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# Involvement of protein solvation in the interaction between a contrast medium (iopamidol) and fibrinogen or lysozyme

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**Abstract**

The interaction between proteins and a radiological commonly-used contrast medium (iopamidol) have been studied by calorimetry. When aqueous solutions of fibrinogen or of lysozyme (20 g/l) are mixed with an aqueous solution of iopamidol (1,3-5 benzendicardoxamid,N,N'-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[(2-hydroxy-1-oxopropyl)amino]-2,4,6-triiodo) in the clinical blood concentration range (26–485 mM), isothermal calorimetry reveals a weak endothermal interaction at a high concentration of iopamidol for both proteins. This endothermal effect does not appear to be due to direct protein–iopamidol association. Differential scanning calorimetry confirms the influence of iopamidol by the change in protein unfolding in the presence of contrast medium, and suggests alterations in the protein solvation as a mechanism. Dilution studies indicate that iopamidol can influence protein solvation even when water molecules are present in a molecular excess of 1000. The influence of iopamidol on the availability of water molecules and the absence of direct interaction with the protein molecules is shown by Raman spectroscopy of two amino acids in the presence of iopamidol. The spectrum of alanine is unchanged at any iopamidol concentration studied, whereas the spectrum lines due to the thiol group of cysteine are shifted in a manner consistent with altered solvation.

*Keywords:* Protein solvation; Amino acids; Fibrinogen; Lysozyme and contrast medium interaction; Isothermal microcalorimetry; Differential scanning calorimetry; Raman studies

## 1. Introduction

Radiological contrast agents are used as diagnostic molecules for the visualization of the vascular system. Clinical symptoms, subsequent to their injection, can be thought of as being relatively bio-incompatible, and the origin of such disorders has been the subject of research [1–3]. A number of studies have attempted to analyze the interactions between CM and plasma proteins [3], and particularly with fibrinogen, since contrast media induce anticoagulant effects and fibrino formation anomalies [4–6]. Functional modifications of these proteins in the presence of non-ionic contrast media (NICM) have been proved conclusively [1], but the formation of protein–NICM complexes in solution has not been demonstrated using classical techniques such as equi-

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librium dialysis [1,7,8]. These methods are efficient for the study of interactions or protein–solute bonds, when the equilibrium constants that characterize such interactions are strong and when they can be determined in ideal solution conditions. But when these conditions are not fulfilled, the study of solute–protein interactions requires a more global thermodynamic approach, involving variations in the state of the solvent and therefore protein–solvent–solute interactions. This type of study has been undertaken by different authors [9,10], who show that protein modifications in the presence of polyhydroxyl solutes (polyethylene glycol, glycerol, sugars) cannot be attributed to direct protein–solute interactions but rather to a modification in the chemical potential of the solvent, due to the presence of the solute which in turn induces modifications in the protein–solvent interactions. The hypothesis that NICM lead indirectly to protein modifications, via the changes they induce in the properties of the solvent, can be put forward in view of: (1) similarities of configuration between these different solutes and the NICM (polyhydroxyl); (2) the lack of demonstrable protein–NICM complex formation for most of the proteins studied, of which functions have been modified; and (3) the radiological injection of 50 to 200 ml of solution at NICM concentration close to the molarity, for which viscosity or osmolarity [11] measurements reveal a non-ideal behaviour, for concentrations starting at 0.2 M.

The present study will attempt to answer the following question: are the protein functional anomalies related to: (1) NICM–protein complex formation, or (2) modifications in the properties of the solvent in the presence of NICM, resulting in the modification of protein solvation?

The experimental approach is based on thermodynamics, allowing an overall analysis of the system under study: the modification of fibrinogen in the presence of iopamidol. The techniques used are isothermal microcalorimetry and temperature-programmed differential scanning calorimetry (DSC). However, the global approach provided by thermodynamics, as well as the molecular complexity of fibrinogen, do not allow a direct interpretation of the molecular phenomena. For this reason, the same type of analysis has been carried out on simpler molecular systems:

(1) The action of iopamidol on a reference protein: lysozyme, which is well characterized from a structural and molecular point of view [12], and which has already been studied in the laboratory [13,14].

(2) The action of iopamidol on two amino acids (AA): alanine and cysteine, which present different polar characteristics. The iopamidol–AA mixtures have been monitored by Raman spectroscopy and have provided molecular information on the atoms or molecular groups involved in the interactions with NICM.

## 2. Materials and methods

### 2.1. Products

The iopamidol solutions (PM = 777) were obtained by dilution in distilled water with a commercially-available iopamiron solution (Schering) at 0.971 M. Lyophilisates of egg-white lysozyme (sigma) and of human fibrinogen (Kabi-Vitrum) were regenerated using distilled water, at concentrations of 20 g/l (respectively, 1.38 mM for the lysozyme and 0.059 mM for the fibrinogen). The amino acid solutions were prepared with lyophilized L-alanine (99% Prolabo) and L-cysteine (99% Merck) regenerated, respectively, at 1.66 M and 0.5 M for the dilution studies, and at 0.18 M for the NICM interaction studies. Except for the iopamidol and amino acids solution which do not contain any salt, the protein solutions contain: 65 mM chloride sodium for the lysozyme solution, and 19 mM citric acid, monosodium salt for the fibrinogen solution. The solutions of amino acids and proteins were filtered (Millipore filter 22 $\mu$m) and adjusted to pH 7.4 with NaOH.

