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Differential isothermal rotating microcalorimeter: realization and evaluation. Application to the study of the interaction between human growth hormone and co-solvents

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Abstract

In order to study interactions in biological sytems, a differential isothermal rotating microcalorimeter has been developed. The small reaction volume (<1 ml) and the high sensitivity of the system (0.5 μW) allows the study of dilute solutions (2–20 mg ml^{-1}) of proteins that are difficult to obtain.

A study of the interaction between biosynthetic growth hormone (hGH) and different molecules (alanine, glycerol, benzylic alcohol and chlorocresol) was carried out. The interaction enthalpy rose to 10 kJ mol^{-1} for alanine and glycerol, to 26.5 kJ mol^{-1} for benzylic alcohol and 51.5 kJ mol^{-1} for chlorocresol, with which protein denaturation is instantaneous. A comparison of these results with those obtained by differential scanning calorimetry (DSC) was made, showing the complementarity of both techniques.

INTRODUCTION

Microcalorimetry allows the study of biological solutions at a molecular level as well as at a cellular level [1, 2]. Differential scanning calorimetry (DSC), in association with other techniques (circular dichroism, NMR, crystallography), provides the characterization of conformational modifications of proteins [3, 4], but under particular conditions (association of low affinity, low quantities of available proteins), few techniques permit investigation into solution equilibria.

From the first principle of thermodynamics which enables a direct relation to be established between the internal energy of a system and the heat developed during its transformation, by measuring these heat quantities calorimetry allows the determination of the heat capacities and

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transformation energies of different elements of the system. Isothermal microcalorimetry has been used to study the different interaction energies of the protein–solvent, co-solvent–solvent and co-solvent–protein systems. The heat quantities considered correspond to the sum of the different events in solution. In theory and in a simple case, we can represent the heat contributions of different events by the equation

$$Q_{\text{total}} = Q_{\text{interaction (A-B)}} + Q_{\text{dilution(A)}} + Q_{\text{dilution(B)}}$$

where A is the protein and B is the co-solvent.

In order to determine the thermodynamic characteristics of protein–co-solvent interactions, we studied first the heats of dilution of the components taken separately, and then the heats of mixing.

The commercial devices used in this determination were flow calorimeters which were used to mix the two liquids: an injection system (LKB 10700, Setaram micro DSC "Batch and Flow"), and a rotating system (Setaram C 80 D). The advantages of this latter system over injection systems are the perfect thermal equilibrium of the two solutions before mixing, the partial resolution of the problem of the viscosity of the solutions, the determination of the thermodynamic parameters during molecular aggregation, and the attainment of chemical equilibrium even in the case of slow kinetic processes.

The disadvantages are the long time to record a measurement (1–2 hours) and the large solution volume, e.g. for the C80D, the cell volume is between 6 and 12 ml, which is a drawback in the study of some proteins, which are difficult to obtain.

To overcome these problems, we have developed a calorimeter of high sensitivity (0.5 μW) using low volumes (<1 ml). These two objectives reached protein–co-solvent interactions in dilute solution (1–10 mg ml) were studied to characterize the associated thermodynamic values.

This system was applied to the study of the interactions between hGH and different adjuvants: alanine, glycerol, benzylic alcohol and chlorocresol. Isothermal calorimetry allows the characterization of the formation or dissociation energies of molecular complexes, and DSC allows the characterization of the final state of complexation. Thus, these two techniques are complementary and the results which are obtained in isothermal calorimetry are correlated qualitatively with the results obtained by DSC of the same biological systems.

EXPERIMENTAL

Isothermal microcalorimeter assembly

The equipment is composed of a calorimeter block and a signal treatment system, both manufactured in this laboratory.

