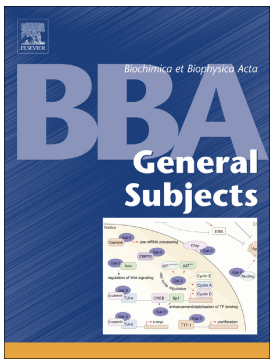

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INFLUENCE OF CYTOSOLIC CONDITIONS ON THE REACTION EQUILIBRIUM AND THE REACTION ENTHALPY OF THE ENOLASE REACTION ACCESSED BY CALORIMETRY AND VAN 'T HOFF

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1.1 Abstract

Background: Thermodynamic methods are finding more and more applications in systems biology, which attempts to understand cell functions mechanistically. Unfortunately, the state variables used (reaction enthalpy and Gibbs energy) do not take sufficient account of the conditions inside of cells, especially the crowding with macromolecules.

Methods: For this reason, the influence of crowding agents and various other parameters such as salt concentrations, pH and temperature on equilibrium position and reaction enthalpy of the glycolytic example reaction 9 (2-Phospoglycerate -> Phosphoenolpyruvate + H_2O) was investigated. The conditions were chosen to be as close as possible to the cytosolic conditions. Poly(ethylene glycol) MW = 20,000 g mol^{-1} (PEG 20,000) was used to analyze the influence of crowding with macromolecules. The equation of state electrolyte Perturbed-Chain Statistical Associating Fluid Theory (ePC-SAFT) was applied to consider the influence of crowding agents on the reaction equilibria.

Results and Conclusions: For the reaction enthalpies and for the equilibria, it was found that the influence of salts and temperature is not pronounced while that of pH and PEG 20,000 concentration is considerable. Furthermore, it could be shown that under identical measurement conditions there are no differences between the van 't Hoff and the calorimetrically determined reaction enthalpy.

General Significance: The results show how important it is to consider the special cytosolic conditions when applying thermodynamic data in systems biology.

Keywords: Biothermodynamics; Glycolysis; Macromolecular crowding; isothermal titration calorimetry (ITC); ePC-SAFT; Enolase

1.2 Symbols

Greek letters

Symbol	Property	Unit
$\varepsilon^{A_iB_i}/k_B$	association-energy parameter	K
γ_i^m	generic activity coefficient of component i on molality base	(kg water)·mol^{-1}
$\gamma_i^{*,m}$	rational activity coefficient of component i on molality base	-
$\gamma_i^{\infty,m}$	generic activity coefficient of component i at infinite dilution on molality base	(kg water)·mol^{-1}
κ^{AiBi}	association-volume parameter	-
σ_i	segment diameter of component i	Å
ν_i	stoichiometric coefficient of component i	-
χ_i	arbitrary concentration measure	-

Latin letters

Symbol	Property	Unit
a_i	activity of component i	-
$\Delta^R c_p$	heat capacity of biochemical reaction	J·mol^{-1}·K^{-1}
$\Delta^R \mathrm{g}$	Gibbs energy of biochemical reaction	J·mol^{-1}
$\Delta^R g'^0$	standard Gibbs energy of biochemical reaction	J·mol^{-1}
$\Delta^R h$	enthalpy of biochemical reaction	J·mol^{-1}
$\Delta^R h'^{0,van\,'t\,Hoff}$	standard enthalpy of biochemical reaction	J·mol^{-1}
$\Delta^R h'^{,cal}$	calorimetric enthalpy of biochemical reaction	J·mol^{-1}
$\Delta^R h'^{,van\,'t\,Hoff}$	van 't Hoff enthalpy of biochemical reaction	J·mol^{-1}
k_B	Boltzmann constant (1.38·10^{-23}·m^2·kg·s^{-2}·K^{-1})	J·K^{-1}
k_{ij}	binary interaction parameter of components i and j	-
K_a'	thermodynamic equilibrium constant of biochemical reaction	-
K_γ'	activity-coefficient ratio of biochemical reaction	(kg water)·mol^{-1}

K'_m	apparent equilibrium-molality ratio of biochemical reaction	mol·(kg water)$^{-1}$
m_i	molality of component i	mol·(kg water)$^{-1}$
m_i^0	initial molality of component i	mol·(kg water)$^{-1}$
m_i^{eq}	molality of component i at equilibrium	mol·(kg water)$^{-1}$
m_i^{seg}	segment number of component i	-
N_i^{assoc}	number of association sites of component i	-
z	valence of an ion	-
R	ideal gas constant (8.314 J·mol^{-1}·K^{-1})	J·mol^{-1}·K^{-1}
T	temperature	K
u_i/k_B	dispersion-energy parameter of component i	K
x_i	mole fraction of component i	-

1.3

1.4 Introduction

Biotechnology benefits on the one hand from the unique properties of microbial cells (complex single-step reactions, high stereo and regioselectivity etc.) but suffers on the other hand from the fact that these cells often behave non-linearly and are difficult to predict [1]. Systems biology tries to remedy this shortcoming by developing models for the underlying metabolic networks. These models are increasingly integrating the thermodynamic state variable of individual reactions and reaction cascades [2, 3] to improve the predictive power of metabolic models. The Gibbs energy for instance is the driving force for biological reactions and determines whether biological reactions or reaction cascades are thermodynamically feasible or not [4-7]. Dynamic balances of the reaction enthalpy allow the stoichiometry of bioprocesses to be determined in real time and these processes to be controlled accordingly [8]. In addition, accurate enthalpies are a prerequisite to describe the temperature dependence of reaction equilibria via the van 't Hoff equation. Unfortunately, the literature values for both the Gibbs energy and the enthalpy are on the one hand very different and contradictory, and on the other hand the measurement conditions are often poorly described by the authors. Finally, literature values for both state variables are often measured in buffers and under conditions, which deviate significantly from the cytosolic conditions inside of the cell [9]. For these reasons, reliable enthalpy $\Delta^R h$ and Gibbs energy $\Delta^R g$ values that are also applicable under cellular conditions are urgently needed.

Therefore, the standard terms $\Delta^R g'^0$ and $\Delta^R h'^0$ must be well known and the influence of the environmental conditions must be available via the activity coefficients γ_i^m (see eq. 1). For reasons of thermodynamic correctness, we use here and in the following the molalities m_i (mol kg^{-1}) instead of the molarities c_i (mol L^{-1}) common in systems biotechnology. The differences between the two quantities result from the temperature dependence of the density of water and the excess volume. The differences are often negligibly small.

$$\Delta^R g' = \Delta^R g'^0 + R \cdot T \cdot ln\left(\prod_i a_i^{\nu_i}\right) = \Delta^R g'^0 + R \cdot T \cdot ln\left(\prod_i m_i^{\nu_i}\right) + R \cdot T \cdot ln\left(\prod_i \gamma_i^{\nu_i}\right) \quad (1)$$

For γ_i^m a suitable G^E model or an equation of state is required. Numerous studies show that this usually works well applying the equation of state "electrolyte Perturbed-Chain Statistical Associating Fluid Theory" (ePC-SAFT) (for instance see [10, 11]). The question arises if ePC-SAFT can account for macromolecular crowding which occurs in living cells. High concentrations of macromolecules such as proteins alter the properties of molecules in a solution in this phenomenon [12, 13].

In contrast to $\Delta^R g'$, the situation is completely different in the case of reaction enthalpy. The reaction enthalpy does not have a direct separation between a concentration-independent and a concentration-dependent expression. Therefore, the dependency on the cytosolic conditions needs to be measured. The reaction enthalpy can be determined either directly using calorimetry or indirectly using the van 't Hoff equation (eq. 2) (for an overview of the two approaches see Figure 1) [14-21]. Both methods should provide the same value if they are performed correctly and under identical conditions.

➔ **Here Figure 1**

In the van 't Hoff method, equilibria are measured at different temperatures and evaluated according to equation 2.

$$\left(\frac{d\ ln(K)}{d\ T}\right)_P = \frac{\Delta^R h}{R \cdot T^2} \qquad \text{with} \qquad K = \prod_i \chi_i^{\nu_i} \quad (2)$$

K, χ_i, ν_{i} stand for the apparent equilibrium constant, any measure for the equilibrium concentration and the stoichiometric coefficient, respectively. If the activity a_i is chosen as a concentration measure, the thermodynamically correct equilibrium constant K_a is obtained. If we integrate eq. 2 and assume a temperature-independent enthalpy, we obtain a linear relationship between *ln(K)* and *1/T* that provides the enthalpy (eq. 3) [17].

$$ln\left(K(T)\right) = A - \frac{\Delta^R \mathrm{h}}{R} \cdot \frac{1}{T} \qquad \text{with} \qquad A = ln\left(K(T_0)\right) + \frac{\Delta^R \mathrm{h}}{R} \cdot \frac{1}{T_0} \quad (3)$$

Various methods such as HPLC and UV/Vis spectroscopy can be applied to determine the equilibrium concentrations and thus the equilibrium constants, depending on the properties of reactants and of the samples. More than half a century ago Benzinger [22] proposed a calorimetric method for the direct determination of the equilibrium constant *K*. Unfortunately, the prerequisites for applying the method (very diluted solutions, measuring back-and-forth reactions and measurable reaction enthalpy) are not fulfilled for the enolase reaction, which is here considered as example reaction.

