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Applications of isothermal titration calorimetry to measure enzyme kinetics and activity in complex solutions

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Abstract

The application of isothermal titration calorimetry (ITC) was tested towards measurements of enzyme kinetics in complex solutions containing high concentrations of proteins. Such investigations are important, due to the increasing interest in biochemical reactions in physiological relevant media as well as the application of enzymes in industrial processes. In contrast to spectral methods, measurements performed with ITC, are independent of the optical properties of solutions, making it possible to measure enzyme kinetics in concentrated solutions of macromolecules. In this study the kinetic properties of hexokinase was investigated in concentrated protein solutions (BSA). It was found, that the quality of the measured kinetic data was independent of protein concentration in the investigated range (0–250 mg BSA ml^{-1}). All results could be accounted for by Michaelis–Menten's approach and both k_{cat} and K_M decreased with increasing protein concentrations. The decrease in K_M with increasing protein concentration was ascribed to an increase in the ratio of activity coefficients between the native enzyme and the enzyme–substrate complex. The decrease in k_{cat} with increasing protein concentrations indicates that crowding by BSA effect the conformational changes/rehydration that accompanies catalysis and/or diffusion of product from the enzyme–product complex. The methodology is discussed together with an analysis of the experimental results.

Keywords: Thermodynamic non-ideality; Macromolecular crowding; Enzyme kinetics; Isothermal titration calorimetry

1. Introduction

Determination of enzyme kinetics and activity are among the most important analytical procedures in biochemistry. The rate of which enzymes catalyze chemical reactions are traditionally measured with a variety of methods including spectroscopy, potentiometric measurements and chromatography. However, common to all of the above approaches are, that they are developed for specific enzymatic reactions. Furthermore, the use of spectral methods when measuring enzyme kinetics in concentrated solutions are limited due to scattering and/or absorption of the light [1–3]. These diluted conditions required for spectral assays, are far from the physiological conditions in cells, where the protein concentration in cytosol can reach 400 mg of protein ml^{-1} [4]. Such high protein concentrations can have massive effect on enzyme kinetics for example through macromolecular crowding effects [3,5–7] and it is therefore desirable to develop methodologies, which can elucidate enzyme kinetics with a minimal perturbance from macromolecules and other added solutes. Such data are needed to test theoretical considerations of the effect of macromolecular crowding on enzyme kinetics along with other possible effects of high solute concentrations [3]. They may also be important in attempts to empirically quantify enzyme activity under certain experimental conditions relevant to, e.g. physiology or biotechnology.

The use of calorimetry to measure the rate of enzymatic reactions has proved very useful and has been known for over three decades [8–12]. The general principle in using ITC is to measure the heat-flow associated with a given chemical reaction. The rate of the enzymatic reaction is proportional to this heat-flow, and thus measured as the primary experimental observable. Since practically all reactions produce or consume heat, enzyme activity can be monitored in real time without the need of probes, substrate analogues, etc. Furthermore, the measurement is independent of the composition of the reaction solvent, as long as complex side reactions with non-linear time dependence do not occur (see Section 4).

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Systematic investigations of the applications of ITC towards systems with high concentrations of macromolecules have not been conducted yet. In a recent review concerning the potential of ITC to measure enzyme kinetics in thermodynamic non-ideal solutions, it is stated that such studies are missing [13]. The use of calorimetry to measure enzyme kinetics in thermodynamic non-ideal solutions has been restricted to crowding by small solutes such as proline, glycine and sorbitol and only up to concentrations of 100 mM [14,15]. The method has also been used to measure enzyme activity in crude tissue extracts [16], and enzyme activity towards insoluble substrates [17]. The above studies indicate the promising potential of calorimetry towards measurements of enzyme kinetics in complex solutions.

The scope of the current work is to investigate the usefulness of ITC to measure enzyme kinetics in solutions containing high concentrations of macromolecules, thus mimicking *in vivo* conditions. Hexokinase was used as a model enzyme and bovine serum albumin (BSA) as model crowding component. Advantages and disadvantages of the method will be discussed together with an analysis of the effect of macromolecular crowding on the kinetic properties of hexokinase.

2. Materials and methods

2.1. ITC

All experiments were conducted using an ITC instrument (VP-ITC, MicroCal, Northampton, USA). The enzyme kinetic was investigated using both the so-called single injection method and the multiple injection method [12]. Briefly, the former detects the time-course of substrate depletion upon one large addition of substrate, while the latter measures the rate of enzyme catalysis during stepwise increase of substrate concentration in an extremely diluted enzyme solution. A brief introduction to the calculations follows.

