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ORIGINAL ARTICLE

Characterization of β-cyclodextrin inclusion complex with procaine hydrochloride by ^{1}H NMR and ITC

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Abstract The inclusion of local anesthetic drug procaine hydrochloride by β-cyclodextrin was investigated by 1D and 2D proton NMR spectroscopy and isothermal titration calorimetry (ITC) at 298 K. The stoichiometry of the complex was determinate by the method of continuous variation, using the chemical induced shift of both host and guest protons. The association constant K, of the obtained complex was calculated and found to be 293.17 M^{-1}. Rotating frame NOE spectroscopy, was used to ascertain the solution geometry of the host–guest complex. The result reveals that the procaine molecule penetrates into the β-cyclodextrin cavity with the aromatic ring. The energetics of complexation process is investigated by ITC technique. The analysis indicates that the complexation of procaine by β-CD is an exothermic process and show that both enthalpy and entropy contribute to the binding process. The obtained value for the association constant is in good agreement with that obtained from NMR.

Keywords Inclusion complex · Molecular interaction · Nuclear magnetic resonance · Isothermal titration calorimetry

Introduction

Local anesthetics are amphiphilic molecules that have hydrophobic and hydrophilic domains that are separated by an intermediate alkyl chain. The hydrophilic group can be tertiary or secondary amine, and the hydrophobic domain is an aromatic residue. They are classified in ester types and amide types; it depends on the group that binds to the aromatic residue. The nature of this bond determines several pharmacological properties for these drugs [1].

Procaine hydrochloride, also called Novocaine, is a synthetic organic compound used in allopathic medicine as well as in neural therapy. Generally used in a 1–10 % saline solution, procaine hydrochloride is administered by injection for infiltration (area flooding as in dental anesthesia), nerve-block, spinal, and caudal anesthesia. Unlike cocaine, procaine is not toxic, addicting, or irritating. It has been displaced somewhat by the chemically related drugs lidocaine and mepivacaine, which produce prompter, more intense anesthesia.

Cyclodextrins (CDs) are cyclic oligosaccharides containing six (α-CD), seven (β-CD) or eight (γ-CD) α-1, 4-linked glucopyranose units with a hydrophilic hydroxyl group on their outer surface and a hydrophobic cavity in the center. The hydrophilic exterior of the CD molecules can make them water soluble, but the hydrophobic cavity provides an environment for appropriate sized non-polar molecules. In aqueous solution CDs are capable of forming inclusion complex with many molecules by taking up a whole molecule or some part or it, into the cavity. These non-covalent complexes offer a variety of physicochemical advantages over uncomplexed molecules including increased water solubility and stability.

The procaine:β-CD system was also investigated by other spectroscopic or thermodynamic methods and the authors [2–4] obtained different values for the association constants in the range 200–350 M^{-1}.

We expect that a regional administration complexed anesthetic with β-CD improve the delivery process, leading to a larger duration of action and an improvement of therapeutic index. This inclusion complex has a strong clinical

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application by the possibility to use procaine in anesthesia in vivo. Recent experimental works with several local anesthetics highlight the interest of such and approach [5–7].

Experimental

In this paper, we report a ^{1}H NMR and ITC studies of the inclusion complex formed between procaine (Fig. 1a) and β-CD (Fig. 1b), in aqueous solution. Analysis of our data by the continuous variation method confirms that the inclusion occurs and the complex has 1:1 stoichiometry. The association constant was calculated by a non-linear least squares regression analysis of the observed chemical shift changes of the procaine and β-CD protons as a function of β-CD concentration.

The energetics of biochemical reaction at constant temperature was measured by isothermal titration calorimetry (ITC). A single well-designed experiment can provide complete thermodynamic characterization of a binding reaction, including the equilibrium constant K, the Gibbs free energy ΔG, the reaction enthalpy ΔH, the entropic effect ΔS and reaction stoichiometry (*n*).

Materials

β-CD (water content 8 mol/mol) was purchased from Sigma Chimie GmbH. Germany and procaine hydrochloride from Alfa Aesar GmbH & CoKG, Germany. Both chemicals were used without any further purification. Deuterium oxide (99.7 % D) was obtained from Heavy Water Plant Romag-Prod, Romania.

