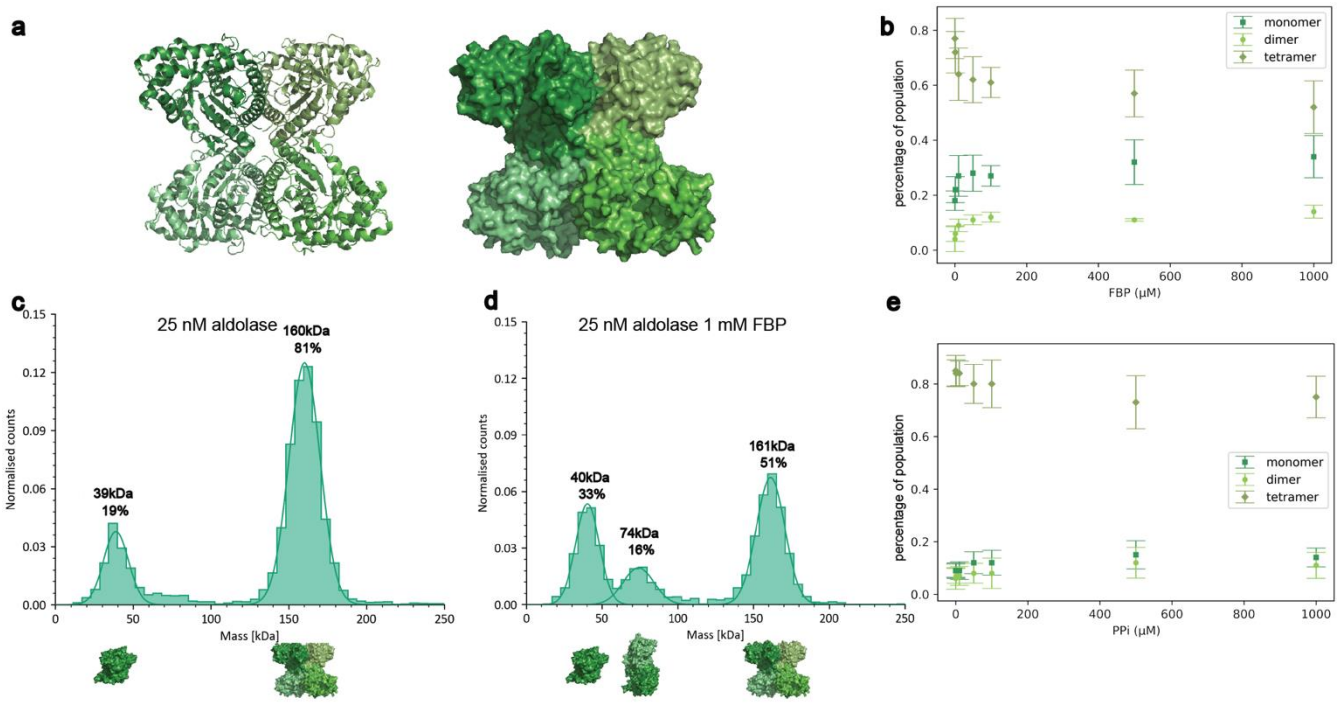

Figure 3. Oligomeric states of aldolase (*Oryctolagus cuniculus*) in the presence of FBP (substrate) and PPi (inhibitor). **a.** Crystal structure (PDB 1ADO) in cartoon representation (left) and surface representation (right). Each monomer is colored in a different shade of green. **b.** Normalized oligomeric states of aldolase as a function of substrate concentration, where normalized oligomeric states as percentage of all oligomeric states are shown. **c.** Histogram of aldolase in HEPES buffer (pH 7.4) and **d.** in the presence of substrate. **e.** Aldolase oligomeric states in the presence of inhibitor.