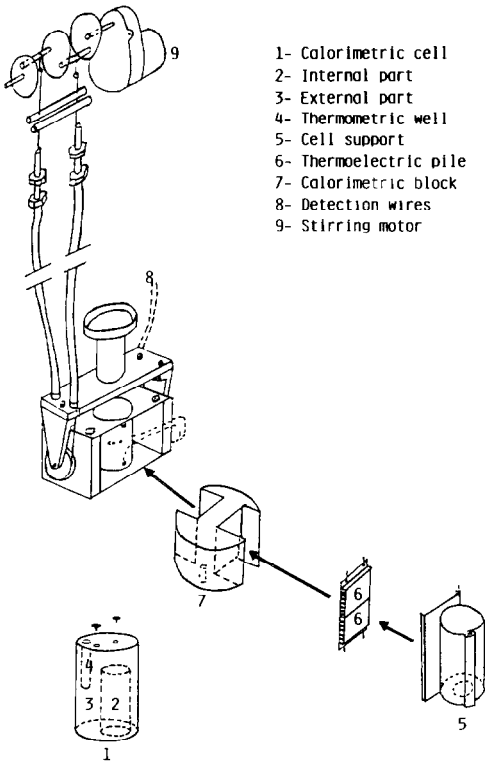

Fig. 1. Schematic view of the microcalorimeter.

Calorimeter block

The calorimeter block is composed of a calorimetric cell consisting of two compartments containing the crucibles which are made of two compartments (internal and external) containing the solutions (Fig. 1). They are removed for filling. Only the experimental crucible has a thermometric well which permits electric calibration. The other is the reference crucible filled with distilled water and connected in opposition. Using a differential system, this enables the thermal power variations dependent on the experimental system and on external perturbations to be accounted for. The calorimeter block also includes four thermopiles (Melcor SC 06-66-06L), comprising 66 thermocouples. Two thermopiles are superposed for each crucible (see Fig. 1). The block is covered by an airtight stopper.

Finally, the block is connected to a rotating engine which can move the crucible over time.

Operating method

The crucibles were filled with approximately 400 μl of solution A in the internal compartment and the same quantity of solution B in the external compartment. For thorough mixing, ten inox milliballs were put in the

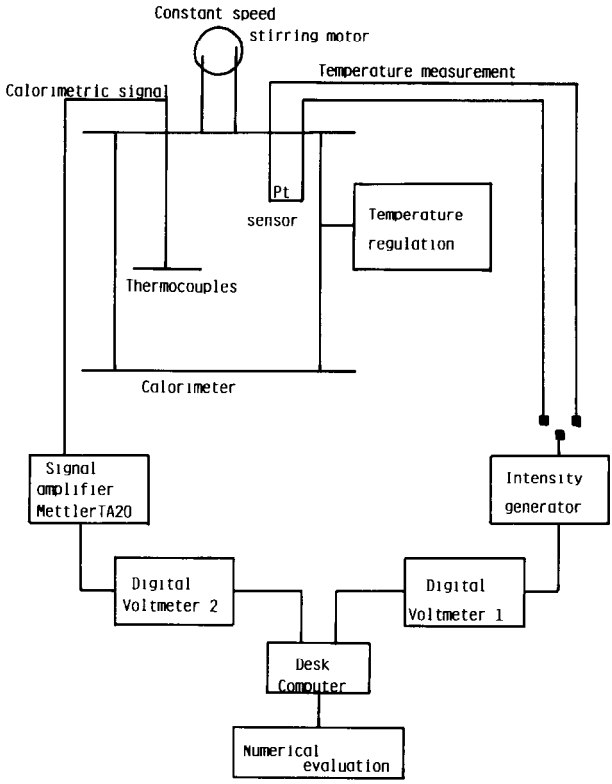

Fig. 2. Instrumental set-up of the calorimetric system.

internal compartment. The crucibles were closed tightly with teflon joints, and the two crucibles were placed in the calorimeter block itself in a thermostated area. Once thermal equilibrium (stabilization of the base line) has been reached, the calorimeter block was rotated continuously until the end of the experiment, i.e. until the base line is recovered.

Signal processing

A synoptic diagram of the system is given in Fig. 2. The acquisition of the results is done with an HP calculator via a numerical voltmeter. The results are processed off-line with an HP 9816 according to a specific program developed in the laboratory, and available with all types of flow calorimeters.