Apparatus

The ^{1}H NMR experiments were performed on a Bruker Avance III, spectrometer operating at 500.13 MHz and equipped with a broadband observe probe. The NMR spectra were recorded in D_2O solution at 298 K and all chemical shifts were measured relative to TMS. For each ^{1}H NMR experiment, between 16 and 256 transients were collected into 65 K data points over a 5,000 Hz spectral window, using a 2 s relaxation delay. The 2D ROESY spectra were acquired in the phase sensitive mode and residual water suppression, using Bruker standard parameters (pulse program roesyphpr). Each spectrum consisted of a matrix of 8 K/4 K data points covering a spectral width of 4,000 Hz. The spectra were obtained with a spin-lock mixing time of 500 ms, relaxation delay 3 s and 8 scans were recorded.

The isothermal titration calorimetric experiments were carried out on a Nano ITC^{2G} calorimeter (TA Instruments, New Castle, Delaware, USA) at 298 K next to the electrically and chemically calibration. The volume of the titration and reference cells was 1 ml. Small aliquots of a solution are injected under computer control into the titration cell at predefined time intervals [8, 9]. The recorded quantity is the rate of heating as a function of time. The individual pulses are integrated to obtain plots recording the ratio of heat to the amount of injected molecules.

^{1}H NMR measurements

In order to study the complexation process between procaine and β-CD in solution by NMR spectroscopy, two stack solutions of the procaine and β-CD in D_2O, both having 10 mM were prepared. Based on these two equimolar solutions, a series of nine samples (i = 1–9) containing both the procaine and β-CD molecules were prepared. This was accomplished by mixing the two solutions to constant volume at varying proportions, so that a complete range $(0 < r < 1)$ of the ratio $r = [X]/([A] + [B])$ was sampled. X = A or B and [A] and [B] are the total concentrations of the host (β-CD) and guest (procaine), respectively. Thus the total concentration $[A] + [B] = [C] = 10$ mM was kept constant for each solution.

For example, taking 0.3 ml for procaine solution and 0.7 ml for β-CD solution, we obtained 1 ml solution with:

$$0.3\text{ml} \times 10\text{mM} = 1\text{ml} \times \text{A};\ [\text{A}] = 3\text{mMprocaine}$$

$$0.7\text{ml} \times 10\text{mM} = 1\text{ml} \times \text{B};\ [\text{B}] = 7\text{mM}\ \beta\text{-CD}$$

$$\text{and}\ [\text{A}] + [\text{B}] = 10\ \text{mM}$$

The same set of samples was used both for the determination of stoichiometry and association constant.

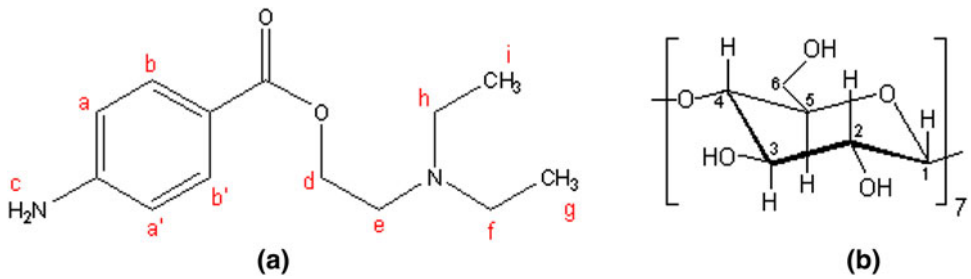

Fig. 1 Chemical structure of **a** procaine and **b** β-CD

Isothermal titration calorimetry

Calorimetric titration experiments were carried out at constant temperature by injecting 90 mM procaine hydrochloride solution into the sample cell containing 9 mM β-CD, keeping the stirring speed fixed at 250 rpm. From a computer controlled 250 µl Hamilton syringe, aliquots of 5 µl have been injected with a 1600 s interval which was sufficiently long for the signal to return to the baseline and to ensure the equilibrium for the system. The heat associated with each injection was calculated by integration of the peaks in the heat flow curve using the calorimeter software. Dilution heat of the host was measured under identical experimental conditions by injecting the procaine into water. The blank effects were subtracted in order to correct for dilution, mixing and injection effects.

