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Differential scanning calorimetric studies on binding of N-acetyl-D-glucosamine to lysozyme

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Abstract

Differential scanning calorimetric (DSC) measurements were performed on the thermal denaturation of lysozyme and lysozyme complexed with N-acetyl-D-glucosamine (GlcNAc) at pH 5.00 (acetate buffer), 4.25 and 2.25 (Gly–HCl buffer). DSC data have been analyzed to obtain denaturation temperature T_d, enthalpy of denaturation ΔH_d, heat capacity of denaturation ΔC_{pd} and cooperativity index η. From these thermodynamic parameters, the binding constant K_L and enthalpy of binding ΔH_L, for the weak binding of lysozyme with GlcNAc have been determined. The values of K_L and ΔH_L at pH 5.00 and 298 K are 42 ± 4 M^{-1} and -24 ± 4 kJ mol^{-1}, respectively, and agree very well with the experimentally determined values from equilibrium and other studies. The binding constant has also been estimated by simulating the DSC curve with varying values of K_L (T_d) until it matches the experimental curve.

Keywords: Binding constant; Thermal denaturation; Differential scanning calorimetry (DSC); N-Acetyl-D-glucosamine; Lysozyme

1. Introduction

The stability of the native globular conformation of a protein is a delicate balance between various interactions. The free energy of stabilization [1] is only 20–60 kJ mol^{-1}. The stability of proteins is affected by the concentration of small molecules such as substrates, coenzymes, inhibitors and activators which bind specifically to the native state [2–4]. Studies on the specific binding of low-molecular-weight compounds to proteins form one of the most active fields of biochemical thermochemistry today. Such studies contribute towards an understanding of the reactions taking place at the active sites of proteins [5].

The determination of the binding constant is therefore important in evaluating the effect of the substrate on enzyme activity, and also, in predicting the stabilization of enzymes by substrates [6]. Various methods [7–15] such as UV difference spectroscopy, NMR, circular dichroism, fluorescence, dialysis and solubility, calorimetry and optical rotatory dispersion techniques have been employed to evaluate the binding constant for the enzyme–substrate reactions. Methods have been described [10–18] for estimating binding constants from thermal data but very few studies have been made to characterize the effect of substrate concentration on protein stability and protein–ligand interaction [19–21] us-

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ing differential scanning calorimetry (DSC). The DSC method of evaluating the binding constant involves model-based assumptions [18]. It is an indirect method since the binding constants are estimated by observing the effect of ligand concentration on the midpoint of the unfolding thermal transition. This method of estimating the binding constant from DSC data has proved to be very efficient for very strong interactions of enzymes and substrates [21]. The aim of the present investigation is to test the efficiency of the DSC method for small binding constant reactions. Hence the present work involves the determination of the binding constant and the binding enthalpy of N-acetyl-D-glucosamine with lysozyme using DSC, and a comparison of the values obtained with those obtained using other methods [7–15].

Analysis of DSC data gives, the cooperativity index η, the denaturation temperature T_d, the enthalpy of denaturation ΔH_d and the heat capacity of denaturation ΔC_{pd}, of lysozyme in an uncomplexed and a complexed form. It is assumed that the protein binds in a 1 : 1 ratio with the inhibitor only in the native state.

2. Experimental

Hen-egg white lysozyme (Type L6876) and N-acetyl-D-glucosamine (Type AB625) were purchased from Sigma Chemical Co. N-Acetyl-D-glucosamine of a specified purity of 99% was dried under vacuum over P_2O_5 for 72 h prior to use. The lysozyme had a stated purity of 95%. The protein sample was exhaustively dialysed against double-distilled water at 277 K for 24 h to remove salts/ions. The protein solution was lyophilized and dried under vacuum prior to use. Millimolar solutions of lysozyme were prepared by dissolving lysozyme in 0.1 M sodium acetate–acetic acid solutions buffered at pH 5.00. A few experiments were also carried out at pH 2.25 and 4.25 using 0.2 M Gly–HCl buffer.