The primary experimental observable is heat-flow measured in μW (μJ s^{-1}). This heat-flow is directly proportional to the reaction rate. However, calculation of the reaction rate requires knowledge of the enthalpy change of the reaction (ΔH_r) and the volume (V) of the reaction chamber. The enthalpy change is calculated by measurements of the total-heat, which is consumed or released and the total number of moles which are converted from substrate to product:

$$\Delta H_r = \frac{1}{n_{sub}} \int_{t=0}^{t=\infty} \frac{dQ}{dt} dt \quad (1)$$

where n_{sub} is the number of moles of substrate which are converted to products and dQ/dt is the heat-flow. Thus, the reaction rate (v) can be calculated:

$$v = \frac{1}{V\Delta H_r} \frac{dQ}{dt} \quad (2)$$

The substrate concentration ([sub]) as a function of time can be calculated using the integrated heat as a function of time:

$$[sub](t) = [sub]_{t=0} - \frac{\int_{t=0}^{t} \frac{dQ}{dt} dt}{\Delta H_r V} \quad (3)$$

Using Eqs. (2) and (3) it is possible to calculate both the reaction rate and substrate concentration as a function of time and finding the kinetic parameters k_{cat} and K_M by fitting to the Michaelis–Menten equation. A more detailed discussion of the above methods can be found elsewhere [12]. The single injection methodology used here is sensitive to a possible product inhibition. To assess this, two types of control experiments were conducted. First, a second injection of substrate was performed after the reaction of the first injection had gone to completion. Secondly, a multiple injection assay was made. In this approach, the enzyme concentration is lower compared to the single injection assay and the amount of product produced hence negligible.

2.2. Enzyme assays

The P2 form (b) of Yeast hexokinase (EC 2.7.1.1, H 6380) was purchased from Sigma–Aldrich. The enzyme was frozen in stock solutions in MilliQ water with 10% glycerol (applichem by >99%). This stock solution was frozen in 100 μl aliquots and had an enzyme concentration of 0.89 μM ($E^{1\%}_{280} = 9.47$) [18]. The enzyme reaction buffer contained 50 mM HEPES (Sigma > 99.5%), 50 mM KCl (Aldrich > 99%), 20 mM $MgCl_2$ (Merck > 99%), 9 mM ATP (Sigma > 97%) and varying amounts of BSA (Sigma A3059 > 99%) (0–250 mg ml^{-1}). The pH in all solutions was 7.0 (±0.2) and experiments were performed at 25 °C. To adjust pH in 250 mg BSA ml^{-1} 22 mM more NaOH was needed compared to pure buffer. Control experiments showed that addition of 22 mM NaCl had no effect on enzyme kinetic, thus the increase in ionic strength due to the buffer effect of BSA was negligible. The concentration of glycerol originating from the enzyme stock solution was negligible (2.5 mM), since only 6 μl of the stock solution was mixed with 2.5 ml enzyme reaction buffer, resulting in an enzyme concentration of 2 nM in all experiments (single injection assay). The BSA was dialysed three times against water (total of 72 h) and freeze dried before use. The concentration of ATP was at saturation level ensuring pseudo 1. order reaction conditions. In the single injection assay, the enzymatic reaction was initiated by injection of 10 μl 112 mM glucose (Fluka > 99.5%) to the calorimetric cell containing the enzyme in the reaction buffer. The heat-flow was measured until the reaction had gone to completion. In the multiple injection assay the glucose concentration was gradually increased by a total of 12 injections; 2 × 5 μl, 8 × 10 μl and 2 × 15 μl 11.2 mM glucose. The enzyme concentration in the multiple injection assay was 0.5 nM.

3. Results

The kinetic parameters k_{cat} and K_M, were derived from ITC measurements of the reaction rate of the phosphorylation of glucose catalyzed by hexokinase in solutions containing up to 250 mg BSA ml^{-1}. Fig. 1A shows raw data from the single injection assay of an experiment without BSA. In Fig. 1B, the raw data from Fig. 1A has been transformed (Eqs. (2) and (3)) into reaction rates and glucose concentrations. The dependence of the reaction rate on glucose concentration followed typical Michaelis–Menten kinetics, and the parameters k_{cat} and