Results and discussions

Determination of the stoichiometry by ^{1}H NMR

NMR is a technique which provides the most evidence of inclusion of a guest molecule into the hydrophobic CD cavity in solution. The inclusion of procaine in β-CD is shown by the change in the chemical shift of some guest procaine and host (β-CD) protons (in the mixture), in comparison with the chemical shifts of the same protons in the free components. Partial ^{1}H NMR spectra of pure components and procaine:β-CD mixture in a 1:1 molar ratio is presented in Figs. 2 and 3.

The absence of new peaks that could be assigned to the complex suggested that complexation is a dynamic process, the included procaine being in a fast exchange between the free and bound states.

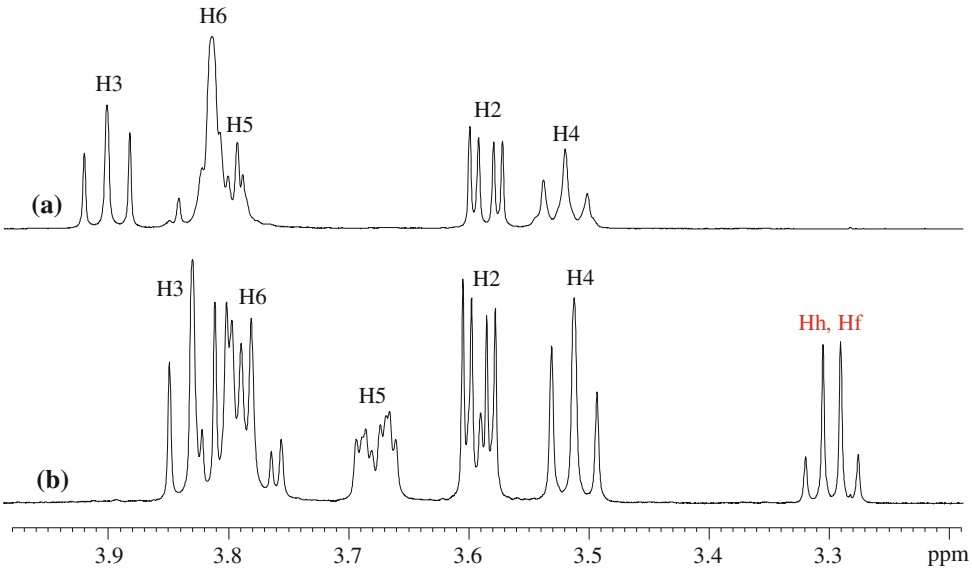

Fig. 2 Partial ^{1}H NMR spectrum of **a** 10 mM β-CD and **b** 5 mM β-CD and 5 mM procaine