In the other method (direct calorimetric measurement) the enthalpy is the result of the integration of the monitored heat flow.

The intrinsic assumption that both methods should provide identical enthalpies for the same reaction is controversially discussed in the literature [21]. In 1995, Naghibi et al. published the surprising result that both methods deliver different enthalpy values [14]. The authors discussed three potential reasons for the observed discrepancy. First, the temperature dependence of the reaction enthalpy (expressed as heat capacity ΔC_p) cannot be neglected. Second, the proton exchange of the buffer with the reactants might play a role and third, the complexity of chemical reactions might be too large to be reproduced by the simple van 't Hoff equation. Therefore, the authors postulated that the calorimetric enthalpy is the "true" enthalpy that covers all possible processes [14]. Also two subsequent papers by Liu confirmed the

discrepancy between calorimetric $\Delta^R h^{',cal}$ and van 't Hoff $\Delta^R h^{',van't\,Hoff}$ enthalpies and explained it with the complexity of the considered reactions [15, 16]. Chaires found that small ΔC_p influence the slope of the van 't Hoff plot and thus lead to errors in $\Delta^R h^{',van't\,Hoff}$ [17]. A turning point came in 2001 with Horn's research using the Ba^{2+} binding to 18-crown-6 ether and 2'-CMP binding to RNase as examples, which showed that if the experimental setup and the analysis were correct and the temperature dependence of the reaction enthalpy is considered, there is no difference between the two methods [19]. One year later Horn further described that in proton-linked systems the enthalpy values can differ if the van 't Hoff method does not consider the ionization of the buffer, whereas the calorimetric enthalpy does [18]. In 2004, Mizoue repeated the measurements of Liu and increased the accuracy of the measurements to define the discrepancies more precisely and found that there are no significant differences between the van 't Hoff and calorimetric enthalpies [20]. He also described that it can be a source of error of the calorimetric enthalpies if the reference measurements (blank measurements where titrant was added to water/buffer or vice versa) were not correctly taken into account. One and a half decades later the topic was taken up again and it was postulated that both values should theoretically agree and large discrepancies indicate experimental errors [21].

This literature survey results in two key questions for this study, which are answered using the example of reaction 9 of glycolysis (the enolase-catalyzed reaction from 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP)). Firstly, are there potential discrepancies between calorimetric and van 't Hoff enthalpies and how can these be explained? Secondly, what influence do cytosolic conditions have on the enthalpy of the reaction? Can the influence of cell-mimicking conditions on biochemical equilibria, especially of crowding agents, be properly considered with the equation of state ePC-SAFT? Some initial studies point to the special importance of macromolecular crowding for thermodynamic state variables [23, 24].

1.5 Materials and Methods

Materials

All substances used in this work are listed in Table S1 in the Supporting Information (SI) and have been used without further purification. The enzyme used in this work was a lyophilized enolase from *Saccharomyces cerevisiae*. The supplier tested the following side reactions (3-phosphoglycerate kinase reaction and glyceraldehyde 3-phosphate dehydrogenase reaction) and found no significant enzymatic activity for both. The final potential side reaction could be the conversion of PEP and bicarbonate into oxaloacetate catalyzed by carboxylases. This reaction was excluded in a previous work [11] using aspartate as a well-investigated inhibitor of the carboxylation [25]. The water used in this work was ultra-pure water freshly generated with a Millipore® purification system (Merck KGaA, Darmstadt, Germany). The water content of the phospho(enol)pyruvic acid monosodium salt hydrate, which was provided by the supplier, was considered in all calculations. All solutions were composed by weight with an analytical balance XS205 (Mettler Toledo GmbH, Gießen, Germany) and a Sartorius microbalance M5P (Satorius AG, Goettingen, Germany) with an accuracy of 0.01 mg. The relative weighing error for the usual quantities between 0.5 and 2 mg is 0.5 - 2 %.

1.6 Measurement of the equilibria

In the following we will use molality instead of the more common molarity used in biochemistry and biotechnology. Molality has the advantage of not being dependent on temperature, pressure and composition. At room temperature and small concentrations of metabolites, crowding agents and salts, the deviation between molality and molarity is small in relation to the experimental errors. All reactions were carried out in triplicates in 1.5-mL Eppendorf Tubes® (Eppendorf AG, Hamburg, Germany), which were placed in a ThermoMixer C (Eppendorf AG, Hamburg, Germany) to maintain the desired temperature and to mix at 350 rpm. All investigated reaction solutions were freshly prepared by weighing. The reaction was carried out in 200 mmol kg^{-1} MOPS buffer and the pH adjusted to the desired value by adding sodium hydroxide. The quantity added was determined gravimetrically. The reactions were started by adding 3-5 U g^{-1} enolase (leading to a reaction time < 30 min until equilibrium was reached). Sampling occurred after reaching equilibrium. This was validated in pre-experiments for 298.15 K. The equilibrium molality of PEP was quantified with an UV spectrometer BioSpectrometer® kinetic (Eppendorf AG, Hamburg, Germany) at 245 nm using High Precision cuvettes (Hellma Analytics, Müllheim, Germany) with a pathway of 10 mm. The details of the determination are described in detail in [11]. The equilibrium molality of 2-PG $m_{\text{2-PG}}^{eq}$ could not be measured and was therefore calculated according to eq. (4) from the molalities of PEP before the reaction m_{PEP}^{0} and at equilibrium m_{PEP}^{eq}.

$$m_{2-PG}^{eq} = m_{PEP}^{0} - m_{PEP}^{eq} \tag{4}$$

1.7 Thermodynamic treatment and van 't Hoff enthalpy

During enolase reaction, 2-phosphoglycerate (2-PG) is converted to phosphoenolpyruvate (PEP) and water (eq. 5).

$$2-PG \;\rightleftharpoons\; PEP + H_2O \tag{5}$$

Following eqs. (5) and (2), equilibrium ratios $K_a^{'}$ or $K_m^{'}$ for activities a_i^{eq} or molalities m_i^{eq}, respectively, can be formulated (eqs. 6 and 7).

$$K_a' = \frac{a_{PEP}^{eq} \cdot a_{H2O}^{eq}}{a_{2-PG}^{eq}} \tag{6}$$

$$K_m' = \frac{m_{PEP}^{eq} \cdot m_{H2O}^{eq}}{m_{2-PG}^{eq}} \tag{7}$$

$K_a^{'}$ has the advantage in comparison to $K_m^{'}$ to be independent of the cytosolic concentrations. However, $K_m^{'}$ can be calculated directly from the measured molalities. Activity coefficients are required to convert K_m' into the more favorable K_a' (eq. 8).

$$K_a' = K_m' \cdot K_\gamma' \quad \text{with} \quad K_\gamma' = \frac{\gamma_{PEP}^{*,m,eq} \cdot \gamma_{H2O}^{m,eq}}{\gamma_{2-PG}^{*,m,eq}} \tag{8}$$

In this work, the generic activity coefficient γ_i^m with standard state '*pure substance*' was used for water. Water is a product of the enolase reaction but also the solvent, meaning it is close to this standard state. For 2-PG and PEP, the rational activity coefficient $\gamma_i^{*,m}$ was advantageously used. Here is the standard state a '*hypothetical ideal solution*', which is defined in this work as a solution of 1 mol kg^{-1} of the substance diluted in water. The rational activity coefficient $\gamma_i^{*,m}$ approaches unity if the substance is infinitely diluted in water. $\gamma_i^{*,m}$ can be calculated with eq. (9) from the generic activity coefficients at present conditions γ_i and at infinite dilution $\gamma_i^{\infty,\text{m}}$.

$$\gamma_i^{*,m} = \frac{\gamma_i^m}{\gamma_i^{\infty,m}} \quad (9)$$

The standard enthalpy of biochemical reaction $\Delta^R h'^{0,van\,'t\,Hoff}$ was calculated from the temperature dependence of K'_a according to the van 't Hoff equation in eq. (3). The standard enthalpy of reaction determined with K'_a is also an activity-based value and therefore independent of the composition of the reaction medium.

The activity coefficients required for the calculation of K'_a and $\Delta^R g'^0$ (eqs. (8) and (9)) were predicted using the equation of state ePC-SAFT, as proposed by Held et al. [26]. ePC-SAFT is based on original PC-SAFT model from Gross and Sadowski [27] which was extended by Cameretti et al. [28]. More information about the ePC-SAFT model are given in the SI in section 2.1-2.3. ePC-SAFT requires five pure-component parameters. Two of them describe the geometry of the hard spheres: the segment number m_i^{seg} and the segment diameter σ_i. The dispersion-energy parameter u_i/k_B including the Boltzmann constant k_B were used to describe dispersive interactions. The association-energy parameter $\varepsilon^{A_iB_i}/k_B$ and the association-volume parameter $\kappa^{A_iB_i}$ were used to describe the associative interactions. In addition to this, the number of association sites N_i^{assoc} has to be set prior to modeling. The ePC-SAFT parameters used for this work are given in Table 1. The parameter estimation is described in the SI in section 2.4. Parameters of MOPS were fitted to osmotic coefficients determined in this work and literature densities [29] (see SI section 2.5, Figure S1 and Table S2).