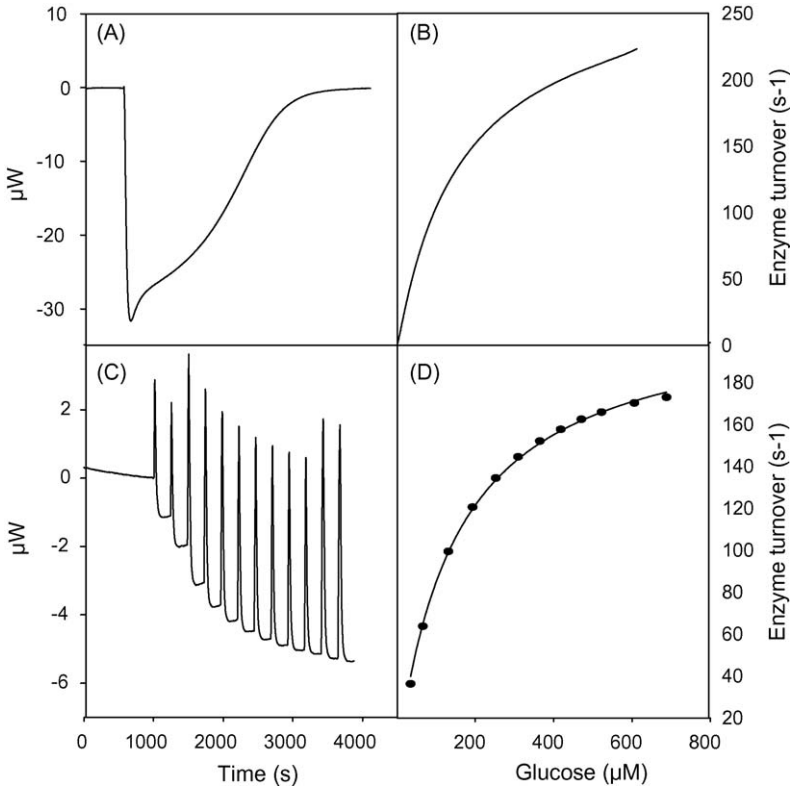

Fig. 1. Hexokinase. (A) Single injection assay: raw data of a calorimetric experiment. After 600 s, 10 μl 112 mM glucose was injected to the calorimetric cell containing the reaction buffer and 2 nM hexokinase at 25 °C. (B) The data from (A) has been transformed into reaction rates and glucose concentrations. (C) Raw data from the multiple injection assay at 25 °C. The glucose concentration in the calorimetric cell was increased by injections of 2 × 5 μl, 8 × 10 μl and 2 × 15 μl 11.2 mM glucose with 240 s between each injection. The enzyme concentration was 0.5 nM. (D) The steady state heat-flow levels after each injection has been transformed into reaction rates and plotted against glucose concentrations.

K_M were found by fitting the curve to the Michaelis–Menten equation using non-linear regression. To ensure that product inhibition was not significant when using the single injection assay, a multiple injection assay was performed. The raw data from the multiple injection assay is seen from Fig. 1C. The sharp peaks correspond to the heat of dilution, when injecting the substrate solution into the enzyme solution in the calorimetric cell. The baseline level between these peaks corresponds to a change in steady state heat-flow reflecting the reaction rate which gradually increases following each injection.

In Fig. 1D, the steady state heat-flow after each injection has been converted to reaction rates, and plotted against the glucose concentration. The enzyme and substrate concentrations were corrected for dilutions when increasing the reaction volume by additional injections. Also, the glucose concentrations in Fig. 1D were corrected for the small amount of glucose phosphorylated during the trial. The kinetic parameters derived from these experiments were respectively $K_M = 176\ \mu M$, $k_{cat} = 284\ s^{-1}$ (single injection assay) and $K_M = 181\ \mu M$ and $k_{cat} = 238\ s^{-1}$ (multiple injection assay). The slightly lower value for k_{cat} in the multiple injection assay compared to the single injection assay was probably due to the lower enzyme concentration in the multiple injection assay, which could result in a higher fraction of enzyme absorbed to surfaces. Kinetic parameters were also derived from two consecutive injections using the single injection assay. This resulted in almost the same kinetic parameters, an approximately 5% decrease in k_{cat} and 9% increase in K_M in the second injection compared to the first injection. Based on the fact that the two different assays gave almost identical kinetic parameters and that the kinetic parameters were almost the same between two consecutive injections, it was concluded that there was no significant product inhibition when using the single injection assay and above glucose concentration. Thus all experiments with BSA were performed using the single injection assay.

In Fig. 2A, raw data from experiments with respectively 25 and 250 mg BSA ml^{-1} are presented. The sharp peak at 600 s corresponds to the heat of dilution, when injecting 10 μl of 112 mM glucose into the concentrated protein solution in the calorimetric cell. The dilution peak was simply removed from the thermogram, when determining the enthalpy change from the enzyme catalyzed reaction, since the heat of dilution disappears within 2 min (data not shown). It is noticeable the rather short response times of modern ITC instruments (15 s for this calorimeter) which strongly facilitates this correction, and consequently lighten the requirement of equality in composition of the titrate and titrand. This is a simplificant experimental advantage for studies of crowding effects, where major composition discrepancies necessarily occur. The sensitivity of the calorimetric method was almost independent of BSA concentration in the whole concentration range examined. All fits to Michaelis–Menten equation resulted in $r^2 > 0.99$.