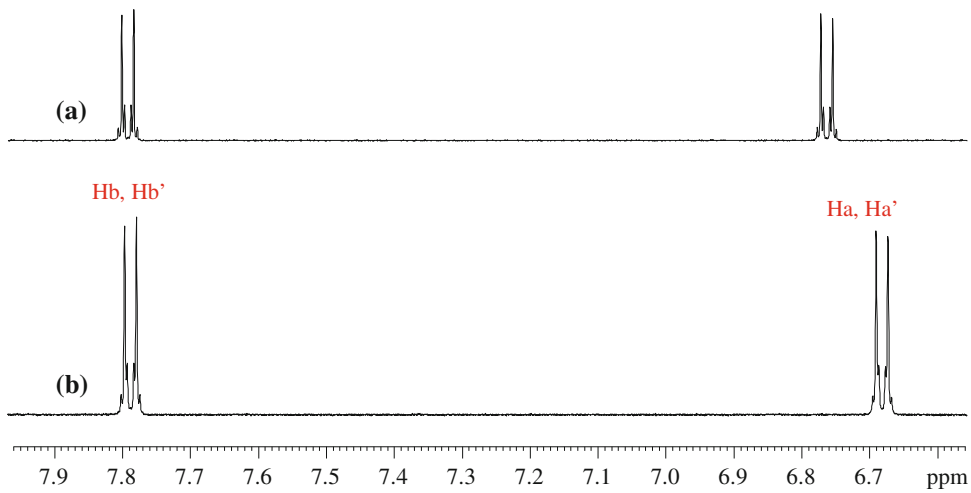

Fig. 3 Partial ^{1}H NMR spectrum of **a** 10 mM procaine and **b** 5 mM procaine and 5 mM β-CD

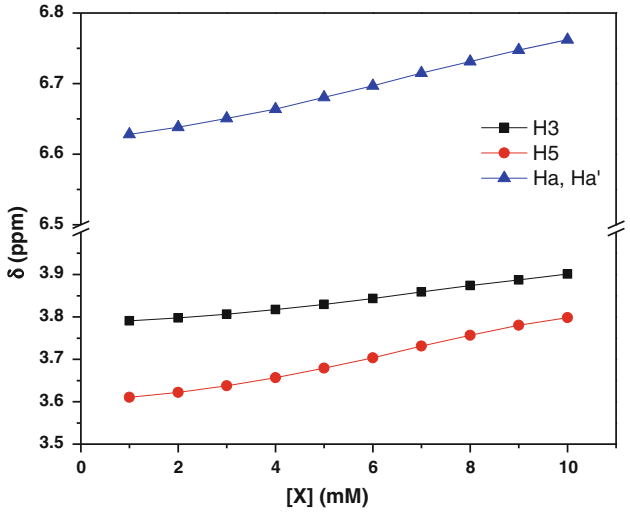

Fig. 4 Chemical shifts variation of some representative β-CD and procaine protons as a function of their concentration, ([β-CD] + [Proc] = 10 mM), where [X] = [Proc] or [β-CD]

The ^{1}H chemical shifts variation for H3, H5 protons of the β-CD and Ha, Ha' protons of the procaine as well as a function of their concentration is presented in Fig. 4.

The stoichiometry of the complex must be calculated before proceeding with any association constant calculations. Determination of the stoichiometry of the procaine-βCD complex, by the continuous variation method was based on ^{1}H NMR spectra obtained for procaine and β-CD mixtures in which the initial concentrations of the two species were maintained constant and the ratio r varied between 0 and 1 (see procedure). A physical parameter directly related to the concentration of the complex (in our case the chemical shift, δ) is measured under these conditions and plotted as a function of r. The maximum value for this parameter well occur at r = n/(m + n), where m and n are, respectively, the proportions of procaine and β-CD in the complex (procaine)$_m$:(β-CD)$_n$. The calculated quantities Δδ[βCD] are proportional to the concentration of the complex [10], and can be plotted against r. The resulting continuous variation plots demonstrate that because r has a maximum value of 0.5 and highly symmetrical shapes (Fig. 5), the complex has 1:1 stoichiometry.

The induced chemical shift variation, Δδ is defined as the difference in chemical shifts in the absence and in the presence of the other reactant for a given ratio r.

ROESY experiments

While the 1D NMR provides unambiguous evidence on the formation of a complex, ROESY experiments provide information on the dynamics and the averaged relative inter- and intramolecular proton distances. In the present study, to gain further information on the inclusion complexation mode and additional insights into the dynamic structure, a 2D ROESY ^{1}H NMR spectrum was acquired. Due to the rapid dynamics of the complexation process, the ROESY effects were only quantitatively used and no conclusions on intermolecular distances were extracted. An expansion of the ROESY spectrum of the procaine-βCD complex is reported in Fig. 6.