➔ **Here Table 1.**

Multicomponent mixtures were described with mixing rules, see eqs. (S3)-(S6) in the SI. Applying ePC-SAFT and knowing K'_a allows predicting K'_m at different reaction conditions. Therefore, the proceeding of the enolase reaction was simulated by stepwise decrease of the substrate and increase of the product concentration. For each step, the activity coefficients of all reacting agents were predicted with ePC-SAFT and K'_γ was calculated. The resulting K'_a was compared with the known value and this procedure was repeated until the reaction equilibrium was reached (i.e. the known and the calculated K_a were equal). This yields the desired value of K'_m.

Calorimetric determination of the reaction enthalpy

An isothermal titration calorimeter (ITC) was used for the calorimetric measurement. Two solutions (for the syringe and for the calorimetric vessel) had to be prepared. The first is a 12 µmol kg^{-1} enolase solution in 0.2 mol kg^{-1} MOPS buffer with 0.15 mol kg^{-1} Na^+-ions, 0.001 mol kg^{-1} $MgCl_2$ and pH 7. The 0.15 mol kg^{-1} sodium-ions were composed of NaOH (for the pH adjustment) and of sodium chloride. The second solution contained 89.5 mmol kg^{-1} PEP in the same buffer as the enolase solution. PEP is a weak acid and the used MOPS-buffer is unable to maintain the pH of 7 during the solvation process. Therefore, a correction of the pH of the PEP solution with a 10 M sodium hydroxide solution was necessary. To make sure that the pH adjustment did not alter the buffer concentrations of the PEP solution, sodium hydroxide was not dissolved in water but in 0.2 mol kg^{-1} MOPS buffer with 0.15 mol kg^{-1} Na^+, 0.001 mol kg^{-1} $MgCl_2$ and pH 7. In order to investigate the influence of the cytosolic conditions on the equilibrium, the measurements were performed under different cell mimicking conditions. For this purpose, the cytosolic condition of interest was varied and the other properties (0.2 mol kg^{-1} MOPS buffer with 0.15 mol kg^{-1} Na^+, 0.001 mol kg^{-1} Mg^{2+}, pH = 7, T = 310.15 K, 0 mol kg^{-1} PEG 20,000) were kept

constant and will be designated in the following as the "basic conditions". For testing the different conditions, triplicate measurements were done. The changed conditions are summarized in Table 2. Only the change of one condition per measurement was allowed, the others remain in the basic conditions.

➔ **Here Table 2**

In order to validate the influence of the ionization enthalpy of the applied buffer system on $\Delta^R h'^{,cal}$, measurements were additionally carried out in a different buffer system: 0.2 mol kg^{-1} Tris/HCl pH 7, 0.15 mol kg^{-1} Na^+, 0.001 mol kg^{-1} $MgCl_2$, 298.15 K.

The determination of the enthalpy of reaction $\Delta^R h'^{,cal}$ was done using titration calorimetry measurements. A MicroCal PEAQ ITC from Malvern Panalytical (Malvern, UK) was used. Single injection measurements were performed, with enolase solution in the titration syringe and PEP solution in the sample cell. To avoid slow diffusion of enzyme into the calorimetric vessel completely before starting the reaction, a buffer plug in the end of the injection needle is recommended for very active enzymes. For the following experiments the influence of enolase diffusion can be neglected, because a very stable baseline was observed and the actual signal is very large. For this reason, the buffer plug has been omitted. The reference cell was filled with water. The setup of the PEAQ-ITC was set to high feedback, reference power of 41.9 µW, stirrer speed of 750 rpm, titration speed of 0.5 µL s^{-1}, baseline recording of 15 minutes and an injection volume of 39.2 µL. The concentration of PEP in the cell after injection was 75 mmol kg^{-1} and the enolase concentration was 2 µmol kg^{-1}. The signal was recorded until it reached the baseline again. The reference measurement was done with buffer in the titration syringe and PEP solution in the sample cell to delete the heat of dilution. The reference signal was than subtracted from the signal of the reaction. The $\Delta^R h'^{,cal}$ was calculated from the integration of the area under the reaction curve (Q) according to eq. (10)

$$\Delta^R h'^{,cal} = \frac{Q}{(m^0_{PEP} - m^{eq}_{PEP}) \cdot m} \qquad (10)$$

$m, m^0_{PEP}, m^{eq}_{PEP}$ stand for the mass of water present in the reaction cell (density = 1 g mL^{-1}, mass fraction (water) = 1), PEP concentration in the calorimetric cell before injection and after reaching the chemical equilibrium, respectively. m^{eq}_{PEP} was calculated from the apparent equilibrium-molality ratio K'_m. The K'_m values of the different reaction conditions were determined using the ePC-SAFT approach. These values were used because it has been proven to be inaccurate to analytically determine m^{eq}_{PEP} from the small reaction volume of the PEAQ-ITC. One additional reason for the uncertainties is the rapid change of the reaction temperature after sampling. Another reason is that when the cell is filled, there is also PEP solution above the cell, which does not participate in the reaction. During sampling after the reaction, this part is inevitably mixed with the reaction mixture from the calorimetric sample. This changes the PEP concentration of the solution leading to incorrect results. Therefore, it is much more accurate to set and measure the equilibria outside the calorimeter in larger reaction vessels and to use these data.

1.8 Results

Van't Hoff Enthalpy of Reaction

Two equilibrium constants (K'_a and K'_m) were used to determine the reaction enthalpy. One is the activity-based and thus concentration-independent equilibrium constant K'_a for the determination of the standard van 't Hoff enthalpy $\Delta^R h'^{0,van't\ Hoff}$. The resulting van 't Hoff plot provides a standard van 't Hoff enthalpy $\Delta^R h'^{0,van't\ Hoff}$ = 25.3 ± 1.1 kJ mol^{-1} (Fig. 2b, red solid line) which was measured at m^0_{PEP}= 10

77 mmol kg^{-1}. The blue dashed line is the result from Ref. [11] and provides $\Delta^R h'^{0,van't\ Hoff}$ = 27 ± 10 kJ mol^{-1} (m^0_{PEP}= 2 mmol kg^{-1}). This result demonstrates that the standard reaction enthalpies obtained are truly concentration independent. Then K'_m was used to determine the enthalpy of the reaction $\Delta^R h'$. Surprisingly, if K'_m is plotted, strong concentration-dependent reaction enthalpies were obtained $\Delta^R h'^{,van't\ Hoff}$ = 26 ± 10 kJ mol^{-1} for m^0_{PEP}= 2 mmol kg^{-1} (Fig. 2a, blue dashed line) and $\Delta^R h'^{,van't\ Hoff}$ = 2.4 ± 1.3 kJ mol^{-1} for m^0_{PEP}= 77 mmol kg^{-1} (Fig. 2a, red solid line).

➔ **Here Figure 2**

Enthalpy of Reaction determined by ITC

As the enthalpies, which were determined applying the van 't Hoff equation, are highly error prone (± 10 kJ mol^{-1}) and in order to further validate the enthalpies of reaction, the heat during enolase reaction was directly quantified via titration calorimetry, which yields the calorimetric enthalpy of reaction $\Delta^R h'^{,cal}$ with eq. (10) (Fig. 3).

➔ **Here Figure 3**

The calorimetrically determined reaction enthalpy for the basic condition is $\Delta^R h'^{,cal}$ = 2.4 ± 0.2 kJ mol^{-1}. One potential reason for the surprising deviation could be the dissociation of the buffer, which does not have to be taken into account for the van 't Hoff approach, but for the ITC analysis. For the sake of clarity here, calorimetric measurements were performed in two different buffer system with basic conditions but at 298.15 K, which differ considerably in the dissociation enthalpy (MOPS: 21.1 kJ mol^{-1}, Tris/HCl: 47.4 kJ mol^{-1} [34]), in order to evaluate this potential source of error [35]. $\Delta^R h'^{,cal}$ = 2.4 ± 0.2 kJ mol^{-1} was measured in MOPS buffer. $\Delta^R h'^{,cal}$ = 2.4 ± 0.04 kJ mol^{-1} was determined in Tris/HCl buffer. The pKa value of Tris is 8.07. This means that under our measurement conditions (pH=7.0) only about 10% of the buffer was dissociated and therefore could only marginally influence the calorimetric measurement. For this reason and because both buffers lead to identical values, it can be assumed that the dissociation of the buffer hardly influences our measurements.

