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Interaction of myo-inositol hexakisphosphate with biogenic and synthetic polyamines†

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Abstract

Within all the eukaryotic cells *myo*-inositol phosphates (Ins*P*s) are an important group of biomolecules that have been potentially related to signaling functions. The most abundant member of this family in nature is InsP_6 (phytate, L^{12-} in its fully deprotonated form). The intricate chemical behavior of this molecule demands a great effort to understand its function in the cell medium. In this work we follow our earlier studies on the interaction of InsP_6 with metal cations, by inclusion of polyamines (both biogenic and synthetic) as potential agents to produce stable adducts. The stability constants of InsP_6-amine adducts and the relevant thermodynamic parameters $\Delta G°$, $\Delta H°$, and $\Delta S°$ were determined at 37.0 °C and 0.15 M ionic strength by means of potentiometric titrations and isothermal titration calorimetry (ITC). The biogenic amines studied were 1,4-diaminobutane (putrescine, put), 1,5-diaminopentane (cadaverine, cad), N-(3-aminopropyl)-1,4-diaminobutane (spermidine, spd), N,N'-bis(3aminopropyl)-1,4-diaminobutane (spermine, spm), and 1-(4-aminobutyl)guanidine (agmatine, agm), while the synthetic models of longer polyamines were 1,19-dimethyl-1,4,7,10,13,16,19-heptaazanonadecane (Me_2hexaen), 1,22-dimethyl-1,4,7,10,13,16,19,22-octaazadocosane (Me_2heptaen), 1,25-dimethyl-1,4,7,10,13,16,19,22,25-nonaazapentacosane (Me_2octaen) and N,N'-bis(3-aminopropyl)-1,3-propanediamine (3,3,3-tet). With the aid of molecular modeling, we also studied the structural aspects of the molecular recognition in operation. The final result is a balance between many parameters including charge of the species, flexibility of the amines, H-bonds in the adduct, and desolvation processes.

Introduction

myo-Inositol plays a fundamental role in eukaryotic cells since it constitutes the structural basis for two major families of important biomolecules: phosphatidylinositols and *myo*-inositol phosphates (Ins*P*s).[1-4] Ins*P*s are a wide variety of ubiquitous molecules with different phosphorylation patterns over the inositol ring, whose biological functions have been so far poorly understood. They participate in a number of complex intertwined chemical processes that have complicated the biochemical and biological research of the cellular roles of Ins*P*s.[5-8] The interaction of *myo*-inositol phosphates with metal cations plays an especially relevant role in the chemical behaviour of these biomolecules.[9-14] Stoichiometry, charge, solubility, relative stability and structural features of the biologically relevant Ins*P*s species influence their biological functions at the cellular level. In this sense, we have studied so far, under simulated physiological conditions, the complex chemical reactivity displayed by the most important Ins*P*s, with special attention to metal cations interaction.[15-23] We have also recently reported the first crystallographic evidence of the interaction of Cu(II) with an Ins*P* in a mixed-ligand complex.[24]

The hexaphosphorylated Ins*P*, InsP_6 (L^{12-} in its fully deprotonated form, Figure 1), is an ubiquitous and abundant molecule in eukaryotic cells.[5,25,26] Most or all of the cellular InsP_6 is located in cytosolic and nuclear compartments, either free or bound to cellular components, including membranes[27], but its biological functions are still not completely understood. The high charge displayed by InsP_6 species under simulated physiological conditions (H_4L^{8-} being the main