Protein concentration was determined spectrophotometrically on a Perkin-Elmer Lambda 3B spectrophotometer. For UV spectroscopic analysis, the protein solutions were diluted to a concentration of 0.1 mass-% with a measured volume of 0.2 M Na_2HPO_4/NaH_2PO_4 buffer at pH 7.00. The optical density was measured at 279 nm for lysozyme and

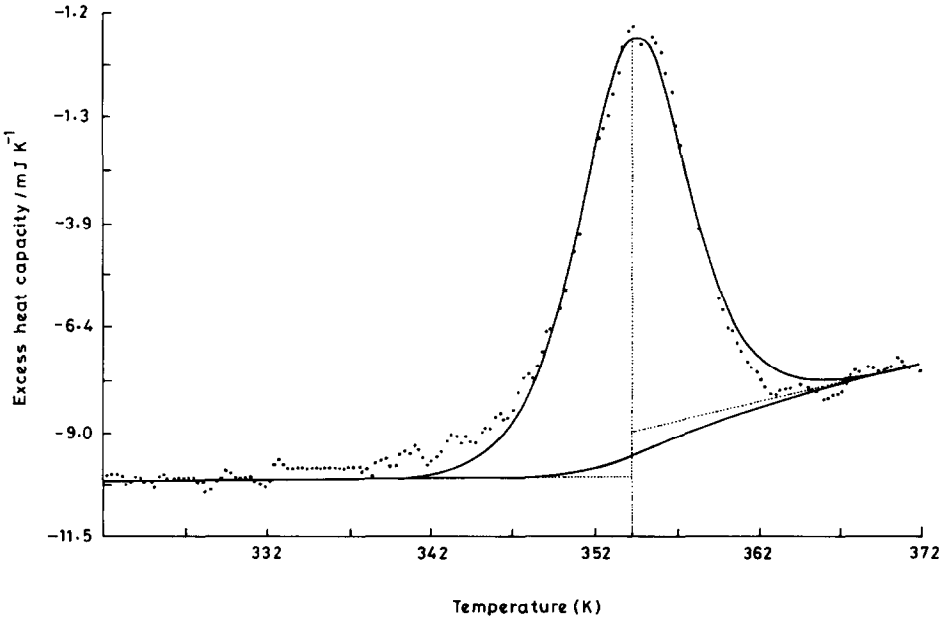

Fig. 1. DSC thermal scan of lysozyme in 0.56 M GlcNAc at pH 5.00 (0.1 M acetate buffer). T_d = 354 K. ΔH = 566 kJ mol^{-1}.

Table 1
Thermodynamic parameters [a] obtained from DSC scans of lysozyme in the presence of varying concentrations of GlcNAc at pH 5.00 (0.1 M acetate buffer)

GlcNA (M)	T_d (K)	ΔT_d (K)	ΔH_d (kJ mol^{-1})	ΔC_{pd} (kJ K^{-1} mol^{-1})	$K_L(T_d)$ (M^{-1})	$K_L(298)$ (M^{-1})	$\Delta H_L(T_0)$ (kJ mol^{-1})
0	350.2	–	510	6.1 ± 0.7	–	–	–
0.0123	350.4	0.2	523	6.1 ± 0.3	7.3	42.5	–
0.0250	350.5	0.3	535	5.8 ± 0.8	7.0	41.0	−23
0.0525	350.8	0.6	542	4.9 ± 0.7	7.0	41.3	−28
0.1097	351.3	1.1	552	5.5 ± 0.2	6.5	39.1	−35
0.1606	351.7	1.5	557	6.8 ± 0.2	6.8	41.7	−37
0.2108	352.0	1.8	558	5.5 ± 0.2	6.9	42.6	−36
0.310	352.5	2.3	559	4.9 ± 0.8	6.9	43.4	−35
0.442	353.0	2.8	563	5.3 ± 0.7	7.1	45.7	−35
0.5565	353.5	3.3	565	6.4 ± 0.6	7.6	49.4	−35
0.6555	353.8	3.6	567	5.6 ± 0.8	8.0	52.7	−34
0.7580	354.2	4.0	569	6.2 ± 0.4	8.8	58.7	−38