1.9 Influence of Cytosolic Conditions

The phenomenon most often neglected by previous thermodynamic equilibrium measurements of metabolic reactions is the presence of high concentrations of macromolecules (crowding). PEG is considered as an example macromolecule. In a first step the influence of PEG with different molecular masses on reaction equilibria at 298.15 K and m^0_{PEP} = 2 mmol kg^{-1} was investigated (Fig. 3). The presence of PEG leads to a significant shift of the reaction equilibrium towards the 2-PG side. In order to describe the influence of PEG on the reaction equilibrium with ePC-SAFT accurately, $k_{PEG,PEP}$ at 298.15 K was adjusted to the equilibrium data for PEG 8,000 and PEG 35,000. With the resulting $k_{PEG,PEP}$, the influence of PEG 20,000 on the reaction equilibrium is predicted with ePC-SAFT as shown in Figure 4 for m^0_{PEP} = 2 mmol kg^{-1}. In a second step, the influence of PEG 20,000 at m^0_{PEP} = 77 mmol kg^{-1} was predicted with ePC-SAFT (right triangle in Fig. 4). This procedure ensures that calculations with ePC-SAFT at substrate concentrations of up to m^0_{PEP} = 77 mmol kg^{-1}, temperatures between 298.15 K and 310.15 K and PEG 20,000 concentrations of up to 12.5 mmol kg^{-1} which have been used in the titration calorimetry measurements, are accurate.

➔ **Here Figure 4**

Figure 5 shows the influence of crowding agents on the equilibrium constant K'_m (m^0_{PEP}= 77 mmol kg^{-1}). This results in values for ln(K'_m) = 5.500 ± 0.015 (T=310.15 K) without PEG (red dashed line) and ln(K'_m) = 4.3 with PEG (grey solid line). This proves that macromolecular crowding shifts the equilibrium position of the enolase reaction.

➔ **Here Figure 5**

Table S5 in SI shows the influence of the different cytosolic conditions on the measured heat (*Q*), the apparent equilibrium constant (K'_m) and the calorimetric reaction enthalpy. The resulting $\Delta^R h'^{,cal}$ values at the cytosolic conditions range from 0.7- 5.6 kJ mol^{-1} (Fig. 6), surprisingly far away from the value at standard conditions $\Delta^R h'^{0,\ van\ 't\ Hoff}$= 25.3 ± 1.1 kJ mol^{-1}.

➔ **Here Figure 6**

1.10 Discussion

Comparison of reaction enthalpy determined by van 't Hoff and ITC

The important state variable $\Delta^R h'$ was determined directly using calorimetry and indirectly using the van 't Hoff equation. We applied the van 't Hoff approach to three different conditions a) the standard state based on activities yielding the concentration-independent $\Delta^R h'^{0,van\ 't\ Hoff}$ = 25.3 ± 1.1 kJ mol^{-1}, b) at elevated PEP concentrations yielding $\Delta^R h'^{,van\ 't\ Hoff}$ = 2.4 ± 1.3 kJ mol^{-1} and c) in the presence of 12.5 mmol kg^{-1} crowding agents (12.5 mmol kg^{-1} ≙ 250 g L^{-1} PEG 20,000) yielding $\Delta^R h'^{,van\ 't\ Hoff}$ = 0.74 ± 10 kJ mol^{-1} (Fig. S2 in SI). Unfortunately, the available literature data for the enolase reaction does not clearly distinguish between the concentration-independent $\Delta^R h'^{0,van\ 't\ Hoff}$ and the concentration-dependent $\Delta^R h'^{,van\ 't\ Hoff}$. Even worse, the measurement conditions are often insufficiently specified in the literature data, making it difficult to evaluate and compare the data. Li et al. published $\Delta^R h'^{,van\ 't\ Hoff}$ = 15.1 kJ mol^{-1} for an estimated ionic strength of I = 0 (calculated value based on experimental data at different temperatures) [9]. In 1957, Wold et al. published $\Delta^R h'^{,van\ 't\ Hoff}$ = 14.65 kJ mol^{-1} [36]. Wold applied another buffer (0.05 M Imidazole) with 0.4 M KCl and 8 mM $MgSO_4$ at pH 7.5 which potentially explains the difference to our values. In 2009, Vojinovic et al. published a value of 0 kJ mol^{-1}, which was determined at conditions of I = 0.3 M, pMg = 3 and 298 K [37]. In a previous work, the standard enthalpy of enolase reaction was determined based on equilibrium measurements and ePC-SAFT modeling [11] and an activity-based value of $\Delta^R h'^{0,\ van\ 't\ Hoff}$= 27 ± 10 kJ mol^{-1} was obtained.

The calorimetrically measured enthalpy is 2.4 ± 0.1 kJ mol^{-1} at the basic condition and ranges from 0.7 - 5.6 kJ mol^{-1} at all conditions tested. These values are much lower, than the standard enthalpy determined by the van 't Hoff method. However, both approaches should yield the same value.

In case of the thermodynamic equilibrium constant K'_a van 't Hoff provides an enthalpy $\Delta^R h'^{0,\ van\ 't\ Hoff}$ which is independent of the cytosolic concentrations but cannot be determined calorimetrically, because it should be measured in a pure solvent at an infinite diluted concentration. Therefore, the direct calorimetric determination provides the enthalpy that is only correct for the

respective measurement conditions. Table 3 compares reaction enthalpies determined by ITC and van 't Hoff which were generated under comparable conditions. These pairs of enthalpies correspond very well. This is also supported by the fact that the calorimetric enthalpy is not temperature-dependent and thus has a negligible specific heat capacity of reaction ($\Delta^R C_p$= 0 J mol^{-1} K^{-1}). The heat capacity is often discussed as a potential cause for differences between reaction enthalpies determined by ITC and van 't Hoff.

➔ **Here Table 3**

Influence of Cytosolic Conditions

When using thermodynamic state variables like $\Delta^R g$ in systems biology, it is often assumed that a biological cell is only filled with buffer. But, cells are always crowded with macromolecules [38, 39]. Fig. 4 clearly demonstrates with the example of the enolase reaction that macromolecular crowding has a strong influence on the apparent equilibrium constant K'_m. In case of the enolase reaction K'_m can be about three times lower than the K'_m without crowding agents. However, using the ePC-SAFT approach to consider the influence of the crowding agent allows to determine a correct K'_a and a prediction of the influence of crowding on K'_m. There are other approaches to cope with crowding for instance using the scaled particle theory [40], but this approach is recently only applied to the total glycolytic pathway and not to a single glycolytic reaction [39]. Furthermore, the authors in [39] estimated the influence of cytosolic salts using the Debye-Hückel limiting law, which often fails at higher salt concentrations because only the long range Coulomb interactions are considered while strong short-range interactions are neglected.

The directly measured enthalpies for the enolase reaction are independent of the temperature but slightly depend on the Na^+ and Mg^{2+} concentrations (an increase of up to 1.2 kJ mol^{-1}). However, pH (an increase of 3.5 kJ mol^{-1}) and the macromolecular crowding (a change by 1.7 kJ mol^{-1}) have the strongest effects on the reaction enthalpy. Macromolecular crowding leads in case of the enolase reaction to a decrease of the reaction enthalpy. Similar behavior was observed in a trypsin-catalyzed reaction analyzing the effects of PEG 600 and PEG 6,000 on the reaction enthalpy [24] and for another glycolytic reaction (hexokinase) using 250 g L^{-1} BSA as crowding agent [23]. It is difficult to say if this is a general trend as we only have three examples and different types of crowding agents were used. Both works [23, 24] do not discuss the observed trend. Three potential explanations for the influence of the PEG 20,000 are imaginable. First, the entropic effect of the crowding agents has to be considered. The macromolecular crowding reduces the volume available for the reaction (excluded-volume), the degrees of freedom for molecular movement und thus the reaction entropy. This thesis is supported by the entropy calculated from the measured Gibbs energy and reaction enthalpy using the Gibbs-Helmholtz equation. The entropy is 34.7 ± 0.8 J mol^{-1} K^{-1} for 12.5 mmol kg^{-1} PEG 20000 and 51.8 ± 0.5 J mol^{-1} K^{-1} for the reaction without PEG. Therefore, the reaction is less entropy-driven in the presence of crowding agents than without them. This effect is partly cancelled out by a less strong obstruction by the enthalpy. However, enthalpy-entropy compensatory effects are not guaranteed for complex systems as considered here (for more details see [41]). Second, the reaction enthalpy could be additionally influenced by the interactions of 2-PG and PEP with the PEG macromolecules. Repulsive interactions of PEG with proteins and inducing macromolecular associations are already described in literature. The repulsion will become stronger with increasing size of the PEG [42]. It has also been detected that PEG induces repulsion

between lysozyme molecules [43]. This supports the above-mentioned idea of the excluded-volume. On the other hand, one study indicates that there is an attractive interaction between PEG and nonpolar or hydrophobic side chains on protein surfaces [44, 45]. These additional interactions with PEG could also contribute to the observed changes in enthalpy.

Third, the addition of macromolecules like PEG influence the dielectric constant of the solution. Following the concept by Debye and Hückel the dielectric constant has an influence on the activity coefficient and therefore on the excess enthalpy (eq. 11).

$$H_i^E = -R \cdot T \frac{\partial ln(\gamma_i)}{\partial T} \quad (11)$$

PEG mixtures have a much lower dielectric constant than water (ε = 81 at 20°C). No exact value is given for PEG 20,000 in literature. For PEG 400, however, a value of 11.6 at 20°C was postulated [46]. The addition of PEG lowers the dielectric constant of the solution, which leads to a change in the electrostatic forces of the system. If the electrostatic forces of the solutions are estimated with the values of PEG 400 and the highest PEG concentration, 12 % stronger electrical interactions take places in mixtures with PEG than in mixtures without PEG. The interplay of entropic effects, interactions of 2-PG and PEP with the macromolecules as well as the influence on the dielectric constant maybe explain the observed effect of PEG on the enthalpy values. From our study, it becomes clear how important the intracellular environment is for both the reaction equilibrium and the reaction enthalpy.