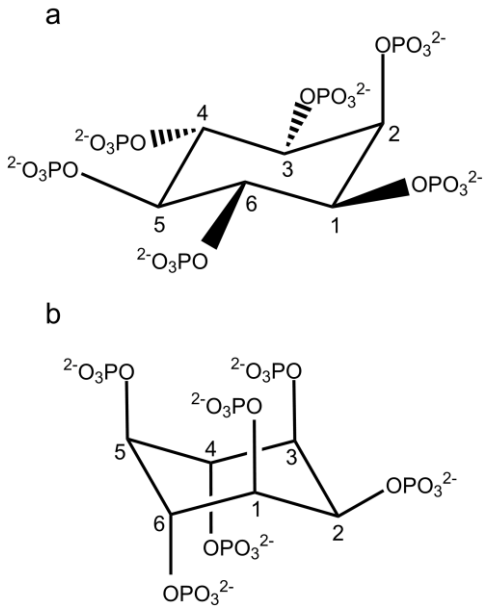

Fig. 1 Structure of InsP_6 (L^{12-}) for both conformations: (a) 1 axial-5 equatorial (1a5e) and (b) 5 axial-1 equatorial (5a1e).

species) implies that strong electrostatic interactions occur with metal cations (M) sharing the same environments. Indeed, it forms 1:1 ligand to metal complexes with mono-, di- and tricharged metal cations. The interaction with InsP_6 is markedly affected by metal cation charge, but it is fairly independent of cation radius, even though smaller cations display relatively higher stability constants and, therefore, give rise to complexes in lower protonation state at each given pH. These features are consistent with InsP_6–M interactions being dominated by electrostatics. Under metal cation excess, polymetallic species are also formed in addition to the 1:1 complexes, in particular $[M_5(H_2L)]$ neutral complexes with dipositive metal ions. For instance, $[Mg_5(H_2L)]$ is the predominant InsP_6 complex under simulated physiological conditions.[22,23]

During the last years, a great effort has been done to describe the chemical behaviour of InsP_6, paying special attention to the interaction with the most relevant biological metal cations. To achieve a complete speciation of this biomolecule in biological media, we should take into account that other cationic species are also present in biological fluids. Biogenic polyamines, for instance, form highly charged protonated species at physiological pH. They interact with many biomolecules, such as nucleotides, present in cellular media in anionic forms. As a matter of fact, polyamines are responsible for the structural modifications observed in the negative fragments of nucleic acids by electrostatic interactions.[28] Accordingly, they are also able to interact with the highly charged anions formed by InsP_6, as shown by previous studies carried out at 20 or 25 °C.[29-31] Nevertheless, the possibility that this interaction might influence the *in vivo* speciation of InsP_6 is still unexplored; the high concentration of biogenic amines, normally found *in vivo* in high excess relative to the InsP_6, makes it worth testing.

For this reason, we performed the speciation of binary systems constituted by InsP_6 and a group of polyamines, both biogenic and synthetic, and determined the stability constants of the relevant InsP_6-amine adducts at 37.0 °C and 0.15 M ionic strength. The biogenic amines are 1,4-diaminobutane (putrescine, put), 1,5-diaminopentane (cadaverine, cad), N-(3-aminopropyl)-1,4-diaminobutane (spermidine, spd), N,N'-bis(3aminopropyl)-1,4-diaminobutane (spermine, spm), and 1-(4-aminobutyl)guanidine (agmatine, agm), while the synthetic models of longer polyamines are 1,19-dimethyl-1,4,7,10,13,16,19-heptaazanonadecane (Me_2hexaen), 1,22-dimethyl-1,4,7,10,13,16,19,22-octaazadocosane (Me_2heptaen), 1,25-dimethyl-1,4,7,10,13,16,19,22,25-nonaazapentacosane (Me_2octaen) and N,N'-bis(3-aminopropyl)-1,3-propanediamine (3,3,3-tet). They are shown in Figure 2. The free energy changes associated to the formation of polyamine adducts were bisected into the corresponding enthalpic and entropic contributions, by means of isothermal titration calorimetry (ITC), to obtain a complete thermodynamic picture of such association processes.