[a] Maximum estimated errors in the various thermodynamic parameters are $T_d = \pm 0.3$ K, $\Delta H_d = \pm 15$ kJ mol^{-1} and $\Delta C_{pd} = \pm 1.0$ kJ K^{-1} mol^{-1}.

the concentration was determined using a value of extinction coefficient $\epsilon_{279} = 2.635$ [22]. Solutions of substrate–lysozyme complex were prepared by dissolving a known mass of N-acetyl-D-glucosamine in a sample of the dialysed lysozyme solution.

Calorimetric measurements were carried out on a Micro DSC-Batch Differential Scanning Calorimeter, (Setaram, France). The DSC was operated at a scan rate of 0.6 K min^{-1} from 298 to 371 K with a sample mass of approximately 0.85 g. For each protein concentration at least three experiments were performed. To analyse a DSC experiment, the instrumental baseline determined with buffer in both cells was subtracted from the results obtained with the sample. All the excess power thermal scans were converted to excess heat capacity vs. temperature scans following the procedure described by Schwarz and Kirchhoff [23]. Thermodynamic functions of protein denaturation, the transition temperature T_d, heat capacity ΔC_{pd} and the enthalpy ΔH_d of denaturation of lysozyme in buffer solution (pH 5.0, 4.25, 2.25) and N-acetyl-D-glucosamine solutions were determined by the least-squares fit of the excess heat capacity data to the two-state model [23].

The two-state model provides a functional dependence of the measured heat capacity on temperature according to the following form:

$$C_{PS} = a + b(T - T_d) + \alpha[\Delta a + \Delta b(T - T_d)]$$

where $a + b(T - T_d)$ is the linear fit of the pre-tran-

Table 2
Thermodynamic parameters [a] obtained from DSC scans of lysozyme [b] in the presence of varying concentrations of GlcNAc at pH 4.25 (0.2 M Gly–HCl buffer)

GlcNAc (M)	T_d (K)	ΔT_d (K)	ΔH_d (kJ mol^{-1})	ΔC_{pd} (kJ K^{-1} mol^{-1})	$K_L(T_d)$ (M^{-1})	$K_L(298)$ (M^{-1})
0	351.5	–	524	6.3 ± 0.5	–	–
0.0232	351.8	0.3	527	4.3 ± 1.0	7.8	48.4
0.1013	352.7	1.3	536	5.9 ± 0.4	8.4	53.9
0.251	353.7	2.2	541	5.3 ± 0.9	9.3	61.6
0.5308	355.0	3.5	546	6.4 ± 0.5	9.7	67.3
0.7498	355.5	4.0	561	7.2 ± 1.1	9.2	64.9

[a] Maximum estimated errors in the various thermodynamic parameters are $T_d = \pm 0.3$ K, $\Delta H_d = \pm 15$ kJ mol^{-1} and $\Delta C_{pd} = \pm 1.0$ kJ K^{-1} mol^{-1}.
[b] Lysozyme concentration 0.14 mM.

sition baseline to T and $\Delta a + \Delta b(T - T_d)$ is the difference between the linear extrapolation of the pre- and post-transitional baselines at T. C_{Ps} is the sigmoidal baseline extrapolated under the transition curve at T and α is the fractional area under the transition curve at T. The transition enthalpy H_d is determined from the area under the transition curve and from the number of moles of lysozyme in the cell.