Conclusion

From the standard Gibbs energy and the standard reaction enthalpy the standard entropy of the reaction can be calculated for the basic conditions to be 51.8 ± 0.5 J mol^{-1}K^{-1} using the Gibbs Helmholtz equation. This means that the reaction is entropy driven, because one molecule of 2-PG breaks down into one molecule of PEP and one molecule of water. A large discrepancy between the standard reaction enthalpy $\Delta^R h'^{0,van't\,Hoff}$ = 25.3 ± 1.1 kJ mol^{-1} and the calorimetrically determined enthalpies $\Delta^R h'^{,cal}$ (0.7 - 5.6 kJ mol^{-1}) was observed. This apparent discrepancy disappears if the enthalpies $\Delta^R h'^{,cal}$ and $\Delta^R h'^{,van't\,Hoff}$ are determined at the same condition concerning: substrate, buffer, crowding agent and pH-value. The other option is to correct the equilibrium-molality ratios to the respective cytosolic conditions using the ePC-SAFT equation and to apply the van 't Hoff approach. Our results are a further indication that it makes no difference in principle whether the enthalpy was determined calorimetrically or via van 't Hoff as controversially discussed by several authors [14-16, 21]. However, there is a very practical reason for preferring the direct calorimetric measurement. The van 't Hoff approach is often error prone, because the slope of the logarithm of the equilibrium constants versus reciprocal temperature is evaluated. A small error in equilibrium constants in the typical range of reversible reaction from 0.1 to 10 in a narrow temperature interval can lead to a significant error in the slope. In addition, direct measurement is much less labor-intensive.

One argument in favor for the van 't Hoff approach is that if activities are applied instead of other concentration measures, a standard enthalpy independent of the concentrations is obtained. It seems to be impossible to measure calorimetrically this standard value, because a calorimetric measurement always requires a certain buffer and substrate concentration, which naturally influences the activity coefficients. A theoretically conceivable extrapolation of the measured reaction enthalpies to an

infinitely diluted substrate and buffer concentration to determine the standard enthalpy experimentally seems to us to be too error-prone. Since only a single glycolytic reaction has been studied in detail here, further work is needed to be able to generalize our conclusions.

The combination of equilibrium measurements and prediction of activity coefficients with ePC-SAFT allows the correct description of the cytosolic conditions especially of the effect of macromolecular crowding. This was clearly demonstrated for the example of reaction 9 of glycolysis (the enolase catalyzed reaction from 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP)) using PEG 20,000 as crowding agent.

Cytosolic conditions have a large influence on the reaction enthalpy $\Delta^R h'^{,cal}$. The intracellular pH and the crowding agent exert the strongest effects. The effect of the crowding agent can be explained entropically because macromolecular crowding reduces the reaction volume and thus the degrees of freedom for molecular movements. The temperature shows no influence on the reaction enthalpy in the investigated range. Temperature independence of the reaction enthalpy is a prerequisite for the application of the van 't Hoff equation (first Ulich approximation). The work should serve all scientists as an explanation of how important it is to choose an environment close to the conditions inside of biological cells to determine thermodynamic data for systems biology. The immense influence of this conditions is shown here. However, in order to recognize the general significance of cytosolic conditions on the thermodynamic state variables, further important metabolic reactions need to be analogously analyzed.

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1.12 Supporting Information

Supporting Information is available online

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Legends to figures

Figure 1: Scheme of the tasks of the present work and the two ways to determine enthalpy. The upper row underlines the importance of considering the cytosolic conditions when measuring the thermodynamic state variables. The lower row compares the two methods for determining the enthalpy of reaction. On the left side down the van ’t Hoff method is shown. The chemical reaction takes place in reaction tubes and then the equilibrium concentrations are determined by analytical methods (here UV/Vis spectroscopy). The equilibrium data are put into a van 't Hoff plot and the enthalpy is calculated from the slope of the obtained curve. On the right side down the enthalpy determination by titration calorimetry is shown. The reaction enthalpy is obtained by integration of the heat flow over time.

Figure 2: van ’t Hoff plot of the equilibrium-molality ratio K'_m (**a**) and of K'_a (**b**). Dashed and solid lines represent linear regression to determine the enthalpy of biochemical reaction with van ’t Hoff equation. Squares represent values from [11] based on measurement at m^0_{PEP} = 2 mmol kg^{-1}, circles represent values based on measurement at m^0_{PEP} = 77 mmol kg^{-1} and reaction conditions listed in table S3 in SI. The residual measuring conditions were the basic conditions.

Figure 3 **a:** Calorimetrically monitored heat flow. Green (dashed) stands for the reaction heat production rate initiated by the titration of 12 µM enolase solution into 89.5 mM PEP solution in the sample cell. Red (dashed) stands for a reference measurement (heat of dilution) with buffer as titrant and PEP solution as titrand. Blue (solid) is the real reaction heat production (the difference between both signals) in order to eliminate the heat of dilution. The other measuring conditions were the basic conditions. **b:** Integration of the heat flow signal over time from a. The integrated area is shown in grey.

Figure 4 Equilibrium-molality ratio K'_m vs mole fraction of PEG x_{PEG} at 298.15 K, pH 7, 1 bar, m^0_{PEP} = 2 mmol kg^{-1} and conditions listed in Table S4 in SI. Square represents measurements without PEG, diamonds represent measurement with PEG 35,000, triangles represent measurements with PEG 8,000. Lines represent ePC-SAFT predictions for PEG 35,000 (left), PEG 20,000 (middle) and PEG 8,000 (right).

Figure 5: Natural logarithm of equilibrium-molality ratio K'_m vs inverse temperature at m^0_{PEP} = 77 mmol kg^{-1}, pH 7 and 1 bar. Circles represent values based on measurement without PEG (detailed reaction conditions in Table S3 lines 4-5 in SI), gray line determined with ePC-SAFT at 22.5 mmol kg^{-1} PEG 20,000 (detailed reaction conditions in Table S6 in SI). The value with PEG is shown with no error because it was calculated with ePC-SAFT.

Figure 6: Influence of different cytosolic conditions on the reaction enthalpy $\Delta^R h'^{,cal}$. For the measurements, only one condition was changed compared to the basic condition.

Legends to tables

Table 1: ePC-SAFT parameters applied in this work with the sources for the respective sets of parameters. For 2-PG the parameters of its isomer 3-PG were used.

Table 2: To determine the influence of cell conditions, the measurements were performed under different conditions. Basic conditions were defined which are close to the cell conditions except for the crowding effect. Basic conditions are: 0.2 mol kg^{-1} MOPS buffer with 0.15 mol kg^{-1} Na^+, 0.001 mol kg^{-1} Mg^{2+}, pH 7 and 310.15 K. Then individual conditions were varied one after the other so that individual influence could be investigated.

Table 3 Comparison of reaction enthalpies determined by ITC and van 't Hoff at pH = 7.0, T = 310.15 K under different conditions. The basic condition is: 0.2 mol kg^{-1} MOPS buffer with 0.15 mol kg^{-1} Na^+, 0.001 mol kg^{-1} Mg^{2+}, 0 mol kg^{-1} PEG 20,000.

Credit Author Statement

Kristina Vogel: Methodology, Investigation, Validation, Formal analysis, Writing- Original draft preparation, **Thorsten Greinert:** Methodology, Software, Investigation, Validation, Formal analysis, Writing- Original draft preparation, **Hauke Harms:** Supervision, **Gabriele Sadowski:** Supervision, Funding acquisition, **Christoph Held:** Conceptualization, Supervision, Funding acquisition, **Thomas Maskow:** Conceptualization, Supervision, Funding acquisition, **All authors:** Writing-Reviewing & Editing

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Table 1: ePC-SAFT parameters applied in this work with the sources for the respective sets of parameters. For 2-PG the parameters of its isomer 3-PG were used.

	m_i^{seg}	σ_i	u_i/k_B	N_i^{assoc}	$\varepsilon^{A_iB_i}/k_B$	$\kappa^{A_iB_i}$	k_{i,H_2O}	z	source
	-	Å	K	-	K	-	-	-	
PEP	12.007	2.200	407.3	2+2	5000	0.1	[a]	-2	[11]
2-PG	3.110	4.660	322.0	5+5	501.2	10^{-4}	[b]	-2	[30]
TRIS	6.373	2.748	302.2	1+1	4787	0.0203	-0.047	-	[31]
TRIS-H^+	10.205	2.408	348.1	4+4	10971	10^{-6}	-0.061[c]	-	[31]
MOPS	15.697	2.271	171.6	2+2	4418	0.001	-0.150	-	this work
water	1.205	[d]	353.9	1+1	2426	0.0451	-	-	[32]
Na^+ [e]	1	2.823	230.0	-	-	-	[f]	+1	[26]
Mg^{2+} [g]	1	3.133	1500	-	-	-	-0.25	+2	[26]
Cl^-	1	2.756	170.0	-	-	-	-0.25	-1	[26]
PEG [h]	M_{PEG} ·0.051	2.900	294.6	2+2	1800	0.02	[i]	-	[33]