### 2.2. Isothermal microcalorimetry

The prototype of rotative isothermal differential microcalorimeter used, has been described in an earlier article [15]. Briefly, the measuring cell has two compartments (internal and external) containing the two different solutions that were filled at a rate of about 400 $\mu$l each. The exact amounts of each solution were weighed. Once the thermal equilibrium

was reached, the calorimetric block, containing a measurement cell and a reference cell (distilled water), was rotated continuously until the base line returns to its original value. The integration of the calorimetric signal ($V$), corresponding to the thermal effect, with relation to time ($t$), indicated the quantity of heat $Q$, using a calorimeter constant ($k = 2.63$ mW/mV), previously determined by electrical calibration for which incertitude was estimated at 1%. Values were then related to a mole of CM or AA.

The calorimetric sensitivity was of the order of 1 $\mu$W, and with an experiment duration of $\Delta t$, the possibility of error was of $10^{-6} \cdot \Delta t$ (J). Electrical calibration and preparation of solutions were carried out for each experiment.

When two solutions with the same solvent, but each having a distinct solute (A and B), are mixed together, the heat observed comes from: (1) the dilution of each solute, in other words an evolution in solvent–solute interaction, and (2) interaction between molecules A and B, possibly in the form of an equilibrium, to form a complex AB. Consequently, our study has dealt, in a first stage, with the energy phenomena observed in the dilution of different, separately-analyzed elements (NICM, amino acids and proteins). These quantities were used, in a second stage, to characterize the NICM–proteins or NICM–amino acids heats of interaction ($Q_{\text{interaction}}$). With the assumption that the heat of dilution was not modified in the presence of the two solutes, the amount of heat measured experimentally during their mixing ($Q_{\text{total}}$) could be decomposed with the following equation:

$$Q_{\text{total}} = Q_{\text{dilution A}} + Q_{\text{dilution B}} + Q_{\text{interaction AB}} \quad (1)$$

where molecules A correspond to proteins or amino acids, and B to contrast medium molecules.

### 2.3. Differential scanning calorimetry (DSC)

The calorimeter used (Mettler TA 2000B equipped with a prototype sensor, which can reach sensitivity lower than 0.1 $\mu$W) has been described in a previous article [14]. Briefly, 120 $\mu$l of protein solution containing different contrast medium concentrations were dropped into the test cell. An identical quantity of distilled water or buffer was poured into the reference cell. The signal was recorded ($\mu$W/g of solution) under a temperature range from 10 to 110°C with a heating rate of 2°C/min. Integration of signal variations as a function of time indicated the quantity of heat $Q$ involved in the protein unfold reaction which was equivalent, in constant pressure conditions, to the transformation enthalpy ($\Delta H$) of the protein (degree of reaction $\alpha$ is taken as 1). The maximum transition temperature ($T_m$) corresponds to the maximum intensity of the transition peak.

The calorimetric sensitivity and the method of error determination were similar to those for the isothermal calorimeter.

Table 1
Dilution heat of iopamidol and amino acids

| Alanine | | | Cysteine | | | Iopamidol | | |
|---|---|---|---|---|---|---|---|---|
| Final concentration (M) | Final molar fraction ($10^{-3}$) [a] | $Q$ dilution [a] (J/mol) | Final concentration (M) | Final molar fraction ($10^{-3}$) [a] | $Q$ dilution (J/mol) | Final concentration (M) | Final molar fraction ($10^{-3}$) [a] | $Q$ dilution (J/mol) |
| 1.66 | 32.1 | 0.0 | | | | | | |
| 0.83 | 16.1 | $-208.5 \pm 3.3$ | | | | 0.97 | 26.0 | 0.0 |
| 0.38 | 7.4 | $-338.3 \pm 6.0$ | 0.51 | 9.5 | 0 | 0.59 | 13.2 | $-1065.1 \pm 12.3$ |
| 0.29 | 5.3 | $-367.2 \pm 7.1$ | 0.27 | 4.9 | NS | 0.46 | 10.1 | $-1375.8 \pm 15.9$ |
| 0.22 | 3.9 | $-373.2 \pm 8.3$ (NS) [b] | 0.11 | 2.1 | NS | 0.25 | 5.0 | $-1937.2 \pm 23.3$ |
| 0.13 | 2.3 | $-382.3 \pm 11.5$ (NS) | 0.03 | 0.5 | NS | 0.11 | 2.0 | $-2262.1 \pm 31.9$ |
| | | | | | | 0.06 | 1.0 | $-2362.7 \pm 41.6$ |
| | | | | | | 0.04 | 0.7 | $-2400.3 \pm 50.8$ (NS) |

[a] The measurement's incertitude was estimated at a maximum of 0.5 mJ. This incertitude and the relative errors, dependent on calibration (see ref. [15]) were indicated.

[b] No significative (NS) difference between two successive dilutions were indicated.

### 2.4. Raman spectroscopy

The analysis of iopamidol–amino acid solutions was carried out using a double-beam spectrometer (CODERG.PHO). The light source was provided by an ionized argon laser (Spectra Physics 165-06), continuously emitting nine spectrum lines, and the most intense ones were used for this work (green line: 514.5 nm and blue: 488 nm). The system has been described in a previous publication [16].

After controlling the solvent (distilled water) Raman spectra, the iopamidol and amino acids (alanine and cysteine) Raman spectra and their mixtures were recorded and compared.

All spectra were recorded at ambient temperature and in identical experimental conditions (resolution = 6 $cm^{-1}$, laser power = 1.1 W).

The amino acid solutions were used at concentrations of 0.5 M and iopamidol solutions at 0.485 M.