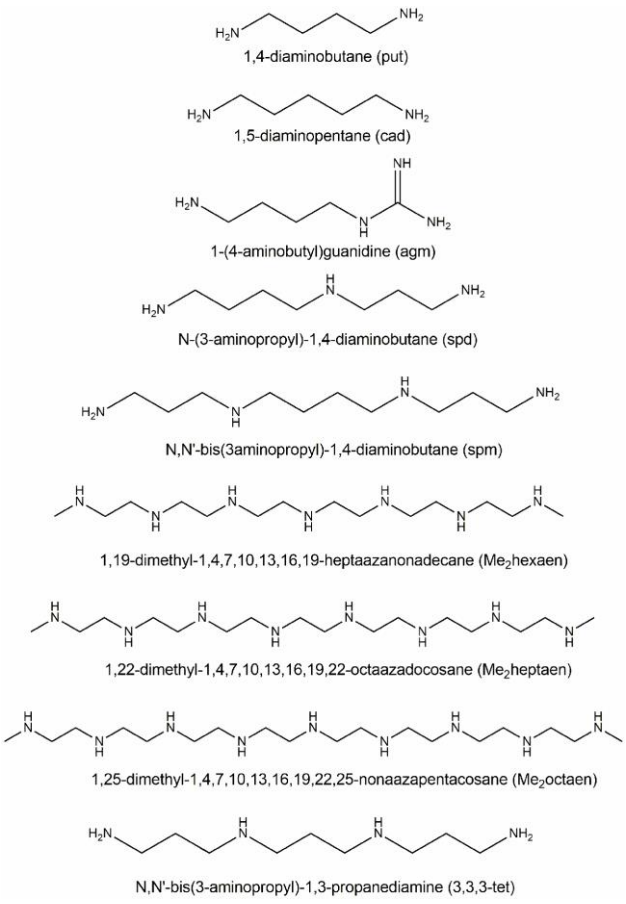

Fig. 2 The studied biogenic and synthetic polyamines.

The second part of this work was to investigate how the structural parameters modulate the strength of the interaction. InsP_6 presents many potential binding points which could interact with the polyamines. The high charge of the InsP_6 anion at neutral pH values (-8) and the possibility to form many hydrogen bonds envisage a complicated interplay of molecular recognition and hydration processes. In order to unravel this issue, we studied the statistical correlation between different structural features of the interacting species and thermodynamic results, and used this information to guide the discussion. We have also performed a computational approach to the structure of the most biologically relevant species, in order to analyse their structural parameters (number, length and geometry of the H-bonds, polyamine conformation, phosphate groups involved, etc.) and provide a more realistic view of some of the main complex microspecies.

Experimental

Materials

InsP_6 solutions were prepared by weighing the dipotassium salt $K_2H_{10}L{\cdot}2\ H_2O$ (Aldrich). Purity and water content of this salt was rechecked by elemental analysis. Biogenic amines solutions were

prepared by weighing putrescine (Aldrich), cadaverine dihydrochloride (Aldrich), spermidine trihydrochloride (Sigma), spermine tetrahydrochloride (Sigma), agmatine sulfate (Sigma). 3,3,3-Tet was purchased from Fluka and used as the tetrahydrochloride salt. Me_2hexaen, Me_2heptaen and Me_2octaen were prepared as previously reported[32] and used as Me_2hexaen·7HCl·1.5H_2O, Me_2heptaen·8HCl·2H_2O and Me_2octaen·9HCl·2.5H_2O.

Potentiometric measurements

HCl and tetramethylammonium hydroxide (Me_4NOH) stock solutions were used throughout the potentiometric measurements. The HCl solution was prepared by diluting concentrated Fixanal (Riedel de Haën) ampoules and were standardised against sodium carbonate. Me_4NOH solution was prepared from $Me_4NOH·5H_2O$ (Sigma) and standardised against potassium biphtalate. All the solutions were prepared with analytical grade water (<18 MΩ cm^{-1}) and were freed of carbon dioxide by Ar bubbling.

The protonation constants of InsP_6 were previously determined under the same experimental conditions.[23] The protonation constants of the polyamines (put, cad, spd, spm, agm, Me_2hexaen, Me_2heptaen, Me_2octaen, and 3,3,3-tet) were measured by at least three potentiometric titrations (*ca*. 120-200 experimental points each) for each system, using total concentrations of polyamines ranging from 0.5 to 5.0 mM.