3. Results and discussion

The thermal denaturation curve for lysozyme in the presence of 0.56 M GlcNAc is shown in Fig. 1. Thermodynamic parameters: denaturation temperature T_d, enthalpy of denaturation ΔH_d and heat capacity of denaturation ΔC_{pd} for lysozyme–GlcNAc systems at pH 5.0, 4.25 and 2.25 obtained from the DSC analysis are listed in Tables 1, 2 and 3, respectively. The maximum estimated error in T_d, ΔH_d and ΔC_{pd} values are ± 0.3 K, ± 15 kJ mol^{-1} and 1.0 kJ K^{-1} mol^{-1}, respectively. The cooperativity η, which is the ratio of the transition enthalpy to the van't Hoff enthalpy for each measurement was determined by the approximate relation $\eta = \Delta H_d^2 / 4RT_d^2 C_d$, where C_d is the height of the transition peak per mole of protein at T_d. The value of η was found to be 1.051 ± 0.031. On reheating, the already scanned solutions of complexed and uncomplexed lysozyme show that the transitions are reversible. The denaturation transition of lysozyme in N-acetyl-D-glucosamine solutions is thus a two-state transition with a stoichiometry of one. The transition temperature T_d and enthalpy of denaturation ΔH_d and heat capacity of denaturation ΔC_{pd} are found to be 350.2 K, 510 kJ mol^{-1} and 6.1 kJ mol^{-1} K^{-1}, respectively, which are close to the values reported in the literature [24,25].

The native conformation of lysozyme has a specific binding site for GlcNAc [26] and the equilibria expected to exist are:

$$PL \underset{-L}{\overset{+L}{\rightleftharpoons}} P \rightleftharpoons P'$$

The binding constant

$$K_L = \frac{[PL]}{[P][L]}$$

where P is the protein in native state, L is the ligand, PL is the protein ligand complex, and P′ is the protein in denaturated state.

When the concentration of P + PL increases relative to the concentration of P′, the protein is by definition more stable. The results in Tables 1–3 indicate that increasing the GlcNAc concentration leads to an increase in T_d and hence in the stability of lysozyme. The magnitude of the stabilizing effect (ΔT_d) of GlcNAc increases with a decrease in pH.

Apart from binding and the formation of a stable lysozyme–GlcNAc complex, which leads to the enhancement of thermal stability, the other factor involved is possibly the structure-stabilizing ability of GlcNAc. This may be attributed to the enhanced water structure in the neighbourhood of the protein

Table 3
Thermodynamic parameters [a] obtained from DSC scans of lysozyme [b] in the presence of varying concentrations of GlcNAc at pH 2.25 (0.2 M Gly–HCl buffer)

GlcNAc (M)	T_d (K)	ΔT_d (K)	ΔH_d (kJ mol^{-1})	ΔC_{pd} (kJ K^{-1} mol^{-1})	$K_L(T_d)$ (M^{-1})	$K_L(298)$ (M^{-1})	$\Delta H_L(T_0)$ (kJ mol^{-1})
0	333.0	–	398	6.2 ± 0.4	–	–	–
0.0235	333.3	0.3	403	6.3 ± 0.6	5.9	19.3	–
0.1030	334.3	1.8	450	4.9 ± 1.1	11.9	40.2	−41
0.2501	337.3	4.3	467	4.9 ± 1.2	22.0	82.5	−42
0.4987	340.8	7.8	478	5.1 ± 1.7	63.1	267.3	−36
0.7497	342.0	9.0	496	6.3 ± 1.4	74.0	326.7	−42
0.9969	343.2	10.2	508	5.4 ± 1.4	99.4	457.8	−46

[a] Maximum estimated errors in the various thermodynamic parameters are $T_d = \pm 0.3$ K, $\Delta H_d = \pm 15$ kJ mol^{-1} and $\Delta C_{pd} = \pm 1.1$ kJ K^{-1} mol^{-1}.
[b] Lysozyme concentration 0.14 mM.

which contributes to protein stability, as explained [27,28], for the stabilizing effect of glycerol and sucrose on the protein's thermal stability. Though the concentrations of GLcNAc used in the present work are not as high, the trend does indicate that the effect of water structure enhancement on the thermal stability of lysozyme probably becomes more prominent at higher concentrations of GlcNAc (> 0.3 M) leading to the observed large T_d values.