## 3. Results

### 3.1. Isothermal microcalorimetry

#### 3.1.1. Dilution enthalpies

The determination of dilution enthalpies was carried out with successive dilution of solutes. The enthalpy of the initial solution (the most concentrated) was taken by convention to be equal to zero. The quantities of heat of the successive dilutions were then added up, in order to obtain the $Q_{dilution}$ values, relative to the initial solution (Table 1).

For iopamidol, by fitting the experimental points to a theoretical curve, we obtained the following result:

$$Q_{\text{dilution NICM}} = -9.08e^{-5}X^2_{\text{NICM}} + 1.19e^{+5}X_{\text{NICM}} - 2490.4 \quad (2)$$

where $X$ is the molar fraction of the NICM.

These dilutions were not significantly modified in the presence of the equivalent salt concentrations used in protein solutions.

These results indicated a peculiar effect between CM and $H_2O$ molecules, which was not observed with protein and cysteine solutions at the concentrations tested. Only alanine solutions developed dilution heat but only for concentrations greater than 0.2 M and with a calorific effect 5 times weaker than for the NICM.

#### 3.1.2. Interaction enthalpies

The results of the interaction enthalpies obtained with amino acids are given using the method of calculating interacting energies as described above in Eq. (1) (Fig. 1). The numerical data are summed in Table 2. The thermal evolution represented in Fig. 1 shows that the experimental thermal effect ($Q_{total}$) was slightly exothermal in the case of the mixture of the two amino acids and the iopamidol. The interaction enthalpies ($Q_{interaction}$) revealed the endothermal character of both types of mixtures.

Table 2
Iopamidol–amino acids interactions

| | Final concentration [a] of iopamidol mmol/l | Final concentration [a] of amino acids mmol/l | $R$ (mol of AA/ /mol of CM) | $Q_{total}$ J/mol CM | $Q_{dilution}$ J/mol CM | $Q_{interaction}$ [b] J/mol CM |
|---|---|---|---|---|---|---|
| Mixture | 124.0 | 62.9 | 0.51 | −27.7 | −150.4 | 122.6 ± 8.2 |
| Alaninie | 79.1 | 110.8 | 1.40 | −72.0 | −259.7 | 187.7 ± 13.8 |
| Iopamidol | 54.1 | 134.8 | 2.49 | −86.6 | −317.2 | 230.6 ± 18.4 |
| | 31.1 | 156.2 | 5.02 | −93.4 | −368.4 | 275.0 ± 26.6 |
| Mixture | 108.0 | 79.0 | 0.73 | −136.1 | −248.7 | 112.6 ± 12.5 |
| Cysteine | 74.0 | 115.5 | 1.56 | −153.9 | −270.8 | 116.9 ± 15.4 |
| Iopamidol | 52.6 | 136.8 | 2.60 | −179.0 | −320.5 | 141.5 ± 19.7 |
| | 39.4 | 149.4 | 3.79 | −196.4 | −350.8 | 154.4 ± 23.9 |

[a] Initial solution's concentrations were at 0.18 M for the amino acid solutions and at 0.2 M for iopamidol solutions.

[b] Confidence intervals were calculated by addition of two incertidudes: one originated from $Q_{total}$ and was determined as in Table 1, and the second originated from predicted values of $Q_{dilution}$.

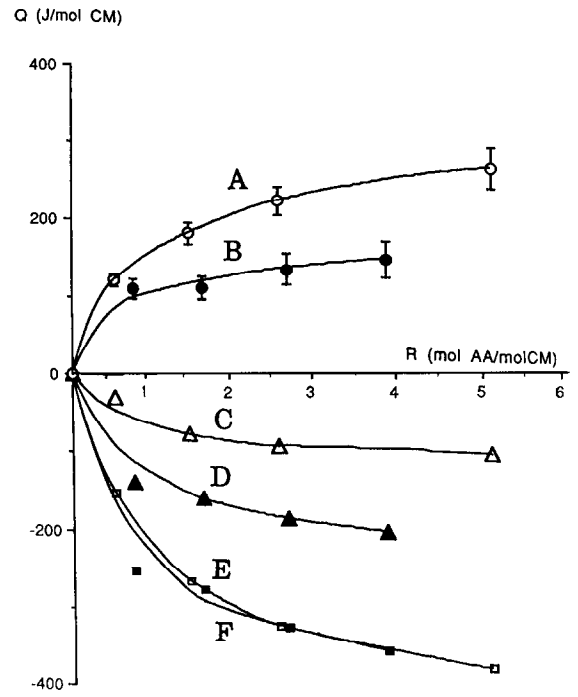


Fig. 1. Experimental calorific effects $Q_{total}$ (triangle), predicted values of heat dilution $Q_{dilution}$ (square), and computed interaction heat $Q_{interaction}$ (circle) for iopamidol–alanine (open symbols) and iopamidol–cysteine (filled symbols) mixtures. (A) $Q_{interaction}$ iopamidol/alanine; (B) $Q_{interaction}$ iopamidol/cysteine. (C) $Q_{total}$ iopamidol/alanine. (D) $Q_{total}$ iopamidol/cysteine. (E) $Q_{dilution}$ iopamidol/alanine. (F) $Q_{dilution}$ iopamidol/cysteine.