The 2D NMR spectrum shows several intermolecular cross-peaks between H3, H5 and H6 protons of β-CD and the protons of aromatic ring of procaine Ha, Ha', Hb and Hb'. In fact the strong correlations were found between Ha, Ha' from procaine and H5, H6 of β-CD. Another strong interaction is observed between Hb, Hb' of the procaine and H5, H3 of the CD. These interactions indicate that the

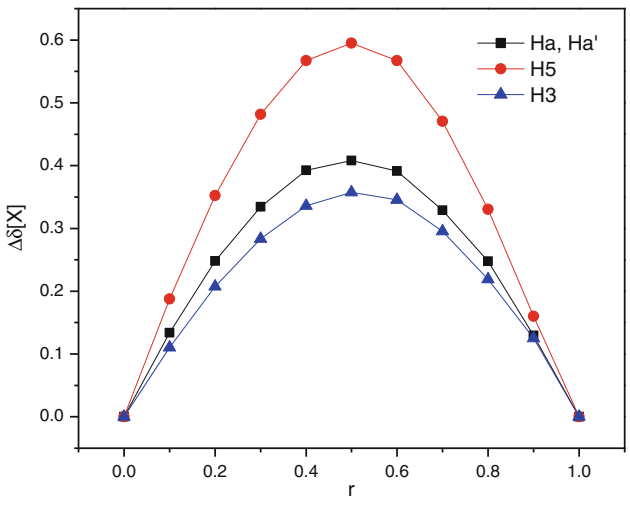

Fig. 5 Concentrations variation plot for: H3 and H5 protons of β-cyclodextrin; Ha and Ha' protons of procaine, where [X] = [Proc] or [β-CD]

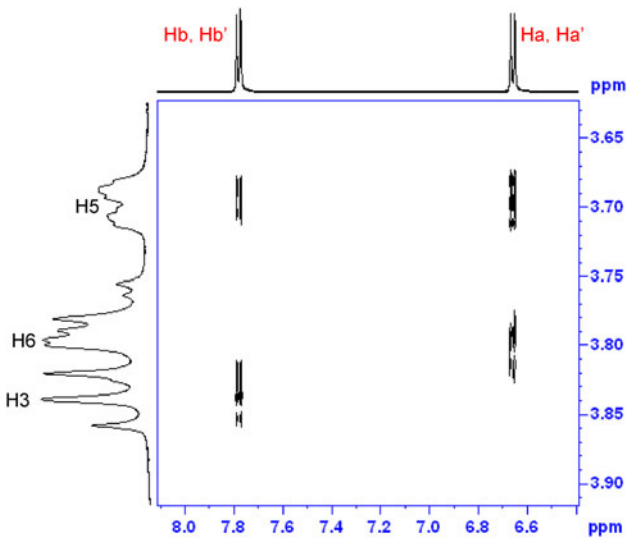

Fig. 6 Expanded region of the ROESY spectrum of procaine:β-CD complex. [proc] = 4 mM; [β-CD] = 6 mM

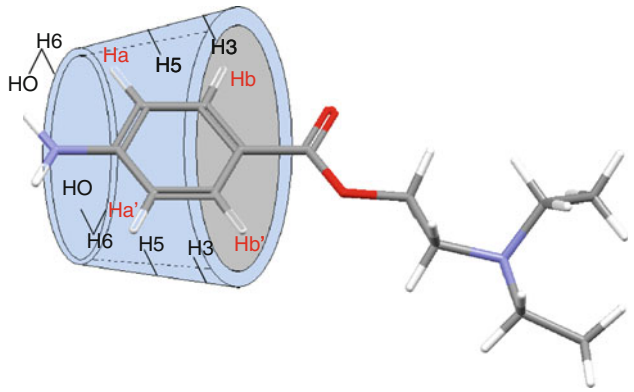

Fig. 7 Structure of proposed procaine:β-CD complex inclusion geometry

procaine molecule is included with aromatic ring into the CD cavity.

Based on these findings, the geometrical structure of the procaine:β-CD inclusion complex, having 1:1 stoichiometry, can be schematically presented as shown in Fig. 7.