3.1. Binding enthalpy, ΔH_L

The binding enthalpy has been estimated from the following relation:

$$\Delta H_d(T_d) = \Delta H_d(T_0) + \Delta C_{pd}(T_d - T_0) - \Delta H_L(T_0)$$

where $\Delta H_d(T_0)$ and $\Delta H_d(T_d)$ are the enthalpies of denaturation of lysozyme in the absence and presence of GlcNAc, respectively, and ΔC_{pd} is the heat capacity of denaturation of lysozyme. The values of $\Delta H_L(T_0)$ thus calculated at pH 5.00 and 2.25 are given in Tables 1 and 3, respectively. The average value of $\Delta H_L(T_0)$ calculated is -35 ± 4 kJ mol^{-1} if the first three values at low concentrations of ligand are ignored.

Using this value of $\Delta H_L(T_0)$ and the reported value [7] of ΔC_{pL} (-220 J K^{-1} mol^{-1}) and T_0 as 350.2 K the value of $\Delta H_L(298)$ at pH 5.00, calculated using the following relation:

$$\Delta H_L(298) = \Delta H_L(T_0) + \Delta C_{PL}(298 - T_0)$$

is -24.0 ± 4 kJ mol^{-1}. This value agrees very well with the value reported by Wadsö and his coworkers (-24.7 kJ mol^{-1}) [7], using titration calorimetry. Literature values of ΔH_L are not available at pH 4.25 and 2.25 for comparison.

The binding constants $K_L(T_d)$ of lysozyme complexed with GlcNAc at pH 2.25, 4.25 and 5.0 are evaluated from Eq. (1), derived by Brandts and Lin [21].

$$K_L(T_d) = \exp\{-\Delta H_d(T_0)/R[1/T_d - 1/(T_0) + \Delta C_{pd}/R(\ln T_d/T_0 + T_0/T_d - 1)] - 1\} / [L]_{T_d} \quad (1)$$

where R = 8.314 J K^{-1} mol^{-1}, T_0 is the transition temperature for lysozyme in the uncomplexed form, T_d is the transition temperature for lysozyme in the complexed form, $\Delta H_d(T_0)$ is the enthalpy of denaturation of lysozyme in the uncomplexed form, ΔC_{pd} is the heat capacity of the solution upon denaturation, $[L]_{T_d}$ is the free inhibitor concentration at T_d, and

$$[L]_{T_d} = L_t - P_t/2 \quad \text{if } L_t \geq P_t$$

where L_t is the total ligand concentration, and P_t is the total protein concentration.

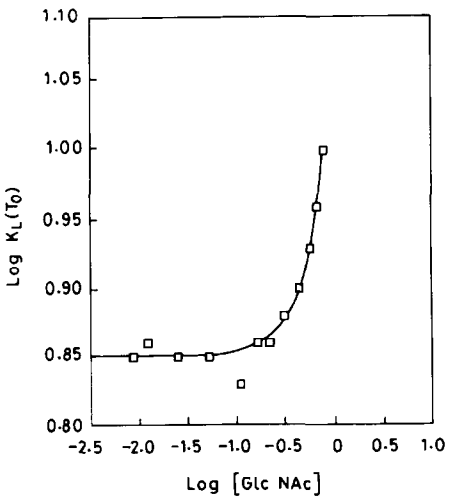

Fig. 2. Variation of binding constant $K_L(T_0)$ with GlcNAc at pH 5.00 (0.1 M acetate buffer).

The values of $K_L(T_d)$, which are listed in Table 1 are found to be small, indicating the weak binding of GlcNAc to lysozyme. The error analysis indicates a maximum of 10% error in the value of the binding constant $K_L(T_d)$.