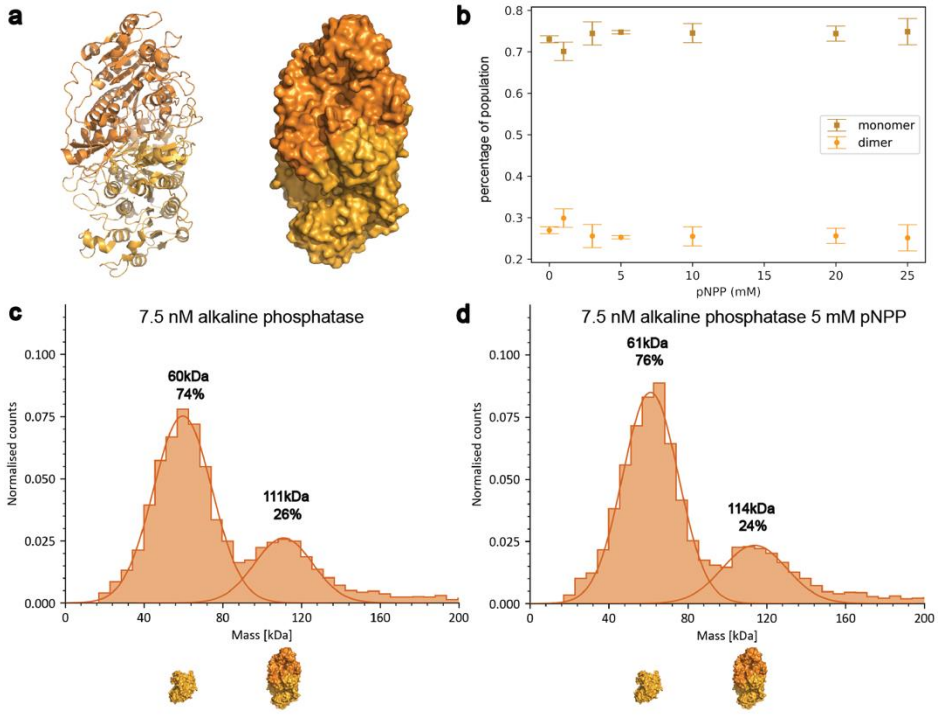

Figure 4. Oligomeric states of alkaline phosphatase (*Bos taurus*) in the presence of pNPP (substrate). **a.** Crystal structure (PDB 1ALK) in cartoon representation (left) and surface representation (right). The two shades of orange represent the two monomers that compose the dimer. **b.** Dependence of oligomeric states of alkaline phosphatase on substrate concentration. **c.** Histogram of alkaline phosphatase in alkaline buffer (see SI) and **d.** in the presence of 5 mM of substrate.

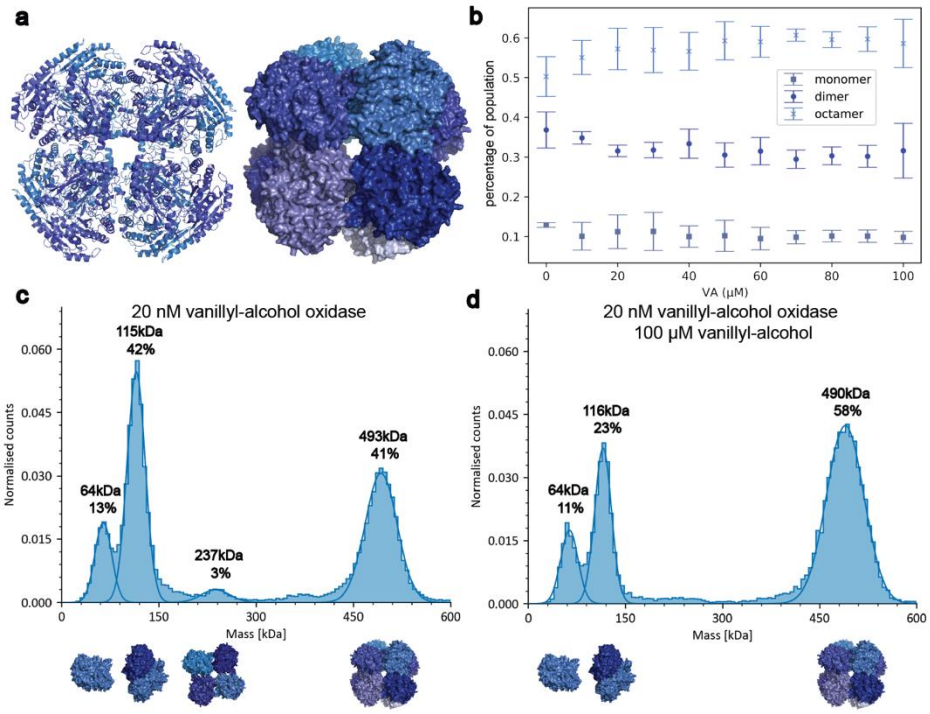

Figure 5. Oligomeric states of vanillyl alcohol oxidase (VAO, from *Penicillium simplicissimum*) in the presence of its substrate vanillyl alcohol. **a.** Crystal structure (PDB 1VAO) in cartoon representation (left) and surface representation (right) The shades of blue represent individual monomers. **b.** Dependence of oligomeric states of VAO on its substrate concentration. **c.** Histogram of VAO in potassium phosphate buffer (pH 7.5) and **d.** in the presence of 100 μM vanillyl alcohol.

ASSOCIATED CONTENT

Supporting Information

Preparation and characterization of enzymes, mass photometry, additional data for hexokinase and aldolase, diffusion enhancement calculations.

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Notes

The authors declare no competing financial interests.

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