Determination of the association constant by ^{1}H NMR

In order to determine the extent of the intermolecular binding between procaine and β-CD, the association constant has been evaluated. The association constant, K for a 1:1 complex can be determined according to the following equation [11]:

$$\Delta\delta^{(i,j)} = \frac{\Delta\delta_c^{(j)}}{2[X]}\left\{[C] + \frac{1}{K} - \left[\left([C] + \frac{1}{K}\right)^2 - 4[A]^{(i)}[B]^{(i)}\right]^{1/2}\right\} \quad (1)$$

where i counts the sample number and j the studied proton. If the studied proton belongs to the guest or host molecule, X = A or B respectively. $\Delta\delta_c^{(i)}$ represents the chemical shift difference (for a given proton) between the free component and the pure inclusion complex. Equation (1) involves no approximations and correlates the total concentrations of the guest and host molecules with the observed difference in the chemical shift:

$$\Delta\delta^{(i,j)} = \delta_{free}^{(i,j)} - \delta_{obs}^{(i,j)} \quad (2)$$

We used a computer software [12] based on an iteration procedure following specific algorithms in order to fit the experimental values of $\Delta\delta^{(i,j)}$ to the appropriate equation. Each iteration sets up a quadratic software to determine the direction of search and the error function:

$$E = \sum_{i,j} (\Delta\delta^{(i,j)} - \Delta\delta_{calc}^{(i,j)})^2 \quad (3)$$

until the search converges.

The treatment of the whole set of protons studied yields one single K value characterizing the inclusion process and a set of calculated $\Delta\delta_c^{(i,j)}$ values. The software is quite flexible since it allows observing the chemical shift variation of the host, guest or both molecules as a function of variable guest or host concentrations.

In our case, we applied Eq. (1) for a set of protons consisting in H3 and H5 of β-CD and Ha, Ha' of procaine. The association constant obtained using the above described procedure is K = 293.17 M^{-1} with E = 4.28 × 10^{-5} and a correlation factor R = 0.9997.

Preliminary indications of the solution structure of the complex can by derived from the proton chemical shift analysis. For β-CD, the observed largest chemical shift changes with increasing procaine concentration were those exhibited by H3 and H5 (up-field shift) of 0.110 and 0.187 ppm respectively. Since H3 and H5 face the cavity of β-CD, their chemical shift changes are most likely due to the presence of the methoxybenzaldehyde moiety in the cavity. On the other hand, the largest chemical shift variation observed for procaine protons (up-field shift) as a function of β-CD concentration occurs at position Ha and Ha' of 0.134 ppm.

Determination of the association constant by ITC

The equilibrium in guest–host reaction is described by the following hypothetical scheme:

$$A + nB \leftrightarrow AB_n$$
$$K = \frac{[AB_n]}{[A][B]^n} \quad (4)$$

where [A], [B], and [AB_n] stand for the concentrations of procaine, β-CD and β- CD-procaine complex respectively.

In an ITC experiment the reaction heat per mole of injected molecules after the ith injection is given by:

$$\frac{q_i}{vB_0} = \frac{\Delta H}{2}\left[1 + \frac{1 - r_i/n - (r_0 + r_i)/nKB_0}{\sqrt{(1 + r_i/n + (r_0 + r_i)/nKB_0)^2 - 4r_i/n}}\right] \quad (5)$$

where $r_0 = \frac{B_0}{A_0}$; $r_i = \frac{B_i}{A_i} = r_0\frac{iv}{V}$, A_0 and B_0 are the initial concentrations of procaine and of β-CD respectively, ΔH is the reaction enthalpy, V is the volume of reaction cell and v is the small volume injected at each titration step [13].

The software supplied with the calorimeter was used to fit thermodynamic parameters K and ΔH to the heat profiles.