[a] $k_{PEP,water}$ = -0.005083 T/K + 1.3316 [11]

[b] $k_{3-PG,water}$ = 0.00203 T/K - 0.7064 [30]

[c] $k_{Tris-H^+,water}$ [11]

[d] σ_{water} = 2.7927 + 10.11 exp(-0.01775 T/K) - 1.417 exp(-0.01146 T/K) [32]

[e] k_{Na^+,Cl^-} = 0.3166 [26]

[f] $k_{Na^+,water}$= -0.007981 T/K + 2.3799 [26]

[g] k_{Mg^{2+},Cl^-} = 0.817 [26]

[h] $k_{PEG,PEP}$ = 011383T/K + 3. 1239 (this work)

[I] $k_{PEG,water}$ = 0.002344 T/K - 0.8338 [13]

Table 2: To determine the influence of cell conditions, the measurements were performed under different conditions. Basic conditions were defined which are close to the cell conditions except for the crowding effect. Basic conditions are: 0.2 mol kg^{-1} MOPS buffer with 0.15 mol kg^{-1} Na^{+}, 0.001 mol kg^{-1} Mg^{2+}, pH 7 and 310.15 K. Then individual conditions were varied one after the other so that individual influence could be investigated.

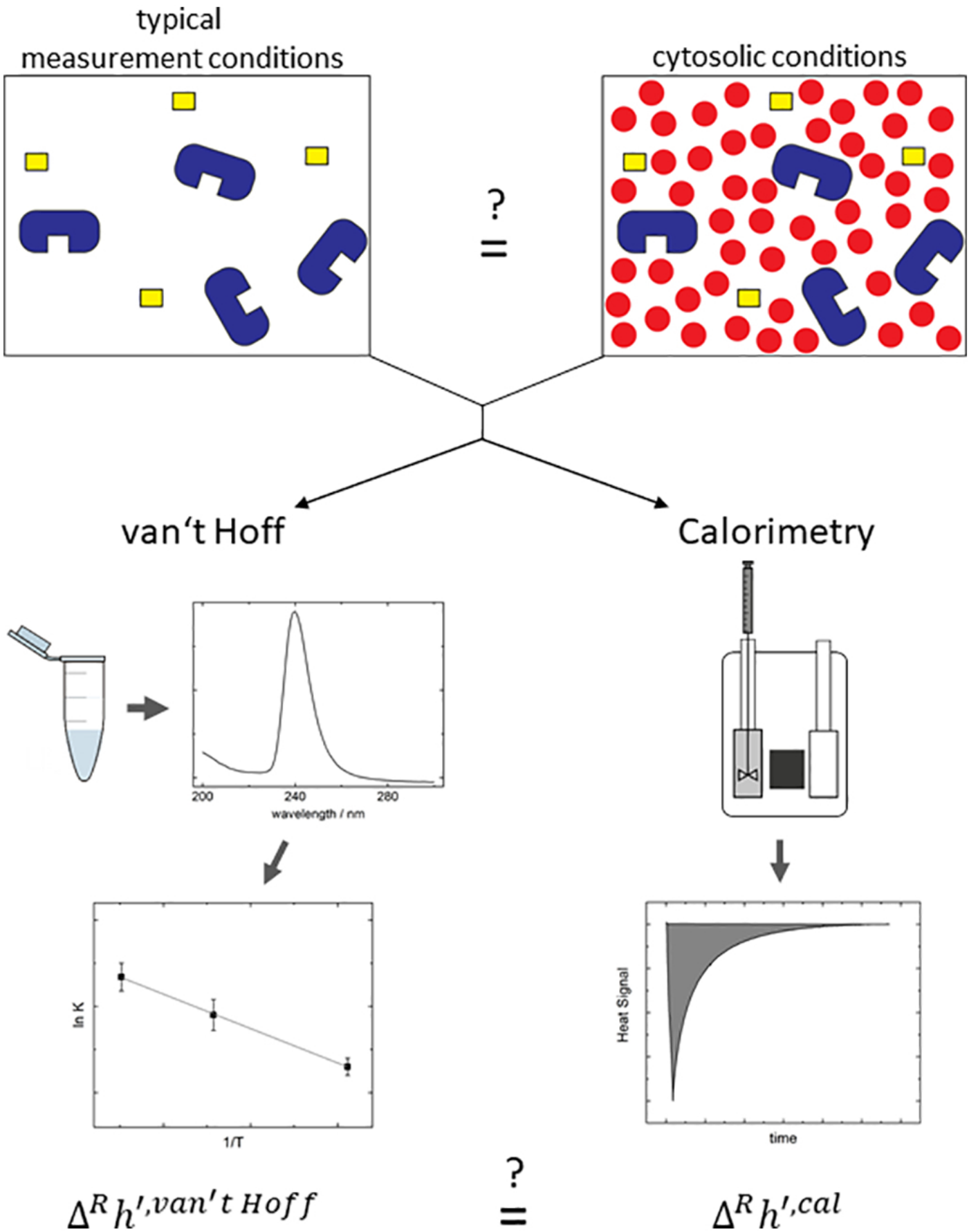
conditions	chemicals	values	unit
temperature	-	298.15, 305.15, 310.15	K
pH	buffer	6, 7, 8	-
Na^{+} concentration	NaOH and NaCl	0.1, 0.15, 0.3	mol kg^{-1}
Mg^{2+} concentration	$MgCl_2$	0.001, 0.008, 0.015	mol kg^{-1}
Crowder concentration	PEG 20,000	0, 5.7, 9.1, 12.5	mmol kg^{-1}

Table 3 Comparison of reaction enthalpies determined by ITC and van't Hoff at pH = 7.0, T = 310.1 K under different conditions. The basic condition is: 0.2 mol kg^{-1} MOPS buffer with 0.15 mol kg^{-1} Na^+, 0.001 mol kg^{-1} Mg^{2+}, 0 mol kg^{-1} PEG 20,000.

Conditions	$\Delta^R h'^{,\ cal}$	$\Delta^R h'^{,\ van't\ Hoff}$	$\Delta^R h'^{0,\ van't\ Hoff}$
	[kJ mol^{-1}]	[kJ mol^{-1}]	[kJ mol^{-1}]
Standard state (i.e. infinite dilution in water)	---	---	27 ± 10 [11]
Basic condition, m^0_{PEP}= 2 mmol kg^{-1}	---	26 ± 10	---
Basic condition, m^0_{PEP}= 77 mmol kg^{-1}	2.4 ± 0.1	2.4 ± 1.3	25.3 ± 1.1
Basic condition, m^0_{PEP}= 77 mmol kg^{-1}, PEG 20,000 = 12.5 mmol kg^{-1}	0.7 ± 0.02	0.74 ± 10	---

Highlights

- The standard reaction enthalpy of 2PG-> PEP + H_2O is 25.3 ± 1.1 kJ mol^{-1}.
- Calorimetric and van ’t Hoff enthalpy are equal under identical conditions.
- The reaction enthalpies depend to a large extent on the cytosolic conditions.
- The greatest influence is the crowding by macromolecules and the pH value.
- ePC-SAFT allows the prediction of cytosolic conditions.


typical
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200
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Heat Signal
time
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=
$\Delta^R h',van't\ Hoff$
$\Delta^R h',cal$