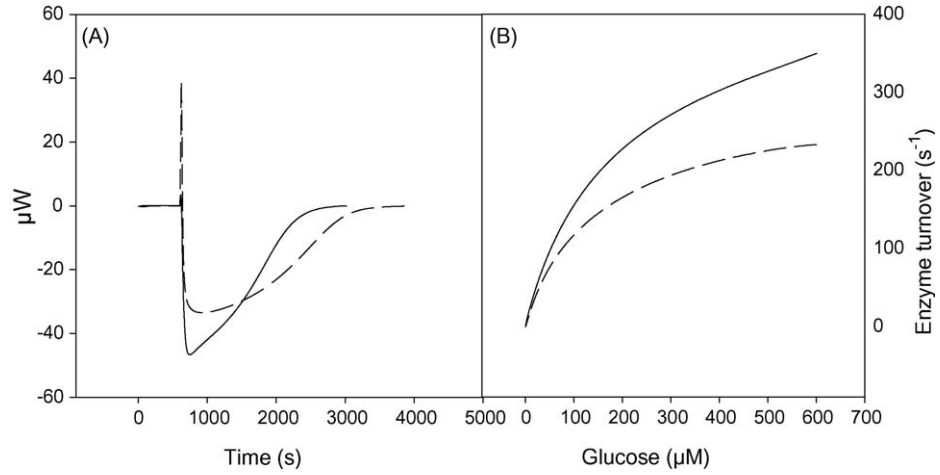

Fig. 2. Hexokinase. (A) Raw data of calorimetric experiments with 25 (solid line) and 250 mg BSA ml^{-1} (dashed line) at 25 °C. (B) The raw data from (A) has been converted into reaction rate and glucose concentration.

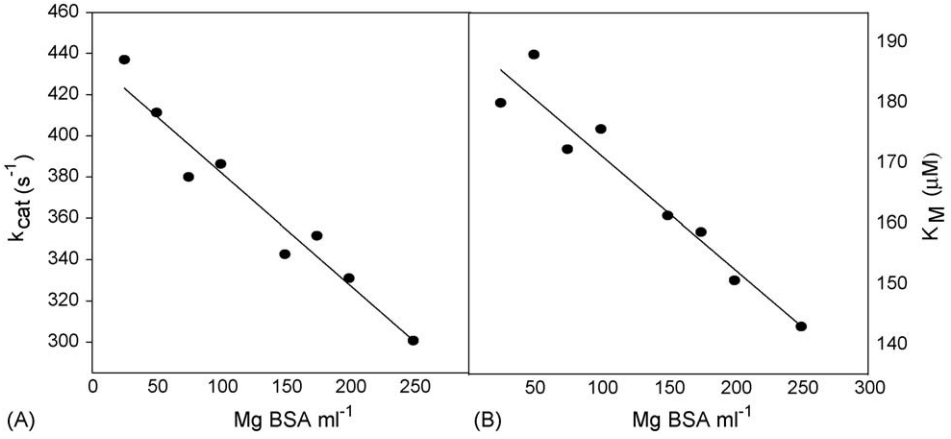

Fig. 3. (A) Hexokinase: k_{cat} as a function of BSA concentration at 25 °C. Every data point represents an average of three measurements. (B) K_M as a function of BSA concentration at 25 °C. Every data point represents an average of three measurements.

In Fig. 3 the kinetic parameters k_{cat} and K_M are presented as a function of the BSA concentration. All data points represent an average of three measurements. Both k_{cat} and K_M decreased approximately linearly with BSA concentration resulting in a reduction in k_{cat} from 440 s^{-1} without BSA (extrapolated) to 300 s^{-1} with 250 mg BSA ml^{-1}.

K_M decreased from 190 to 140 μM over the same BSA range. This corresponds to a 30% decrease in k_{cat} and a 25% decrease in K_M. The K_M values in the absence of BSA, were in the same range as found in other studies. Thus, Fromm and Zewe found K_M for glucose at 167 μM [19] and Hogget and Kellett the dissociation constant, K_d, of glucose to be around 300 μM [20] and Tood and Gomez found K_M and k_{cat}, respectively, of 72 μM and 270 s^{-1} using calorimetry [12]. The specificity constant k_{cat}/K_M did not change significantly over the entire concentration range of BSA.