The calculated values of $K_L(T_d)$, at each T_d, as a function of [GlcNAc] (the free ligand concentration), are shown in a log–log plot in Fig. 2. It can be seen that the apparent value of the binding constant is almost constant up to about 0.3 M of [GlcNAc] and it increases thereafter. The limiting value of $K_L(T_d)$ is found to be 7.0 ± 0.4 M^{-1}. At higher concentrations, the binding constant keeps on increasing. This reiterates that at higher concentrations, the effect of GlcNAc on the solvent structure becomes more pronounced and stabilizes the native state.

The magnitude of binding of GlcNAc to lysozyme decreases with an increase in temperature. The effect of pH on $K_L(T_d)$ may be seen from results in Tables 1–3. The values of $K_L(T_d)$ obtained at pH 4.25 are

nearly the same as those estimated at pH 5.00. But the data at pH 2.25 indicate a much large value of $K_L(T_d)$. This pH dependence may be attributed to the ionic effect on the pK_a of the ionizable groups involved in the binding.

The denaturation temperature of lysozyme in the absence of GlcNAc, T_0, is taken as the reference temperature and the binding constant at temperature T_0, $K_L(T_0)$ for each scan has been calculated using Eq. (2) where T is substituted by T_0:

$$K_L(T_0) = K_L(T_d)\ \exp[-\Delta H_L/R(1/T_0 - 1/T_d) + \Delta C_{PL}/R(\ln T_0/T_d + 1 - T_0/T_0)]$$

where $T_0 = 350.2$ K, $\Delta H_L(T_0) = -35$ kJ mol^{-1}, and $\Delta C_{PL} = -220$ J K^{-1} mol^{-1} (ref. [7]).

ΔC_{PL}, the value of the heat capacity of binding, is taken from the literature since DSC data does not give precise values of ΔC_{PL} [21]. The average value of the binding constant at T_0 the denaturation temperature of lysozyme, $K_L(T_0)$ was 7.2 ± 0.4 M^{-1}.

If the heat capacity, ΔC_{PL}, and the enthalpy, ΔH_L, of binding at temperature T are known, then $K_L(T)$ the binding constant at temperature T can be obtained from $K_L(T_d)$, the binding constant at temperature T_d according to the following equation [21]:

$$K_L(T) = K_L(T_d)\ \exp[-\Delta H_L(T)/R(1/T - 1/T_d) + \Delta C_{PL}/R(\ln T/T_d + 1 - T/T_d)] \quad (2)$$

The binding constant at 298 K, is calculated using Eq. (2) and substituting 298 K for 'T'. The values of the binding constant K_L at 298 K are listed in Table 1.

We observe that the estimated average value of the binding constant at 298 K for lysozyme–GlcNAc complex at pH 5.00 is 42 ± 4 M^{-1} up to a 0.3-M concentration of GlcNAc. This value is compared with the reported values obtained from different methods in Table 4. There is a good agreement of $K_L(298)$ at pH 5.0 (obtained from isothermal calorimetry, NMR, UV and CD methods) with our estimate from DSC data. This shows that DSC can also be used to estimate the binding constant at T_d within an error of 10% for a weak binding system such as lysozyme with GlcNAc.

3.2. Simulation method for the estimation of K_L

A method has also been described in the literature [21], to simulate the DSC curves in order to estimate the binding constant and binding heat. In this method using the experimentally determined input parameters T_0, $\Delta H_d(T_0)$, ΔC_{pd} at T_0, ΔC_{PL}, ΔH_L, P_t and L_t, and an assumed value of $K_L(T_0)$; the concentrations of free ligand GlcNAc [L], lysozyme [P], denatured lysozyme [P'] and the lysozyme–GlcNAc complex [PL] are determined at any temperature T using the set of equations as described by Brandts and Lin [21]. Excess enthalpy is then calculated using the following equation:

$$H_{xs}(T) = [P']/P_t[\Delta H_d(T_0)] + \Delta C_{Pd}(T - T_0) + [PL]/P_t[\Delta H_L(T_0) + \Delta C_{PL}(T - T_0)] \quad (3)$$

The value of ΔH_L (-35 kJ mol^{-1}) was used which was estimated earlier. The value of ΔC_{PL} (-220 J K mol^{-1}) was taken from the literature [22].