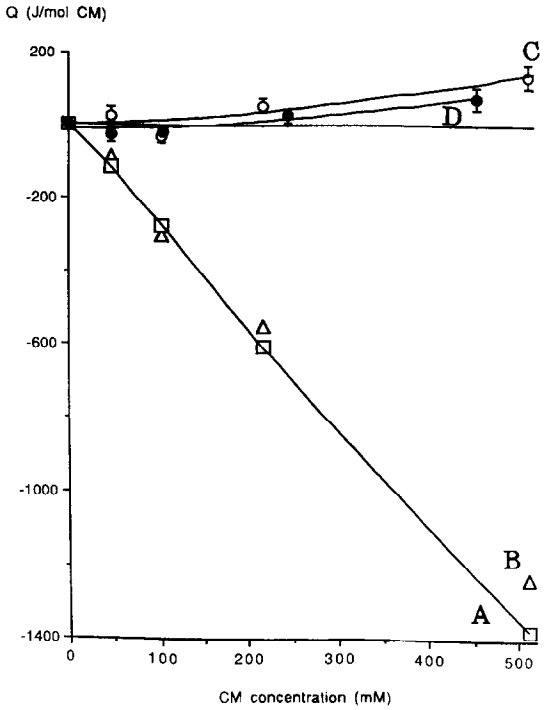


Fig. 2. Endothermal resultant of $Q_{interaction\ (NICM-protein)}$ obtained with a high concentration of iopamidol mixed with fibrinogen (open symbols) and lysozyme (filled symbols). (A) $Q_{dilution}$ (of NICM). (B) $Q_{total}$ (iopamidol/fibrinogen). (C) $Q_{interaction}$ (iopamidol/fibrinogen). (D) $Q_{interaction}$ (iopamidol/lysozyme).

The heat of interaction of iopamidol–protein mixtures is given in Fig. 2 and the concentrations used are listed in Table 3. The resulting endothermal effects were observed for the highest concentrations of iopamidol with both proteins tested. These effects were significant at iopamidol concentrations of 242 and 485 mM, and were more noticeable in the case of fibrinogen than for lysozyme (Table 3).

Table 3
Iopamidol–protein interactions

| | Final protein concentration (mM) | Final CM concentration (mM) | $Q_{total}$ (J/mol CM) | Qdilution (J/mol CM) | $Q_{interaction}$ [a] (J/mol CM) | $R$ (mol CM/ mol protein) |
|---|---|---|---|---|---|---|
| Lysozyme + iopamidol | 1.38 | 512 | −1176.8 | −1254.3 | 77.5 ± 30.9 | 371.0 |
| | 1.6 | 218 | − 622.1 | − 652.6 | 30.5 ± 19.7 | 136.3 |
| | 1.46 | 103 | − 252.3 | − 237.4 | −14.9 ± 15.5 | 70.5 |
| | 1.38 | 48 | − 156.7 | − 132.9 | −23.8 ± 24.2 | 34.8 |
| Fibrinogen + iopamidol | 0.069 | 454 | −1231.9 | −1372.7 | 140.8 ± 33.3 | 6579.7 |
| | 0.066 | 246 | − 547.1 | − 606.1 | 59.1 ± 17.8 | 3727.3 |
| | 0.055 | 105 | − 297.8 | − 269.6 | −28.8 ± 16.2 | 1909.1 |
| | 0.061 | 47 | − 84.0 | − 111.9 | 28.0 ± 23.6 | 770.5 |

[a] Confidence intervals were calculated like in Table 2.

In all the experiments which were carried out, we did not observe any exothermal interaction enthalpy which would indicate an association mechanism between amino acids or proteins and iopamidol, and which could be used for the classical calculation of an equilibrium constant. Thus it was necessary to confirm that the proteins were modified in the presence of CM after obtaining the results.

### 3.2. Differential scanning calorimetry

The results of the DSC experiments, carried out in this study to help understand the overall effect of contrast medium on fibrinogen and lysozyme are shown in Fig. 3, and the characteristic parameters of the signals obtained are shown in Table 4.

Since the thermal evolution of iopamidol in solution did not show any apparent variation in calorific capacity ($C_{p_{appar.}} = dH/dT$) within the temperature range studied (10 to 110°C), it will be unambiguous to quantify the variation of thermal protein unfolding in presence of CM.

In the absence of iopamidol, lysozyme revealed an endothermal effect of 490 kJ per mole of lysozyme, with a maximum temperature $T_m$ of 77°C. For fibrinogen, we observed two endothermal peaks,

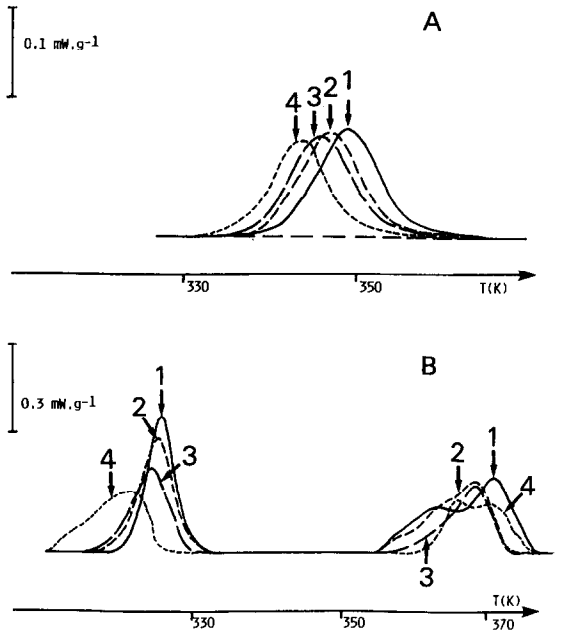


Fig. 3. DSC curves of thermal transformation of the lysozyme and domains D and E of fibrinogen, according to increasing quantities of iopamidol. (A) Lysozyme 2% + iopamidol (× mM): 1 — $R = 0$ (0 mM); 2 — $R = 85$ (120 mM); 3 — $R = 170$ (242 mM); 4 — $R = 342$ (485 mM). (B) Fibrinogen 2% + iopamidol (× mM). 1 — $R = 0$ (0 mM); 2 — $R = 462$ (60 mM); 3 — $R = 923$ (242 mM); 4 — $R = 3700$ (485 mM).