The enthalpy change of the reaction (ΔH_r) also showed dependence to the BSA concentration.

As illustrated in Fig. 4 the heat released by the phosphorylation of glucose increased from 45 to 51 kJ mol^{-1} over the investigated range of BSA concentrations.

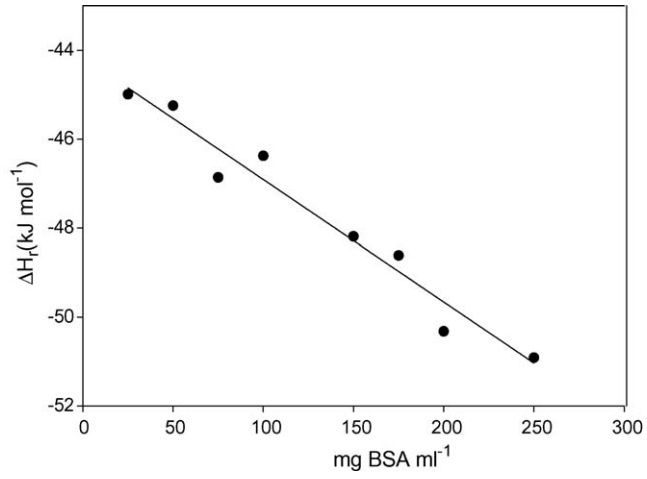

Fig. 4. Hexokinase: ΔH_r as a function of BSA concentration at 25 °C. Every data point represents an average of three measurements.

4. Discussion

Studies of enzyme activity and kinetics have traditionally been carried out in diluted systems, which most often can be treated as thermodynamic ideal solutions (i.e. solutions which are close to Henry's law behavior). Clearly, a detailed insight into enzyme kinetics under *in vivo* conditions is crucially important, and a number of theoretical and experimental reports [5,7,21–24] have concluded that the non-ideality arising from excluded volume/osmotic stress, may significantly influence enzyme kinetics. Experimental backup of these theoretical considerations, however, has been very sparse, perhaps due to problems with absorption and scattering of light in the spectroscopic methods commonly used. In this contribution, it is shown that ITC is an obvious candidate, for studying enzyme kinetics in such systems.

4.1. Experimental considerations

The sensitivity of the method was essentially unchanged when adding BSA up to 250 mg ml^{-1}. Furthermore, the heat of dilution when injecting the diluted substrate solution into the concentrated protein solution in the calorimetric cell only disturbed the signal over a period of about 2 min, due to the fast response time of the power compensated ITC. Thus, it is not necessary to subtract the heat of dilution from the thermogram, since the data points for the first few minutes can be removed without significant error in ΔH_r or the kinetic parameters. This alleviates the limitations of having different compositions of solutions in the injection syringe and the calorimetric cell, an important advantage in studies of complex solutions.

As discussed above hexokinase kinetics were studied by two fundamentally different calorimetric approaches: Single injection and multiple injection assay. The advantage of the multiple injection assay compared to the single injection assay is, that the amount of product formed is negligible. Hence it is assured that product inhibition does not significantly influence the measurements. However, due to the low enzyme concentration, the heat-flow is quite low, especially for reactions with a low enthalpy change, which makes the multiple injection method particularly sensitive to baseline drifts. In the current application where the content in the calorimetric cell is characterized by high protein concentrations, this problem may become accentuated due to side reactions such as chemical modification of labile side chains or protein aggregation. The single injection method is much less prone to uncertainties related to shifts in the baseline levels. This is mainly because this method has a post-reaction baseline and this allows for correction of effects from side reactions which are constant or depend linearly with time. In any event, baseline levels should be checked without enzyme present just injecting the substrate into the reaction media. In the present study a small linear decrease in baseline level was observed at the highest protein concentrations, probably due to protein aggregation in the concentrated media (data not shown). However, this was not a problem for evaluation of kinetic parameters, because it could be readily corrected for by linear interpolation between the pre- and post reaction baseline levels. In all instances, measurements of enzyme kinetics followed Michaeles–Menten kinetics with $r^2 > 0.99$.