Calibrations and performances

Calibrations

An electric calibration was performed by Joule effect with a calibration resistance being placed in the experimental crucible. From the heat $Q = UIt$ released in the crucible and the corresponding surface signal S, the

TABLE 1

Calorimetric sensitivity (K) determination

Experiment	S (μV s)	Q (mJ)	K (mW mV^{-1})
1	3479	9.86	2.83
2	5514	14.79	2.68
3	7613	19.72	2.59
4	11030	29.58	2.68
5	23247	59.17	2.54
6	4596	11.83	2.57
7	4468	11.83	2.65
8	39033	98.60	2.53
9	38968	98.56	2.53
10	1106	2.96	2.67

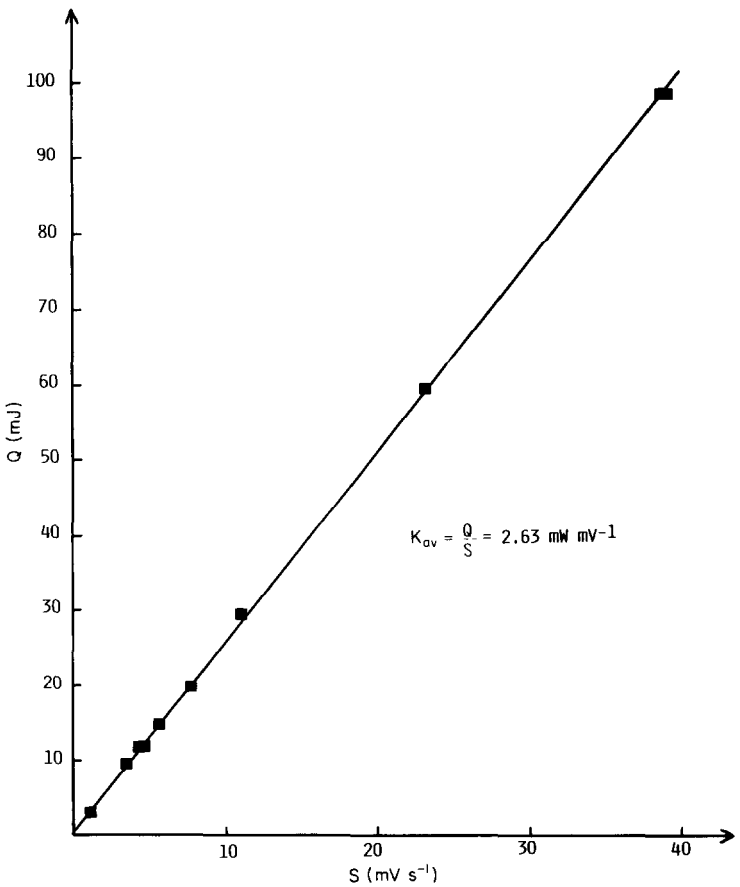

Fig. 3. Isothermal calibration of the calorimeter.

determination of the calorimetric sensitivity $K = Q/S$ ($mW\,mV^{-1}$) is possible. The results are given in Table 1 and are represented as $Q = f(S)$ in Fig. 3. There is a linear relation between 0 and 100 mJ. At 25°C, the average sensitivity is $2.63\,mW\,mV^{-1}$ or $380\,\mu V\,mW^{-1}$. For example, in the calibration given in Fig. 4, the calorimeter rotated 1 turn min^{-1} and the energy was 12 mJ.

Performances

Figure 5 shows the perturbation caused by the mixing of the two liquids (here distilled water). Before the calorimeter rotation (part AB), the background noise was 0.3 μV, and 1 μV during the rotation (part BC). An electrical calibration of about 1 mJ was then performed while maintaining the rotation.

The calorimetric sensitivity was $2.63\,mW\,mV^{-1}\,s^{-1}$. We can estimate the detection threshold as $\pm 0.4\,\mu W$ without rotation and $\pm 1.5\,\mu W$ with a continuous rotation.

Dilution enthalpy of $SrCl_2$

To test the acquisition and processing system, the dilution enthalpy of strontium chloride ($SrCl_2$) in distilled water was measured. The results