The equilibrium constant obtained from ITC measurement was K = 288.70 ± 14.51 M^{-1} which corresponds to a Gibbs free energy ΔG = −3.35 ± 0.02 kcal/mol. The reaction enthalpy of the host–guest inclusion complex is ΔH = −2.95 ± 0.09 kcal/mol. This value is small and

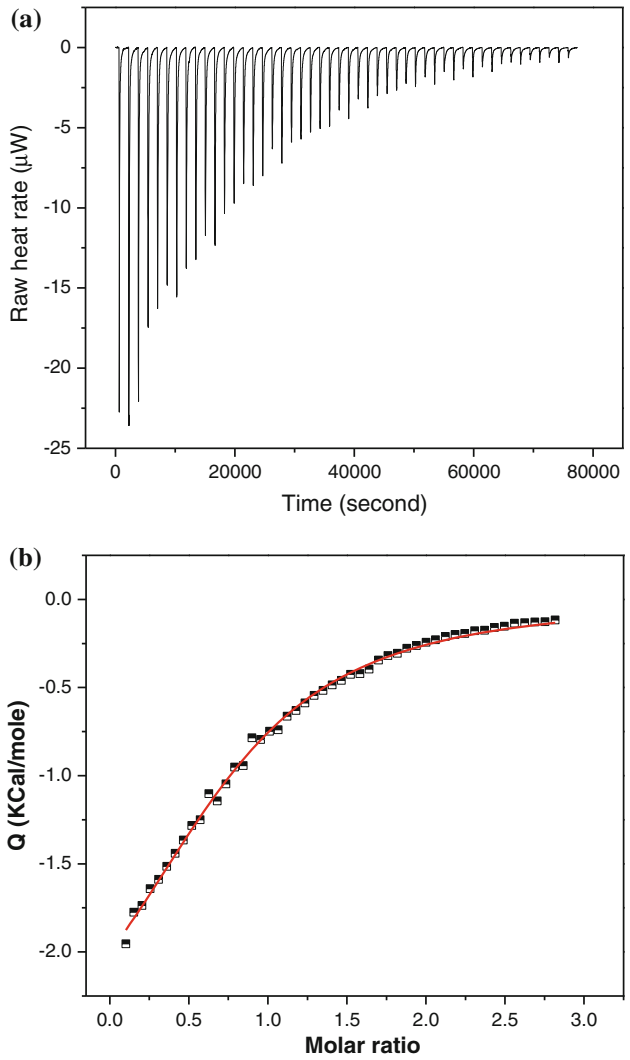

Fig. 8 Calorimetric titration of β-CD with procaine. **a** Raw data obtained from 48 injections of 5 μl aliquots of 90 mM Proc into 9 mM β-CD at 25 °C. **b** The incremental heat/mol of added ligand as a function of molar [Proc]/[β-CD] ratio. The *solid line* is obtained by fitting the experimental data

negative, which point out that the formation of host–guest inclusion complexes is a weak exothermic process. These features are usually found [2, 14] for association between small guest molecules and an apolar cavity in water. The entropic effect of the complexation process is $\Delta S = 1.34 \pm 0.38$ cal/mol·K and bring an additional contribution to the negative Gibbs free energy. The negative sign of the enthalpy change $\Delta H < 0$ combined with the opposite sign of entropy change $\Delta S > 0$ confirm that both hydrophobic and electrostatic interactions contribute to the binding process. The analysis of the thermodynamic data reveals that the driving force of the binding process is a hydrophobic interaction with the released water molecule from the β-CD cavity, which has positive contribution to entropy (Fig. 8). Calorimetric measurements of the interaction of procaine with β-CD have shown that the stoichiometry of the host–guest complex is 1:1 ($n = 0.831$).

Conclusions

The Procaine-CD system in aqueous solution has been studied by ^{1}H NMR. Analysis of our data by the continuous variation method indicates that the inclusion occurs and the complex has 1:1 stoichiometry.

The complexation-induced chemical shift of H3 and H5 protons of β-CD are those expected as a result of interaction with those protons of the procaine Ha, Ha' Hb and Hb' which are oriented towards the β-CD cavity, thus confirming a procaine/β-CD interaction. The association constant K for the inclusion complex was evaluated from the observed difference in chemical shifts for procaine and β-CD protons.

The ROESY experiment indicates that the procaine molecule is included with aromatic ring into the CD cavity.

The microcalorimetry allowed us to study inclusion reaction between β-CD and procaine and to determine all the thermodynamic parameters of the CD complexation.

For this type of inclusion complex, the association constant K obtained by ^{1}H NMR and ITC are in good agreement and both methods sustain a 1:1 stoichiometry.

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