4.2. Analysis of experimental results

An explanation of the decrease in K_M with increasing BSA concentration could simply be a decrease in the ratio of activity coefficients between the enzyme–substrate complex and the native enzyme with increasing BSA concentrations (Eq. (5)):

$$E + S \rightleftarrows ES \tag{4}$$

$$K_0 \frac{\gamma_{ES}[ES]}{\gamma_E[E]\gamma_S[S]} \sim \frac{\gamma_{ES}}{\gamma_E} K_c \tag{5}$$

where K_0 is the equilibrium constant of Eq. (4), expressed by activities (a) ($a = \gamma C$), K_C the concentration dependent equilibrium constant expressed in molarities, γ the activity coefficient, and E, S, ES, respectively, the enzyme, substrate and enzyme–substrate complex. Note that γ_S equals 1 since it has been shown, that the activity coefficient does not change significantly for glucose in concentrated PEG solutions [2], which most likely also applies to concentrated protein solutions. To test this, theoretical calculations were performed using the preferential interaction parameter $\Gamma_{\mu glu} = (\partial m_{glu}/\partial m_{BSA})_{\mu glu}$ for glucose in BSA solutions. The interaction parameter of glucose ($\Gamma_{\mu glu}/m_{glu}$) was estimated to be $-15\,m^{-1}$, based on values of trehalose ($-22\,m^{-1}$) and glycerol ($-9\,m^{-1}$) (m is the molal concentration of the carbohydrate) [25]. These calculations suggest that the activity coefficient of glucose should increase 5% when transferred from water to 21 w/w BSA solution. Thus the activity coefficient has been approximated to be constant ($\gamma = 1$) at all BSA concentrations.

A decrease in γ_{ES}/γ_E would be expected, if the volume and thereby the total surface area of the enzyme–substrate complex is smaller than the native enzyme [6]. The activity coefficients of the enzyme and enzyme–substrate complex are most often calculated using statistical mechanics. Such statistical calculations have been performed by Winzor and Wills [21], who have been revising the osmotic stress hypothesis accounting for the increase in binding constant between hexokinase and glucose upon addition of PEG [2,26]. They concluded that a decrease in the activity coefficient of the enzyme–substrate complex compared to the free enzyme, could also account for the increase in binding constant between glucose and hexokinase upon addition of PEG. Using Eq. (6), they calculated the difference in virial coefficients between the enzyme–PEG interaction ($B_{E,M}$) and enzyme–substrate complex–PEG interaction ($B_{ES,M}$):

$$\ln \frac{K}{K_M} = (B_{E,M} - B_{ES,M}) m_M \rho_s \tag{6}$$

where K and K_M are, respectively, the Micaelis–Menten constant in the absence and presence of crowding solutes, m_M the molality of the crowding solute and ρ_S is the density of the solution. This equation predicts a limiting linear dependence of $\ln(K/K_M)$ as a function of $m_M\rho_S$ as long as second virial coefficients are sufficient to describe the non-ideal interactions. If the second virial coefficient of the native enzyme is estimated from the stokes

radius, the second virial coefficient of the enzyme–substrate complex can be calculated. Based on an equation which relates the excluded volume of PEG and a spherical protein with the second virial coefficients, Winzor and Wills [21] calculated the radius of the enzyme–substrate complex. These calculations predicted a 2.5% decrease in surface area of the enzyme–substrate complex compared to the native enzyme. Analogous calculations were performed in this study using Eq. (7) [27], which states that the excluded volume of two spherical molecules is directly proportional to the second virial coefficients of the two molecules as long as electrostatic (specific) interactions do not occur:

$$V_{i,j} \times N_A = \frac{4\pi}{3}(r_i + r_j)^3 N_A = B_{i,j} \quad (7)$$

In Eq. (7) $V_{i,j}$ is the excluded volume of the to spherical molecules, N_A the Avagadros number, r_i and r_j are, respectively, the stokes radii of the two spherical molecules i and j. Using the data from Fig. 3, with the concentration (w/v) of BSA converted into molar concentrations, and stokes radius of BSA and native hexokinase of 3.7 nm [28] and 3.4 nm [21], respectively, the surface area of the enzyme–substrate complex should be 12% less than the native enzyme to account for the decrease in K_M. This calculated decrease in surface area of hexokinase upon binding of glucose seems to be very high, and is probably overestimated due to electrostatic interactions (neglected in Eq. (7)) between hexokinase and BSA, which both carry net negative charges at pH 7.0. To obtain a better estimate charge-charge contributions needs to be added to Eq. (7) using the Debye–Hückel terminology as discussed in [13]. This analysis, however, is beyond the current scope and probably also requires systematic studies of the reaction at different pH-values (i.e. different protein net charges). Another potential problem using this analysis is the assumption that K_0 (the dissociation constant) equals K_M, where K_M is defined as $(k_2 + k_{-1})/k_1$. Here k_1 and k_{-1} represent the rate constants for the forward and reverse reaction of the binding step of glucose and k_2 equals k_{cat} which is the 1. order rate constant for the breakdown of the enzyme–product complex. K_0 equals K_M in the range where $k_{-1} \gg K_2$. Considering the results of Hoggett and Kellett where k_{-1} was measured to be $1100\,s^{-1}$, k_{cat} might have an influence on K_M. This is not considered using only changes in activity coefficient on estimating the effect of crowding on K_M. In contrast to the above observation the group of Rand found that there was no difference between K_0 and K_M using PEG as crowding agent [2]. If k_{-1} is assumed to be $1100\,s^{-1}$ in the present study, this would result in a difference between K_M and K_0 of 25%. Furthermore K_M would decrease 10% from 0 to 250 mg ml^{-1} BSA due to the decrease in k_{cat}. Nevertheless, it is concluded that the current analysis shows the same trends concerning the effect of crowding on the binding constant between glucose and hexokinase as in the study of Winzor and Wills [21].