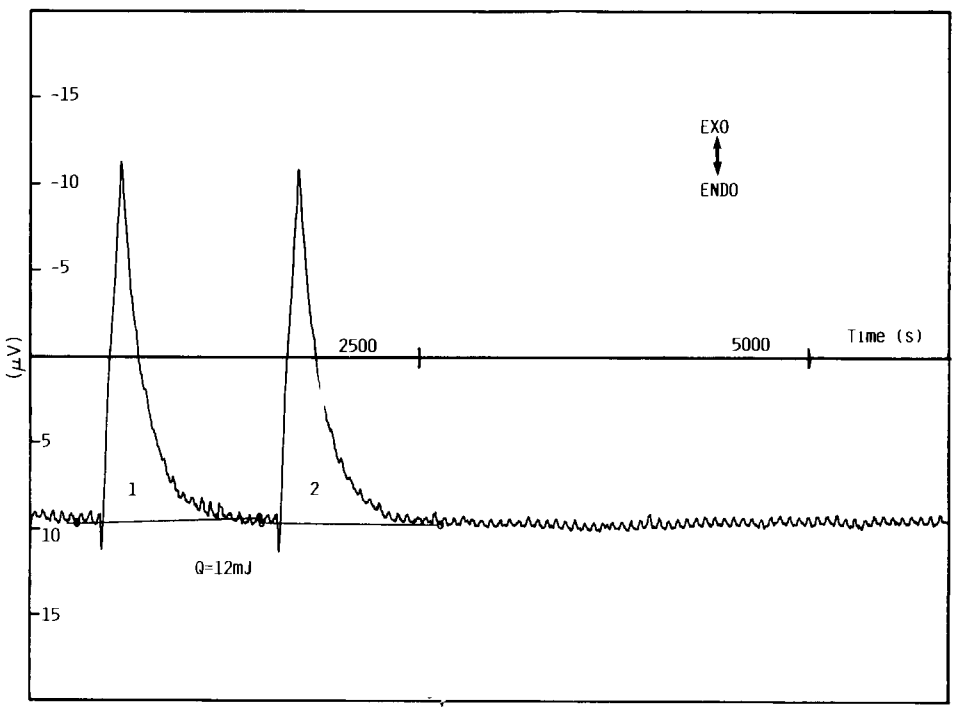

Fig. 4. Experimental calorimetric curves.

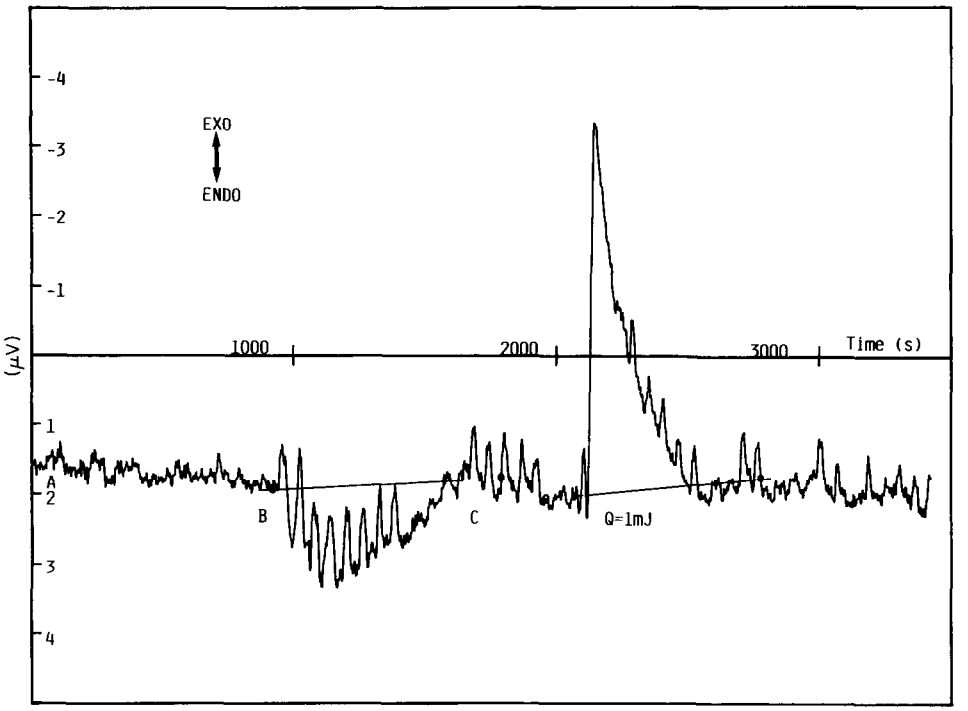

Fig. 5. Evaluation of the thermal noise with and without stirring.

obtained are compared with those in the literature [5] in Table 2 and Fig. 6. An excellent correlation was obtained.

APPLICATION TO THE STUDY OF THE hGH–CO-SOLVENT INTERACTION

Introduction

The characterization of physico-chemical interactions between proteins of industrial interest and their environment is essential in order to optimize their stabilization. The isothermal microcalorimeter was used here to study the interactions between hGH and different co-solvents.