Table 4
Variation in $\Delta H$ and $T_m$ of the thermal transformation of proteins in the presence of iopamidol

| Mixtures developed | $R$ (mol NICM/mol protein) | $\Delta H$ (kJ/mol protein) | $T_m$ (°C) |
|---|---|---|---|
| Iopamidol + fibrinogen 2% (domain D) | 0 | 2037 | 52.9 |
| | 2015 | 2102 | 52.2 |
| | 4062 | 1759 | 51.1 |
| | 8080 | 1676 | 51.0 |
| Iopamidol + fibrinogen 2% (domain E) | 0 | 3022 | 98.6 |
| | 2015 | 2167 | 95.5 |
| | 4062 | 1659 | 95.3 |
| | 8080 | 1626 | 94.7 |
| Iopamidol + lysozyme 2% | 0 | 491 | 76.9 |
| | 3 | 485 | 76.5 |
| | 10 | 474 | 76.4 |
| | 41 | 456 | 75.7 |
| | 85 | 428 | 72.7 |
| | 170 | 397 | 71.7 |
| | 342 | 379 | 71.0 |

already described by Privalov and Medved [17] as corresponding to the thermal transformation of fibrinogen domains D and E, respectively, of 2037 kJ/mol and 3022 kJ/mol and with maximum temperatures of 52.94 and 98.64°C.

Fig. 3 illustrates that the lysozyme and fibrinogen underwent the same kind of modification in the presence of iopamidol, i.e., a drop in $T_m$ and $\Delta H$ in proportion to the contrast medium concentration.

According to the calculation of the relative percentage of changes in the protein transformation enthalpy ($\Delta H$) with the upper concentrations of iopamidol, the effects were more significant for the fibrinogen domain E than for the domain D (−46% and −18%, respectively). The effects observed for the lysozyme corresponded to intermediate values (variation in $\Delta H$ of −23%).

In view of the more compact structure of lysozyme and the domain D of fibrinogen compared to domain E, and with a probable difference of solvation between the more or less globular protein forms, we suggest, with the quantitative differences observed, some molecular explanations concerning the protein accessibility of CM or the protein solvation changes in the presence of CM. Nevertheless, the macroscopic data obtained by calorimetry must be correlated with molecular data in order to put forward a realistic molecular interpretation.

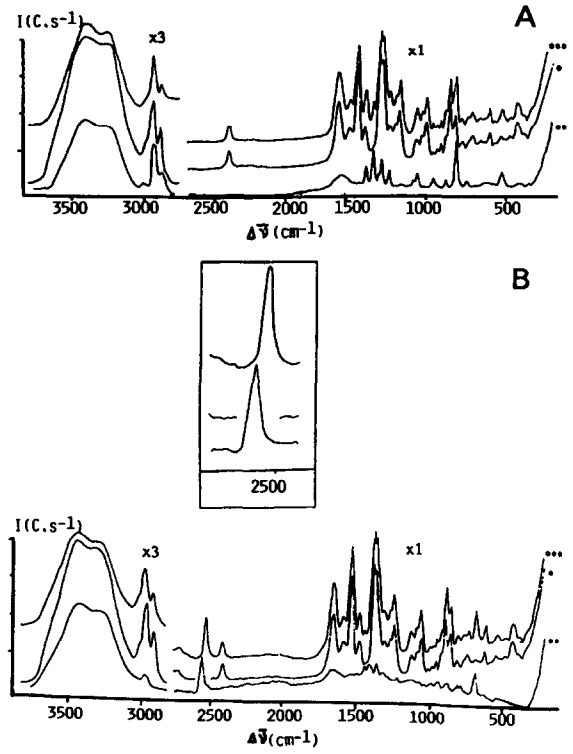


Fig. 4. Raman spectra of iopamidol (*), of amino acids (**), and iopamidol–amino acid mixtures (***) (alanine (A) and cysteine (B)). (Spectra corresponded to the variation in intensity of photon impact per second ($C \cdot s^{-1}$) according to the frequency $\Delta\bar{\nu}$).

### 3.3. Raman spectroscopy

Raman spectra of iopamidol, alanine and cysteine, and of their mixtures are shown in Fig. 4. The results show a shift of 10 $cm^{-1}$ in the peak, located around 1585 $cm^{-1}$ for the cysteine solution spectra, compared to the mixture with iopamidol. This peak can be attributed to the valency vibration of the SH bond of cysteine. However, no modification was observed for the NICM spectra, in any of the mixtures made, and the iopamidol–alanine mixture spectra did not appear to be different to the reference spectra.

## 4. Discussion

### 4.1. Iopamidol–solvent interactions

Our results (Table 1) show that iopamidol dilutions exhibit low but significant energy levels (from −1 to −2 kJ/mol of iopamidol). This quantity is slight compared to hydrogen bond interactions (an order of −5 kJ/mol). However, the quantities measured by calorimetry may correspond to the resultant of endothermal and exothermal effects. The data can thus be interpreted in two ways:

(1) The observed energy changes can be attributed to a break-up of the associated forms of NICM, due to an increase in the associations with available water molecules. This interpretation was set out in a previous paper [8] based on the determination of the osmotic coefficient of the concentrated NICM solutions. In view of our results, it supposes that the NICM dissociation will be effective at high dilution (< 50 mM).