Figure 1

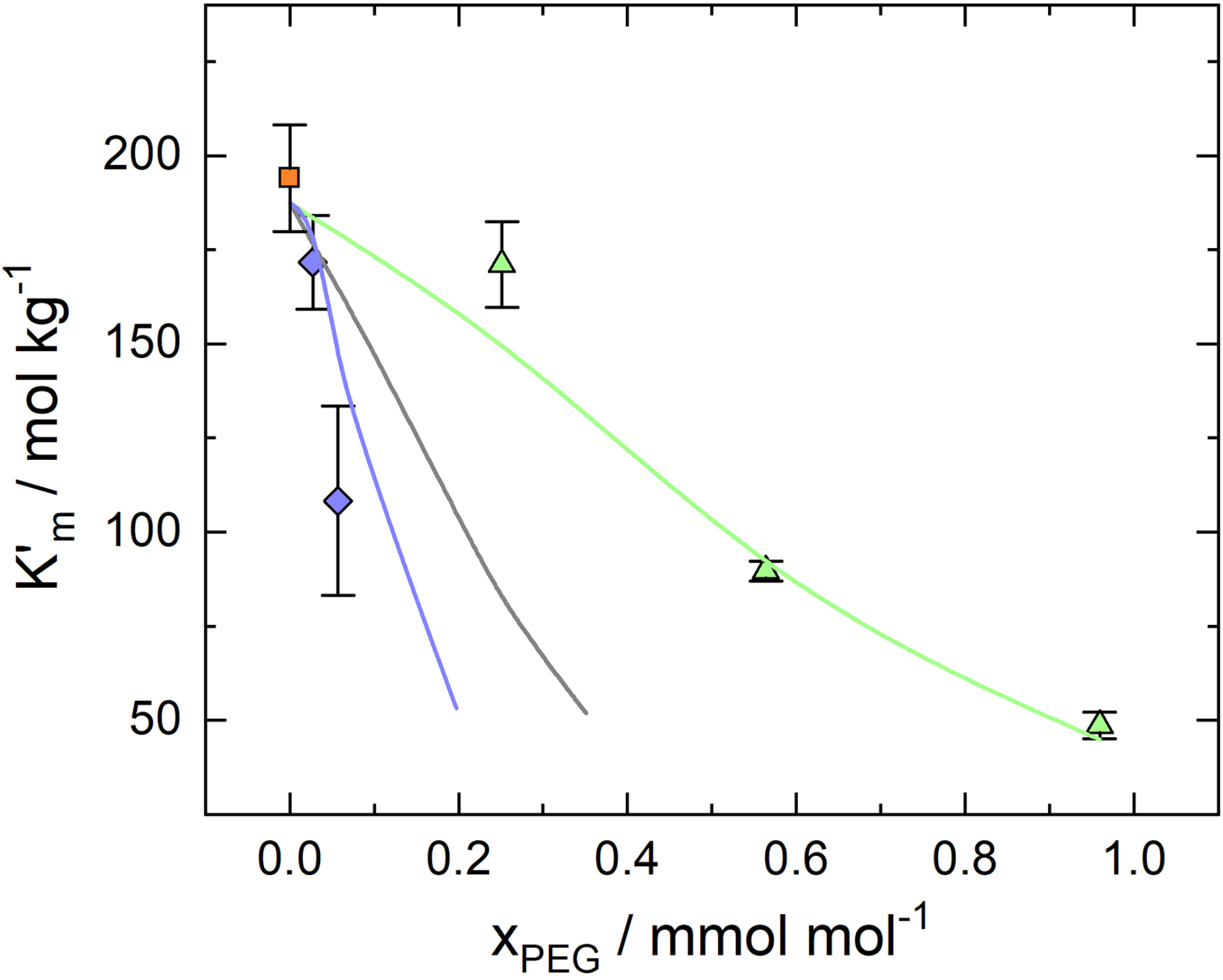

Figure 2

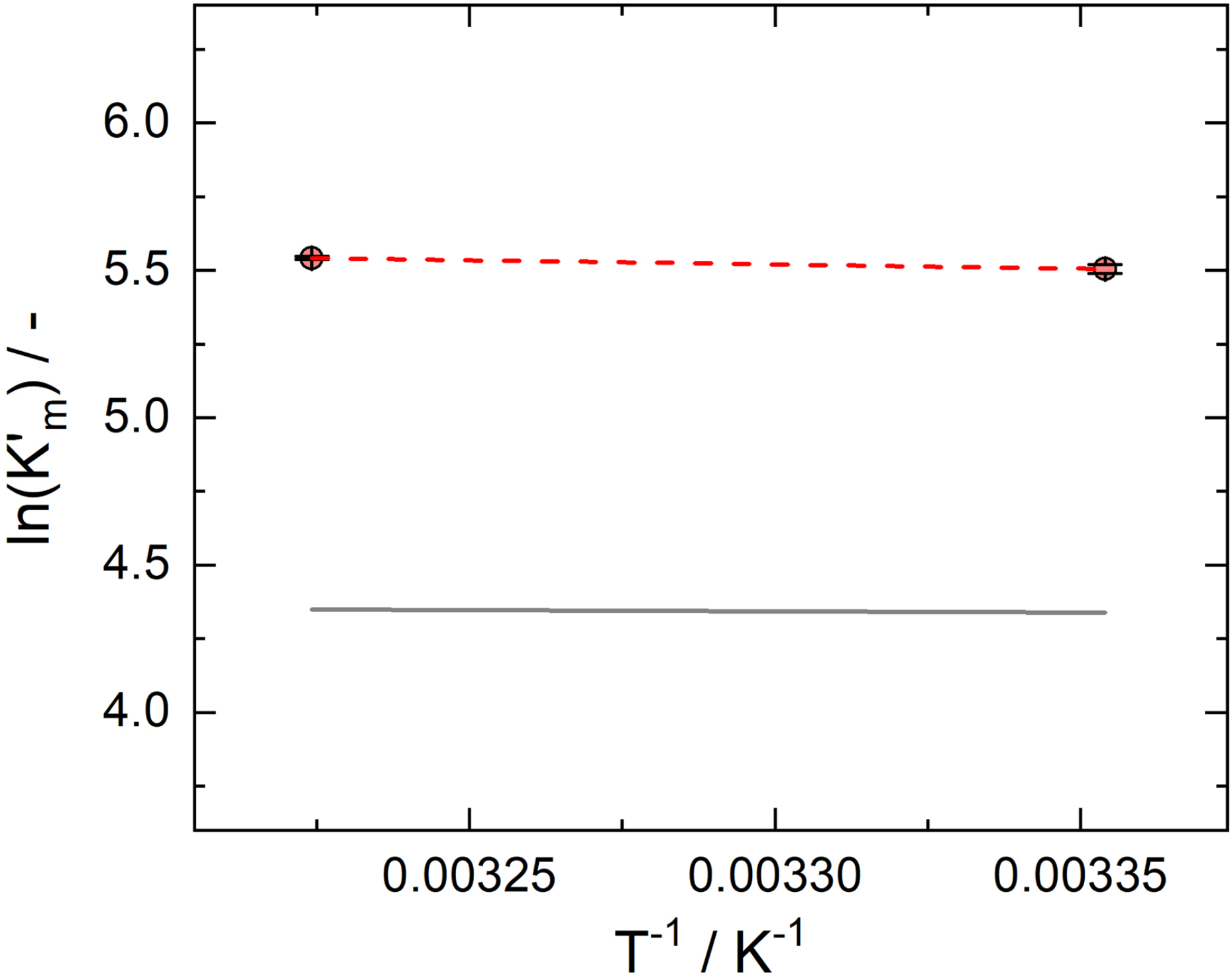

Figure 3

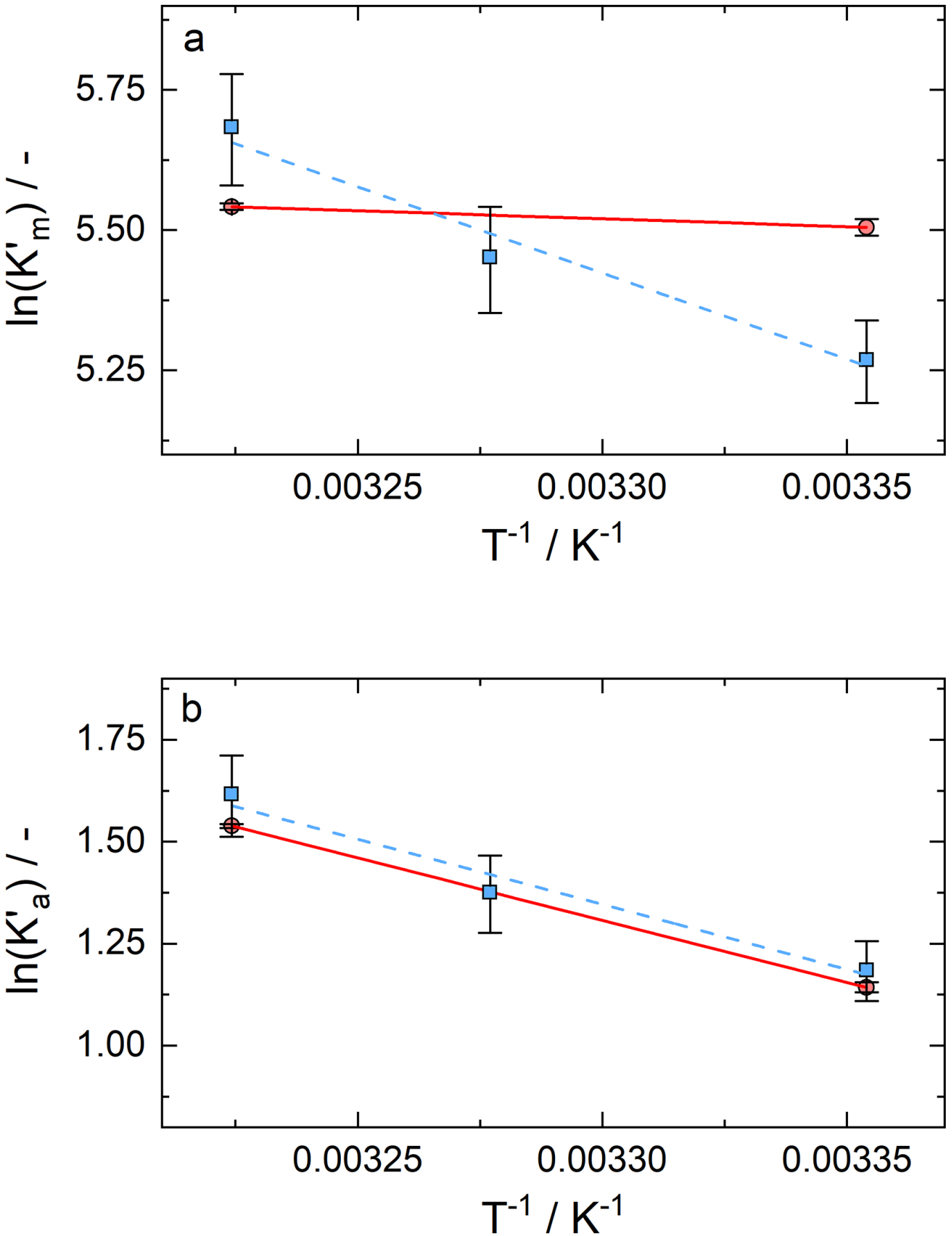
a
5.75
5.50
5.25
ln(K'm) / -
0.00325
0.00330
0.00335
T-1 / K-1
b
1.75
1.50
1.25
1.00
ln(K'a) / -
0.00325
0.00330
0.00335
T-1 / K-1