Assuming that

$$\Delta H_{\text{total}} = \Delta H_{\text{interaction(A–B)}} + \Delta H_{\text{dilution(A)}} + \Delta H_{\text{dilution(B)}}$$

to calculate the interaction enthalpies, the dilution enthalpy was first determined separately for A and B (respectively hGH and co-solvent). Then the enthalpy resulting from the mixing of A and B was measured in order to deduce the interaction enthalpy ($\Delta H_{\text{interaction(A–B)}}$)

$$\Delta H_{\text{interaction(A–B)}} = \Delta H_{\text{total}} - (\Delta H_{\text{dilution(A)}} + \Delta H_{\text{dilution(B)}})$$

However, with an equilibrium $A + B \rightleftharpoons AB$, the enthalpy variations (ΔH)

TABLE 2

$SrCl_2$ dilution enthalpy

Experimental values		Reference values	
$SrCl_2$ (mmol)	Q dilution (J mol^{-1})	$SrCl_2$ (mmol)	Q dilution (J mol^{-1})
2.000	0	2.000	0
0.871	−453	0.630	−778
0.480	−851	0.313	−1192
0.241	−1249	0.158	−1598
0.120	−1799	0.086	−1820
		0.032	−2565
		0.016	−2983

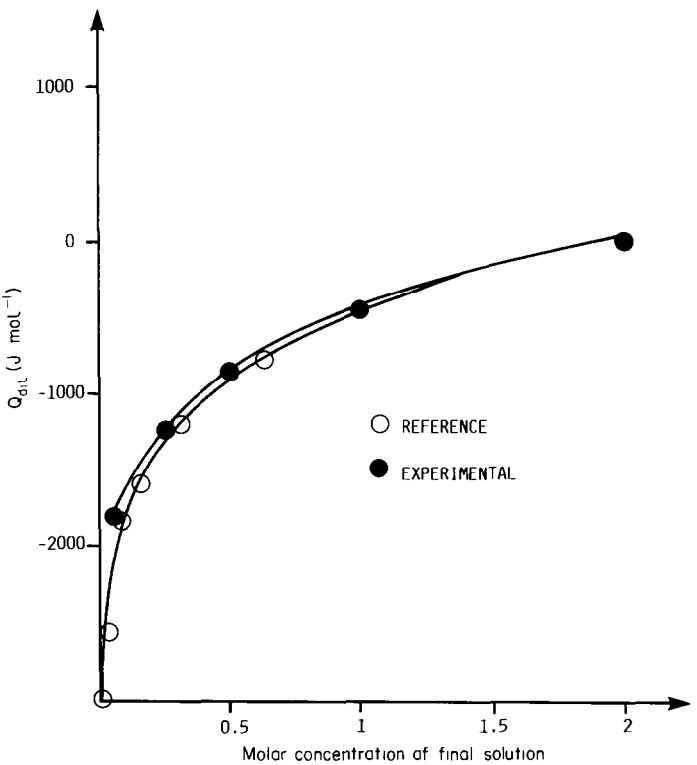

Fig. 6. Determination of the enthalpy of dilution of $SrCl_2$ in water.

were correlated with the heat Q_m, i.e.

$$Q_m = \alpha \Delta H$$

where α is the degree of reaction of the system. Because α is an unknown quantity, the results are expressed in relation to the heat (Q) or to the enthalpy variation (ΔH), α being arbitrarily taken as unity.

Dilution enthalpy conservation was also assumed because the dilution enthalpy functions of the newly formed complexes were not determined. In this work, the aim was to characterize the interactions between hGH and some different co-solvents.

Products

Frozen hGH solutions were purchased from the Centre de Recherche SANOFI ELF BIO Recherche of Labège. The hormone concentration was 5.5 mg ml^{-1} in the presence of ammonium hydrogen carbonate buffer (50 mM, pH 8.0). The different co-solvents (99% purity) were purchased from Aldrich Chimie (benzylic alcohol, chlorocresol, glycerol) and Sigma (alanine). In order to minimize the dilution effects which would screen the weak interaction energy, the co-solvent concentrations were between 10 and 20 mg ml^{-1}.

Dilution effect determination

The results of the dilution energies are given in Table 3. In all cases, the enthalpies were small or below the detection limits.

Interaction enthalpy

The values related to the protein molarity are listed in Table 4.