(2) Another interpretation is that the heat of dilution can correspond only to weak NICM–solvent interactions. The phenomena observed can be repre-

sented by a succession of $n$-equilibria, each characterized by an enthalpy ($\Delta H$) and a degree of reaction ($\alpha$), where W stands for the water molecule:

$$\mathrm{NICMW}_i + \mathrm{W} \Leftrightarrow \mathrm{NICMW}_{i+1}\ (\Delta H_i, \alpha_i) \qquad (3)$$

$$Q = \Delta H_{\text{dilution}} = \sum_1^n d\alpha_i\, \Delta H_i \qquad (4)$$

However, the data obtained in this study do not allow us to analyze correctly the characteristics of the $\mathrm{NICMW}_i$ complexes, because the enthalpies measured represent the total sum of the different processes.

With the density data ($d = 1.41$) of an iopamidol solution at a concentration $C = 754.5$ g/l (0.971 M), it is possible to determine the apparent specific partial volume ($\bar{V}_i$) of the iopamidol, when the density of the solvent ($d_0$) and the concentration of the solution ($C$) is already known: $\bar{V}_i = 1/d_0 - 1/C\ (d/d_0 - 1)$. The molar partial volume of the iopamidol is thus determined at 0.355 l/mol. If we approximate iopamidol to be of spherical shape, we can also estimate the molecular surface developed by the iopamidol as 340 Å$^2$. In the same way, if the water molecule corresponds to a sphere with a radius of $r = 1.4$ Å, the surface developed by this sphere, projected onto a plane is approximately 5 Å$^2$. Given the relation between these two surfaces, 68 water molecules would be required if a molecule of iopamidol is to be covered by a mono-layer of water molecules. Although these geometrical approximations are debatable, we can conclude that the influence of the iopamidol extends beyond the first layer of solvation because the quantities of heat recorded evolve in a nearly linear fashion (Eq. (2)) with the decrease in molar fraction, even beyond a ratio $R$ (mol $H_2O$/mol iopamidol) = 1000.

In conclusion, the determination of physico-chemical properties of iopamidol solutions should consider variations in the state of the solvent. This is indicated by the results of the NICM dilution, the number of free water molecules being reduced when NICM molecules are present. This reduction thus decreases the molar fraction of free water and consequently the chemical potential of the solvent. If the molar fractions of the iopamidol ($X$) are to be determined precisely, the complexes formed with the iopamidol and the solvent ($\mathrm{NICMW}_i$) must be taken into account:

$$X = \Sigma n_i(\mathrm{NICMW}_i) / \left[\Sigma n_i(\mathrm{NICMW}_i) + \left(N - \Sigma n_i(\mathrm{W}_i)\right)\right] \qquad (5)$$

*(where $\Sigma n_i$ = the number of iopamidol molecules and $N$ = the number of water molecules).*

### 4.2. Interactions between NICM and amino acids

The determination of an equilibrium constant with two solutes has been achieved using a calorimetric process in which the interactions between solute molecules were strong enough to be able to disregard the energy variations linked to modifications in the interactions with the solvent (e.g., antigen–antibody reactions where $\Delta H$ is of the order of $-40$ kJ/mol) [18]. In contrast to this data, the different quantities of heat that were detected in our experiments (NICM–AA and NICM–protein interactions) were too low for the energy variations linked to the solvent evolution to be disregarded.

The different results concerning amino acid–iopamidol mixtures can be interpreted in two different ways:

(1) The endothermal behaviour (Fig. 1) can be associated, in conditions of equilibrium (prior hypothesis), with a break-up of the $\mathrm{NICMW}_i$ complexes that can initially be found in the solution, leading to the formation of a new NICM–amino acid complex.

As described earlier for iopamidol dilution, the NICM–solvent solution can be defined as a thermodynamic system presenting a series of equilibria (Eq. (3)). The same can be said in theory for the amino acid–solvent solutions. Before the two solutions (NICM and amino acids) are mixed, two series of equilibria can thus be found, represented by the following equations:

$$\mathrm{NICMW}_{i-1} + \mathrm{W} \Leftrightarrow \mathrm{NICMW}_i$$

$$\text{and } \mathrm{AAW}_{j-1} + \mathrm{W} \Leftrightarrow \mathrm{AAW}_j \qquad (6)$$

When the two solutions are mixed, the resulting endothermal phenomena can thus be due to a

NICM–AA complex formation with a liberation of water molecules:

$$NICMW_i + AAW_j \Leftrightarrow NICMAAW_{i'j'} + [(i+j) - (i'+j')]W \quad (7)$$

The interpretation of the endothermal resultant can be compared to that of different writers [19,20] on the subject of a so-called, entropic-dominant interaction or hydrophobic interaction. However, this interpretation is debatable from two points of view.

(a) The molecular interpretations of an entropic effect in terms of hydrophobicity are contested by different scientists [21,22].

(b) Raman spectroscopy has not revealed any change in NICM spectra, with or without amino acids (see Fig. 4). This does not provide absolute proof of the absence of $NICMAAW_{i'j'}$ compounds, because such a complex may exist in a low proportion with respect to $NICMW_i$, and/or may imply energy changes that are so weak they cannot be observed with the Raman process. However, such observations do not point to a $NICMAAW_{i'j'}$ complexation.