Figure 4

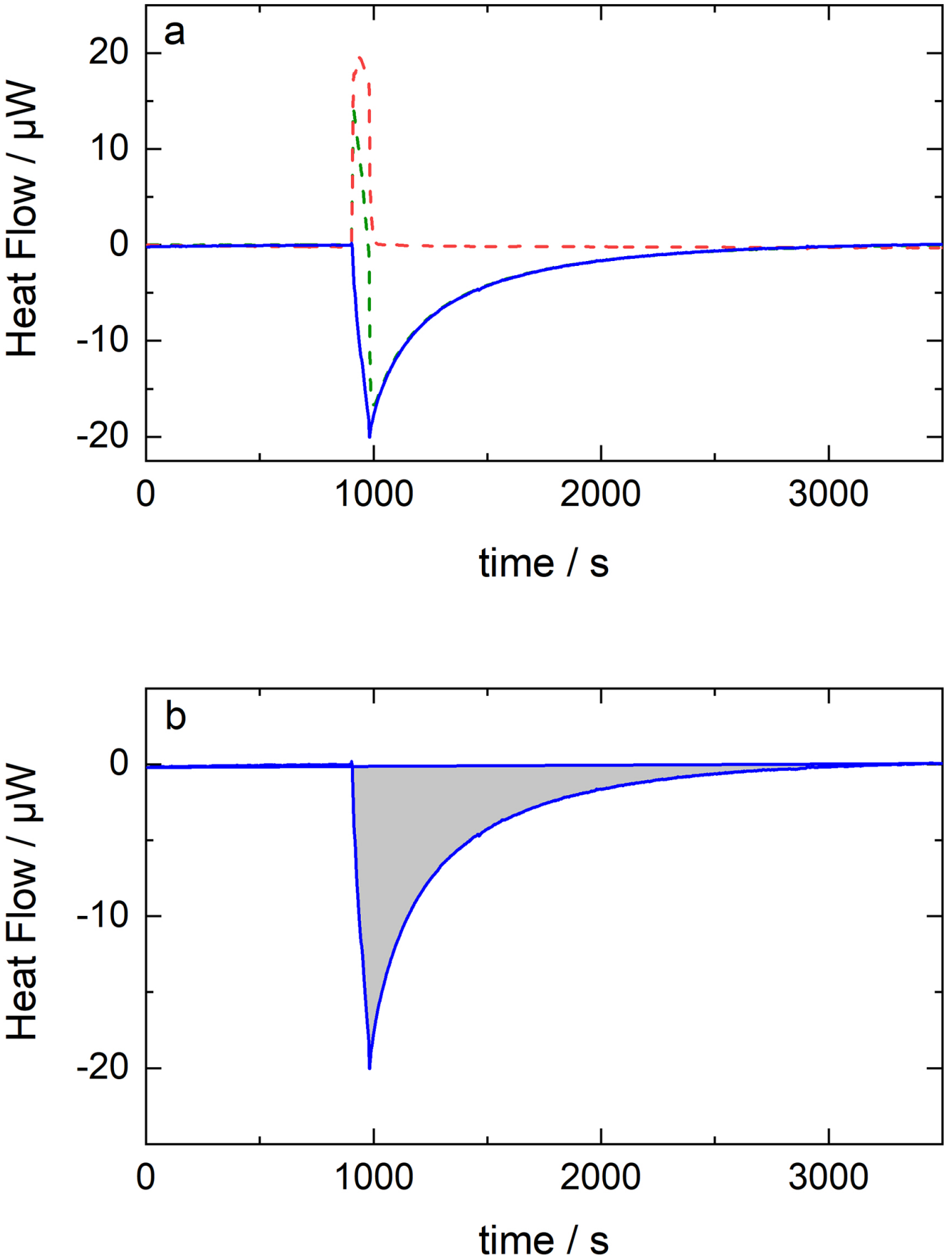
a
20
10
0
-10
-20
Heat Flow / µW
0
1000
2000
3000
time / s
b
0
-10
-20
Heat Flow / µW
0
1000
2000
3000
time / s

Figure 5

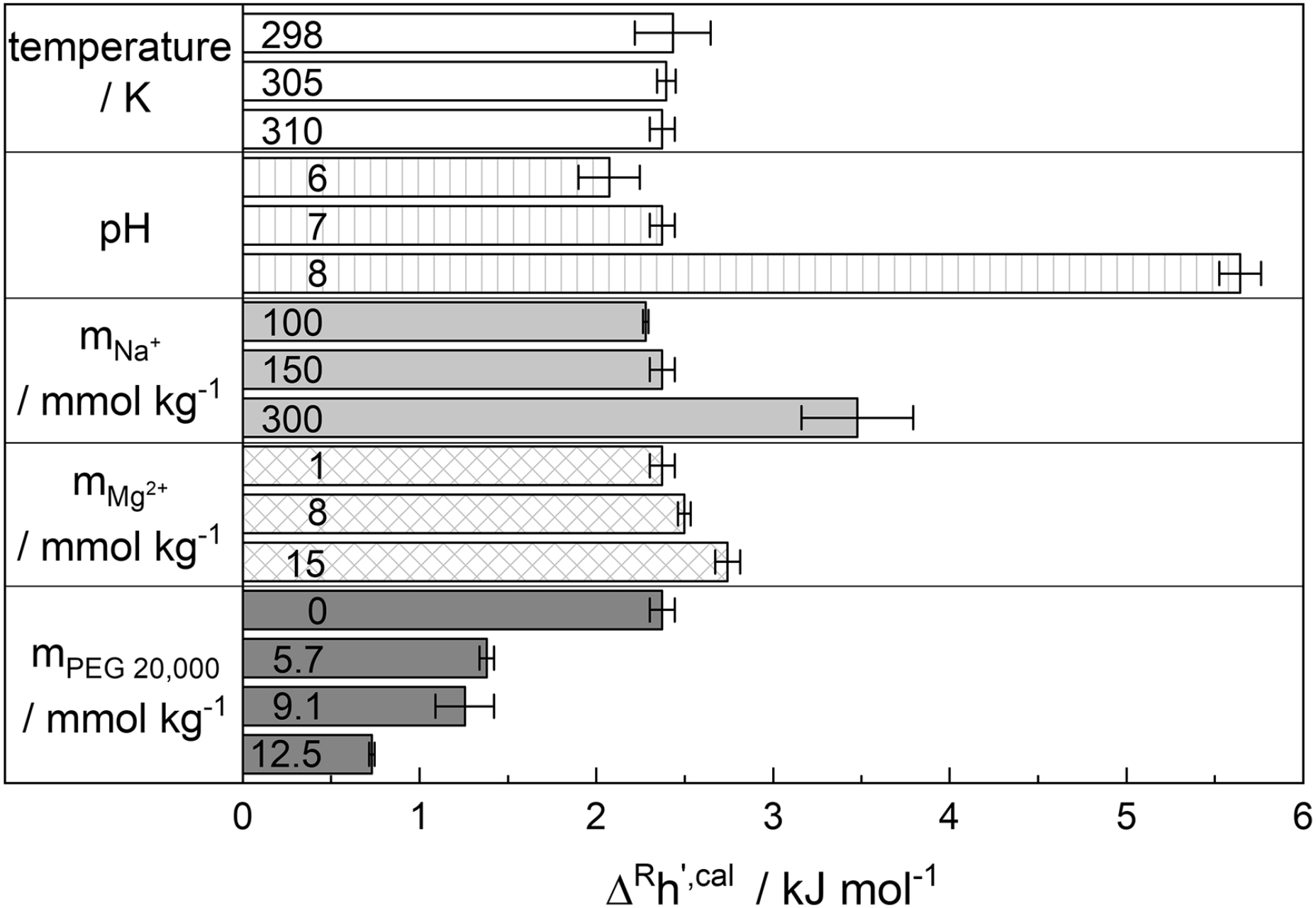
temperature / K
298
305
310
pH
6
7
8
m_{Na^+} / mmol kg^{-1}
100
150
300
$m_{Mg^{2+}}$ / mmol kg^{-1}
1
8
15
$m_{PEG\ 20,000}$ / mmol kg^{-1}
0
5.7
9.1
12.5
0
1
2
3
4
5
6
$\Delta^R h^{',cal}$ / kJ mol^{-1}

Figure 6