TABLE 3

Different co-solvent dilution enthalpies

Products	Initial concentration (mg ml^{-1})	Final concentration (mg ml^{-1})	ΔH_{dil} (J 10^{-3})
hGH	5.5	2.7	undetectable
Alanine	1.4	0.7	+0.3
Glycerol	17	8.5	undetectable
Benzylic alcohol	10	5	+0.3
Chlorocresol	3	1.5	−0.2

TABLE 4

hGH interaction enthalpies with different co-solvents

Solvent	Isothermal (25°C) ΔH (kJ mol^{-1})	DSC T_{denat} (°C)	Ref. 6 ΔH_{denat} (kJ mol^{-1})
Water ppi	–	79.9	174
Water + alanine (16 mg ml^{-1})	−9.1	79.2	188
Water + glycerol (17 mg ml^{-1})	−11.6	80.4	187
Water + benzylic alcohol (10 mg ml^{-1})	−26.5	70.0	147
Water + chlorocresol (3 mg ml^{-1})	−51.5	Unfolding	

Alanine and glycerol

The interaction energies of alanine and glycerol were not significantly different, being weakly exothermic. In a previous study [6], DSC results showed that the enthalpy (ΔH) and the temperature (T_m) of the protein denaturation corresponding to the thermal power maximum, were higher in the presence of these molecules, i.e. a significantly protein stabilization.

Benzylic alcohol and chlorocresol

The interaction enthalpies of benzylic alcohol and chlorocresol (see Table 4) are higher than for alanine and glycerol and always exothermic. The strong interactions destabilized the protein; this was confirmed by the DSC results, showing that T_m and ΔH decrease rapidly with rising concentration of benzylic alcohol and that the chlorocresol immediately unfolds the protein.

DISCUSSION AND CONCLUSION

The utilization of the differential isothermal rotating microcalorimeter has solved problems often found in the biological field. Because of its high sensitivity (0.4/1.5 μW), the calorimeter can accommodate small volumes (<1 ml) with low protein concentrations. Such characteristics are already known in injection calorimeters. However, the rotating calorimeter eliminates the viscosity problem found in injection systems and also permits a better thermal equilibrium between the two solutions before mixing, and a better equilibrium in solution after the mixing, owing to the continuously moving block.

The prototype control demonstrated with the $SrCl_2$ dilution has con-

firmed the functioning of the calorimeter. The enthalpic calibration has a sensitivity similar to that found in DSC.

The characteristics of this device are ideal for biological applications in which study conditions are very specific, for example when proteins are difficult to obtain and purify.

The biosynthetic hGH study revealed the weakness of the interactions with alanine and glycerol. Interactions with benzylic alcohol and chlorocresol were more energetic and for the latter, protein denaturation was effective at 25°C.

In comparison with studies of biochemical specific interactions between antigens and antibodies [7], the interaction energies detected with our experiments were lower by a factor of 1000. Hence, this interaction system has been characterized by a low affinity constant. Classical biochemical techniques, e.g. equilibrium dialyses, are probably less adapted to this kind of study than calorimetric or physicochemical techniques. Biochemical research on proteins using techniques such as DSC have studied the thermal "protein denaturation" phenomenon. The enthalpic variations of proteins observed when the temperature was raised, were explained by different mechanisms; these are still not really understood. The mechanisms cited include the breaking of intramolecular bonding or interactions which modified the second and tertiary protein structure, the modification of the interface between protein surface and solvent, and the transfer of apolar groups from the interior of the protein to its surface [8, 9, 10].

Even though the observed thermodynamic modifications were not always demonstrated at chemical or conformational levels, they represent a way of characterizing the stabilization or destabilization of proteins in particular solutions.

A comparative study between the results from microcalorimetery and DSC has demonstrated the qualitative correlation between the two techniques and, therefore, their complementarity. Assumptions can now be applied to the thermal denaturation steps and their relation to the structural variations of proteins [11]. However, with DSC, we found it impossible to determine if protein destabilization was the consequence of direct interaction between co-solvent and proteins, or the consequence of a modification of the protein environment, i.e. a preferential interaction between co-solvent and solvent [12]. Isothermal calorimetry, however, permitted such discrimination, as the reaction heats for direct protein co-solvent interactions are distinct from the dilution heats of co-solvent and protein.

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