The structural difference between the two amino acids is relative only to the thiol group of cysteine ($-CH_2-SH$), which is absent in alanine ($-CH_3$). The Raman spectra of alanine are not modified by the addition of iopamidol as well as the $CH_2$ cysteine group. Therefore, the interaction of NICM with amino acids is not likely to occur in non-polar groups and the existence of hydrophobic interaction cannot be ascertained.

On the other hand, the cysteine spectra show a modification in the spectrum lines that can be attributed to S–H bonding. As the spectra are not modified at the level of C–S bonds, it can be said that iopamidol only interacts with the hydrogen atom of the S–H bond. The modification observed in the S–H line is a frequency shift towards low frequencies (10 $cm^{-1}$). This shift corresponds to a significant change in the hydrogen surrounding. Since the intensity of the S–H line does not show any variation, it can be concluded that the S–H bond is not broken. As the lines corresponding to iopamidol remain unchanged one can rule out the existence of direct interaction between the iopamidol and the SH group of the cysteine. We can put forward the tentative explanation that this amino acid, which can form a hydrogen bond by his thiol group, is particularly sensitive to variations of the solvent.

(2) With these remarks, it is possible to put forward a further interpretation of the results. If the dilution of the two solutes induces a significant decrease of the available or free water molecules, then the real NICM and amino acids molar fractions, resulting from the mixing experiments, will be different from the case of dilution experiments in pure water. Consequently, the dilution heat observed with the mixture will be less than the dilution heat measured during dilution experiments. These difference could explain the endothermal behaviour resulting from the difference between the real mixing dilution $Q_{dilution}$ (which could correspond to the $Q_{total}$ in Eq. (1)), and the $Q_{dilution}$ calculated with dilution experiments.

In conclusion, this study shows that the presence of the two solutes (amino acids and NICM) in the solution does cause a real, specific phenomenon to occur, involving water molecules, and the difference observed in Raman spectroscopy probably results from a modification of the amino acid solvation rather than from a direct interaction between the two solutes.

### *4.3. Iopamidol–lysozyme and iopamidol–fibrinogen interaction*

The endothermal resultants observed in the case of the proteins with a high concentration of iopamidol (Fig. 2) can be interpreted as above in the case of the amino acids, in terms of a reduction in the heat of iopamidol dilution in the presence of proteins.

With the high NICM concentrations used, and with the non-ideal behaviour of the solutes, there may be competition between proteins and iopamidol for the available water molecules. The calculation of the molar fractions of iopamidol, necessary to deduce $Q_{dilution}$ in the determination of $Q_{interaction}$, was based on the independence of the different molecules in solution (iopamidol, protein, $H_20$). If the free water molecules must only be taken into account for the determination of the final molar fractions, the water molecules that are 'associated' with the proteins can be ignored in computing the molar fractions of iopamidol.

A characterization test was carried out to evaluate the difference between the molar fractions used without consideration of the molecular associations ($X_{\text{iop calculated}}$ corresponding to the quantities weighted and permitted with the dilution experiments to determine $Q_{\text{dilution}}$), and the molar fractions ($X_{\text{iop estimated}}$) which could be estimated by the results of the experiment with the assumption that the energy modification ($Q_{\text{total}}$), is only due to the dilution of iopamidol (i.e. $Q_{\text{interaction}} = 0$ and $Q_{\text{total}} = Q_{\text{dilution}}$). The difference between the calculated molar fractions and the estimated molar fractions concerns the water molecules which are not available for the NICM dilution and are 'associated' with the proteins.

This method has been applied numerically to the mixture of lysozyme and iopamidol at the highest concentration (0.485 M). The results are shown in Table 5.

The difference in available water molecules can thus be represented by: 44.8–41.8 = 3 mmol $H_2O$; in proportion to 1.38 $\mu$mol of lysozyme, it gives: $2200 \pm 800$ moles $H_2O$ per mole of lysozyme, or $2.6 \pm 1.0$ g $H_2O$/g lysozyme. Through crystallography determinations, the quantities of water 'associated' with the lysozyme never exceed 230 molecules of water per molecule of lysozyme [23], and by the dynamics of the Kerr effect and dielectric relaxation, 410 molecules of water were detected [24]. The same type of calculation with the fibrinogen and the iopamidol produces the following result: $11.5 \pm 2.6$ g $H_20$/g fibrinogen, whereas the fibrinogen solvation was usually evaluated at 1 g $H_2O$/g of fibrinogen [25].

These results show that, when determined through calorimetric techniques, the quantities of water molecules involved in protein solvation are about 10 times higher than when determined by the other techniques. This disproportion was emphasized previously by Parsegian and co-workers [26].

The accessible surface area of lysozyme [27] is evaluated at about 6600 Å$^2$. Using the method to estimate the water molecule surface mentioned above, the water molecules amount to 2200 which seems a realistic number; it corresponds approximately to a double-layer of water. A part of the water molecules detected here, might correspond to the water molecules which interact more weakly with the protein than those detected by the other techniques.

Thus, as in the case of amino acids, the distribution of water molecules in the protein solution is changed in the presence of NICM molecules. Consequently, the state of the protein surface can be modified. This modification seems especially probable because the DSC results, obtained in the laboratory and in the course of this work, can be discussed and interpreted in the following way.