Table 4
Comparison of the estimated binding constant from DSC for the lysozyme-GlcNAc system with literature values

Method	pH	T (K)	Ionic strength in buffer	K_L (M^{-1})	Ref. No.
DSC	5.0	298	0.1 acetate	42 + 4	Present work
	5.0	350	0.1 acetate	7.1	Present work
Isothermal calorimetry	5.0	298	0.1 acetate	39	[7]
UV	5.0	301	0.11	17–25	[9]
Dialysis and solubility	5–5.3	298	Crystalline protein 5% NaCl	46–63	[10]
ORD	5.4–6.1	304	0.1 M NaCl	60	[11]
NMR	5.5	298	0.1 M citrate	40	[12]
UV	5.3	298	0.1	42	[13]
CD	5.5	298	0.1	40	[14]
FI	5.4	298	0.1 M NH_4Ac	40	[15]

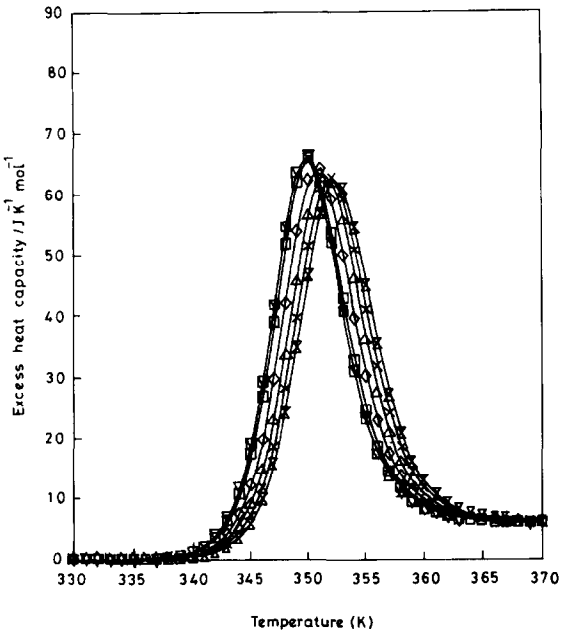

Fig. 3. Simulated DSC curves at 0.21 M GlcNAc (pH 5.00, 0.1 M acetate buffer), at different $K_L(T_0)$ values: (▽) 1, (□) 10, (◇) 50, (△) 100, (×) 150, (⧖) 200; and (⊠) experimental curve.

The excess enthalpy was numerically differentiated to obtain the DSC parameter excess heat capacity C_{Pxs},

$$C_{Pxs}(T) = \frac{dH_{xs}}{dT}$$

The DSC curve is then simulated using C_{Pxs} as a function of temperature. The simulated curve is then analysed to obtain the T_d and $\Delta H_d(T_d)$ values using the two-state model with various values of $K_L(T_0)$ (1–200) until the simulated curve matches with the experimental curve in terms of T_d and $\Delta H_d(T_d)$ [21]. The experimental curve was found to be closest to the simulated curves with $K_L(T_0)$ ranging between 1 and 10 (Fig. 3). The average value of 5 M^{-1} compares well with the values of $K_L(T_0)$ of 7.1 M^{-1} and 6.35 M^{-1} calculated from DSC data using Eq. (2) and extrapolating the value of Wadsö and coworkers [22] for 298 K to T_0, respectively. This indicates that the simulation method can be used to obtain reasonable estimates of the binding constant values of the weak binding systems.

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