The decrease observed in k_{cat}, when increasing the BSA concentration is most likely linked to a decrease in the diffusion rate of product from the enzyme–product complex, and the conformational changes and rehydration that accompanies when hexokinase returns to the substrate free conformation. Such a decrease in k_{cat} is expected with macromolecular crowding but was not observed when PEG was used as a crowding component [2]. It cannot be excluded that direct protein interactions can contribute the observed decrease in k_{cat}, but the effect of the excluded volume should nevertheless increase the binding constant since hexokinase undergoes a significant reduction in the hydrodynamic volume upon binding of glucose.

The specificity constant k_{cat}/K_M was found to be constant over the entire concentration range of BSA at a value of $2.2 \times 10^6\,l\,mol^{-1}\,s^{-1}$ which is close to the binding rate of glucose at $2 \times 10^6\,l\,mol^{-1}\,s^{-1}$ found by Hogget and Kellett [20]. This binding rate is 2 orders of magnitude lower than what would be expected from a diffusion limited reaction. Thus the invariance of k_{cat}/K_M may reflect that the reaction is limited by the binding rate of glucose.

The reason for the decrease in ΔH_r with increasing protein concentrations is not known. It may rely on changes in hydration from substrate to product when increasing the BSA concentration. Further investigation of this should include measurements of heat of solvation for both reactants and products.

It is interesting to note, that hexokinase is a homodimer, where the enzyme in solution exists in equilibrium between monomers and dimers [29]. It should be expected, that macromolecular crowding would result in a shift in this equilibrium towards dimers [5]. However, since the K_M for the dimer (P2) form of hexokinase is 10-fold higher than the monomeric form [30], and since a decrease in K_M with increasing BSA concentrations was observed, it is concluded that the effect on enzyme kinetics by macromolecular crowding is not governed by perturbation of the association-dissociation equilibrium. To investigate this further, experiments where the substrate concentration was raised 10-fold compared to the K_M of the monomer were performed (data not shown). No further increase in activity at these high substrate concentrations was found. This suggest that hexokinase is predominantly monomeric within solutions crowded to the same extent comparable to the cytosol in yeast.

5. Conclusion

In conclusion it has been demonstrated that ITC is an adequate technique for the investigation of enzyme kinetics in complex reaction media, including solutions mimicking *in vivo* conditions. Further systematic work along these lines appears to have a significant potential to fill some of these experimental gaps within this area [13]. This includes both empirical investigations of enzyme kinetics under specific conditions and experimental back-up for the evolving theoretical treatise of biomolecules in crowded systems.

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References

[1] G.D. Dzingeleski, R. Wolfenden, Hypersensitivity of an enzyme reaction to solvent water, Biochemistry 32 (1993) 9143–9147.

[2] R.P. Rand, N.L. Fuller, P. Butko, G. Francis, P. Nicholls, Measured change in protein solvation with substrate binding and turnover, Biochemistry 32 (1993) 5925–5929.

[3] R.J. Ellis, Macromolecular crowding: obvious but underappreciated, Trends Biochem. Sci. 26 (2001) 597–604.

[4] S.B. Zimmermann, A.P. Minton, Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of *Escherichia coli*, J. Mol. Biol. 222 (1991) 599–620.

[5] A.P. Minton, J. Wilf, Effect of macromolecular crowding upon the structure and function of an enzyme: glyceraldehyde-3-phosphate dehydrogenase, Biochemistry 20 (1981) 4821–4826.

[6] A.P. Minton, Molecular crowding: analysis of effects of high concentrations of inert cosulutes on biochemical equilibria and rates in terms of volume exclusion, Meth. Enzymol. 295 (1998) 127–149.

[7] A.P. Minton, The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media, J. Biol. Chem. 276 (2001) 10577–10580.