The characteristic analysis of the thermal phenomena observed on a protein by DSC has not allowed a correct interpretation in terms of the molecular mechanism. Most of the work dealing with this subject [28] has put forward the hypothesis of a reversible phenomenon to explain the endothermal effects. However, this hypothesis has not been proven by experiments. According to the work previously carried out in the laboratory on lysozyme, it has been possible to set a temperature threshold ($T_c$ = 70°C) up to which the thermal transformation of the protein shows a reversible character. Beyond this temperature, the experimental irreversibility of the endothermal phenomenon is been observed [14] and it allows us to provide the following hypothesis for the states of lysozyme: $N' \Leftrightarrow N \rightarrow U \Leftrightarrow U'$ where N and N′ represent the two forms in equilibrium of the native lysozyme structure and, U and U′ repre-

Table 5

| Quantity of heat considered (J/mol NICM) | Molar fractions corresponding | Number of moles deduced | | |
|---|---|---|---|---|
| | | Iopamidol (mmol) | Lysozyme ($\mu$mol) | $H_2O$ (mmol) |
| $Q_{\text{total}} = Q_{\text{dilution}} + Q_{\text{interaction}}$<br>$Q_{\text{dilution}} = -1254$ | $X_{\text{iop calculated}} = 1.13 \times 10^{-2}$ | 0.512 | 1.38 | 44.8 |
| $Q_{\text{total}} = Q_{\text{dilution}}$<br>$Q_{\text{total}} = -1177$ | $X_{\text{iop estimated}} = 1.21 \times 10^{-2}$ | 0.512 | 1.38 | 41.8 |

sent two forms in equilibrium of the unfolded structure. By analogy with the parallel data obtained by circular dichroism and by DSC on the human growth hormone (HGH) [13], it is possible to attribute a significant part (about 40% in the case of HGH) of the endothermal phenomenon to a modification in the protein medium and not to a change in its secondary structure. It can be suggested from this data that the modifications in thermal behavior observed in HGH and those which take into account states N′ and U′ of the lysozyme, can be attributed to equilibria between the proteins and the solvent molecules.

This interpretation is confirmed by the fact that the ranges of temperature used in these experiments (10 to 110°C) do not in theory allow us to assume that stable bond modifications, of the covalent bond type (disulfide bridge, peptide bond), are involved in the transformations observed. On the other hand it could be said that they correspond to the hydrogen bond, ionic bond, or Van der Waals type of interaction.

In quantitative terms, the variations in the transformation enthalpy of the lysozyme, or of the fibrinogen domains D and E (Fig. 3) in the presence of iopamidol, are weak when compared to the results observed with the pH variation in protein solutions [13], or those obtained with lysozyme solutions containing concentrations of sodium dodecyl sulfate at 0.5% (work now being developed). In this latter case, the lysozyme does not show a thermal transformation peak in the same temperature range as that studied in this work.

According to this data, it is possible to put forward the hypothesis that proteins in the presence of NICM show a decrease in their 'pool' of water molecules, and/or that the structure of the protein surface and its solvation layer are simply modified.

This interpretation allows the comprehension of the protein functionality changes induced by contrast media without detectable NICM–protein association.

## 5. Conclusion

According to the data on NICM dilution, as obtained by isothermal microcalorimetry, we are not able to consider the observation of thermal effects for high dilutions as an ideal one. Indeed, to be ideal it would need to be characterized by a mixture enthalpy of zero. The quantities of heat observed, indeed, correspond to modifications in the solute–solvent interactions, and they can be detected above a ratio of 1000 water molecules per molecule of iopamidol. The iopamidol molecules probably impose a specific organization on the water molecules in their proximity, beyond the first solvation layer. It can be assumed that the influence of these molecules on the water molecules will have a repercussion on other molecules of the solution.

The results obtained by isothermal calorimetry concerning the proteins can be compared with the results for the iopamidol–amino acid interaction. Indeed, the formation of a NICM–protein complex does not really provide an explanation for the endothermal effect that is observed for a high concentration of NICM and the absence of a significant thermal effect for lower concentrations.

Taking the current interpretations of the endothermal effect observed in DSC with the proteins [27,28], it can be said that part of their thermal transformation enthalpy is connected to changes in the solvation layer of the protein. Then, it can be suggested that protein–solvent surface interactions represent temperature-dependent equilibria ($\Delta G = \Delta H - T\Delta S$), which are modified by an increase in temperature towards a state of desolvation. With this hypothesis, the equilibrium effects previously defined in the evolution of lysozyme according to the thermal cycles [14] may correspond to 'solvation states' for the forms N and U, and 'desolvation states' for forms N′ and U′ of the lysozyme. The results thus observed for the NICM acting on lysozyme and fibrinogen may tend towards a modification in the solvation layer of the proteins. Indeed, by DSC, the modifications in transformation enthalpy for both proteins in the presence of iopamidol are slight when compared to all the effects recorded on the proteins (pH effect and SDS effect) and are only noticeable for high NICM concentrations. For these same concentrations, it has been shown by isothermal calorimetry that the endothermal effects are probably connected to a bad estimation of the free water molecules that are available for the dilution of iopamidol, leading to an error in the determination of the real molar fractions. By the same process, no exothermal effect of

complexation can be observed for the iopamidol. By analogy with the results obtained with amino acids which show the same endothermal effect, not accompanied by modification of the Raman spectra of iopamidol, it would be reasonable to suggest that there is no iopamidol–protein complex formation, whereas there is probably competition between the two compounds for the available water molecules in solution. This hypothesis allows all the results obtained by calorimetry (isothermal and DSC) to be understood, and tends towards the preliminary results obtained by dialysis, in equilibrium with non-ionic NICM, where an increase in the NICM concentration (1 to 2%) was observed in the non-protein compartment. This latter data, which should be confirmed by further experiments, gives rise to the hypothesis of the preferential hydration [10] of the NICM when they are mixed, corresponding to partial 'desolvation' of one or two elements when they are in competition for available water molecules

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