[8] A.E. Beezer, T.I. Steenson, H.J.V. Tyrrell, Application of flow-microcalorimetry to analytical problems. II. Urea–urease system, Talanta 21 (1974) 467–474.

[9] H.D. Watt, Microcalorimetric procedure for evaluating the kinetic parameters of enzyme-catalyzed reactions: kinetic measurements of the nitrogenase system, Anal. Biochem. 187 (1990) 141–146.

[10] P.E. Morin, E. Freire, Direct calorimetric analysis of the enzymatic activity of yeast cytochrome C oxidase, Biochemistry 30 (1991) 8494–8500.

[11] B.A. Williams, E.J. Toone, Calorimetric evaluation of enzyme kinetic parameters, J. Org. Chem. 58 (1993) 3507–3510.

[12] M.J. Todd, J. Gomez, Enzyme kinetics determined using calorimetry: a general assay for enzyme activity? Anal. Biochem. 296 (2001) 179–187.

[13] T.G.A. Lonhienne, D.J. Winzor, A potential role for isothermal calorimetry in studies of the effects of thermodynamic non-ideality in enzyme-catalyzed reactions, J. Mol. Recogn. 17 (351) (2004) 361.

[14] T.G.A. Lonhienne, D.J. Winzor, Calorimetric demonstration of the potential of molecular crowding to emulate the effect of an allosteric activator on pyrovate kinase kinetics, Biochemistry 41 (2002) 6897–6901.

[15] T.G.A. Lonhienne, P.E.B. Reilly, D.J. Winzor, Further evidence for the reliance of catalysis by rabbit muscle pyruvate kinase upon isomerization of the ternary complex between enzyme and products, Biophys. Chem. 104 (2003) 189–198.

[16] L. Sica, R. Gilli, C. Briand, J.C. Sari, A flow microcalorimetric method for enzyme activity measurements: application to dihydrofolate reductase, Anal. Biochem. 165 (1987) 341–348.

[17] T. Lonhienne, E. Baise, G. Feller, B. Vassilis, C. Gerday, Enzyme activity determination on macromolecular substrates by isothermal titration calorimetry: application to mesophilic and psychrophilic chitinases, 1545 (2001) 349-356, in press.

[18] J.J. Schmidt, S.P. Colowick, Chemistry and subunit structure of yeast hexokinase isoenzymes, Arch. Biochem. Biophys. 158 (1973) 458–470.

[19] H.J. Fromm, V. Zewe, Kinetic studies of yeast hexokinase, J. Biol. Chem. 237 (1962) 3027–3032.

[20] J.G. Hoggett, G.L. Kellett, A fluorescence temperature-jump study of the kinetics of the binding of glucose to the monomer forms of yeast hexokinases P-1 and P-2, Eur. J. Biochem. 68 (1976) 347–353.

[21] D.J. Winzor, P.R. Wills, Thermodynamic nonideality of enzyme solutions supplemented with inert solutes: yeast hexokinase revisited, Biophys. Chem. 57 (1995) 103–110.

[22] R.P. Rand, V.A. Parsegian, D.C. Rau, Intracellular osmotic action, Cell. Mol. Life Sci. 57 (2000) 1018–1032.

[23] S.N. Timasheff, In disperse solution "osmotic stress" is a restricted case of preferential interactions, PNAS 95 (1998) 7363–7367.

[24] S.N. Timasheff, Protein-solvent preferential interactions, protein hydration, and the modulation of biochemical reactions by solvent components, PNAS 99 (2002) 9721–9726.

[25] E.S. Courtenay, M.W. Capp, C.F. Anderson, M.T. Record Jr., Vapor pressure osmometry studies of osmolyte-protein interactions: implications for the action of osmoprotectants *in vivo* and for the interpretation of "osmotic stress" experiments in vitro, Biochemistry 39 (2000) 4455–4471.

[26] C. Ried, R.P. Rand, Probing protein hydration and conformational states in solution, Biophys. J. 72 (1997) 1022–1030.

[27] A.G. Ogston, On the interaction of solute molecules with porous networks, J. Phys. Chem. 74 (1970) 668–669.

[28] S. Tayyab, M.A. Qasim, Influence of succinylation of bovine serum albumin on its conformation and bilirubin binding, Biochim. Biophys. Acta 913 (1987) 359–367.

[29] J.S. Easterby, M.A. Rosemeyer, Purification and subunit interactions of yeast hexokinase, Eur. J. Biochem. 28 (1972) 241–252.

[30] F. Womack, S.P. Colowick, Catalytic activity with associated and dissociated forms of the yeast hexokinases, Arch. Biochem. Biophys. 191 (1978) 742–747.