Then, the behaviour of InsP_6 in the presence of the polyamines was analysed analogously through at least three potentiometric titrations (*ca*. 100-150 experimental points each) for each system, concentrations of InsP_6 ranging from 0.5 to 3 mM, and InsP_6 to amines total molar ratios varying from 0.5:1 to 3:1. The pH interval from 2.0 to 10 was covered.

In each potentiometric experiment, the solutions were poured into a 50 mL titration cell. After thermal equilibrium was reached, hydrogen ion concentrations were determined by successive readings, each performed after a small incremental addition of stock Me_4NOH solution. The titrant addition and e.m.f. measurements were carried out using an automatic titrator Mettler-Toledo DL50-Graphix.

The ionic strength was kept almost constant throughout the titrations by using solutions containing 0.15 M Me_4NCl and relatively low initial concentrations of the reactants (the sum of these reactants initial concentrations did not contribute more than 5 % on the total ionic strength). A tetraalkylammonium salt was selected as supporting electrolyte to avoid the possible interference of alkali metal ions that are known to form relatively stable complexes with inositol phosphates.[19-21,23] On the other hand, amine complexes with chloride in water are quite weak: the variation of the protonation constants in the presence and absence of chloride is very low.[33] Furthermore, in the

interaction studies, when highly charged anions of inositol phosphates are present, chloride anion is not expected to interact with the polyamines to a significant extent.

Pre-saturated argon (free of CO_2) was bubbled through the solutions during the titrations to eliminate the effect of atmospheric carbon dioxide, and the temperature was kept at 37.0 (±0.1) °C. Equilibrium attainment after each titrant addition was verified by controlling the deviation of successive e.m.f. readings and performing back titrations. Independent stock solutions were used in some selected titrations to check reproducibility. The cell electrode potential $E°$, and the acidic junction potential were determined according to[34] from independent titrations of the strong acid with the titrant solution. In this way, the pH scale was the free concentration scale. The calibration in the alkaline range was checked by recalculating K_w values for each system. The obtained values (average log K_w = -13.30) were always checked to assure they were in agreement with previously reported data under the same experimental conditions.[35-40] Data were analysed using the HYPERQUAD program[41], and species distribution diagrams were produced using the HySS program.[42] The fit of the values predicted by the model to the experimental data was estimated on the basis of the σ parameter, corresponding to the scaled sum of square differences between predicted and experimental values. Many other possible stoichiometries were tried for each system, and final models were selected on the basis of the σ parameter, the model confidence level estimator, chi-square, and the internal consistency of data reflected in standard deviations of the formation constants.[41]

Isothermal titration calorimetry

The enthalpies of InsP_6 protonation, amine protonation and complex formation were determined in 0.15 M NMe_4Cl solution at 37.0 (±0.1) °C by means of isothermal titration calorimetry (ITC) using a TAM III (TA Instrument) microcalorimeter equipped with a precision Lund syringe pump coupled with a 0.250 cm^3 gas-tight Hamilton syringe. The microcalorimeter was checked by determining the enthalpy of reaction of strong base (Me_4NOH) with strong acid (HCl) solutions. The value obtained (-54.0(2) kJ/mol) was in agreement with literature values.[43] Further checks were performed by determining the enthalpies of protonation of ethylenediamine.

In a typical experiment, a Me_4NOH solution (0.10 M, addition volumes 15 μl) was added to acidic solutions of the InsP_6 (5×10^{-3} M, 1.5 cm^3), containing equimolar quantities of the amine in the binding experiments. Corrections for heats of dilution were applied. The corresponding enthalpies of reaction were determined from the calorimetric data by means of the HypΔH program.[44] Analysis, performed by the same program, of the alkaline strokes of the calorimetric

titrations carried out in the absence of polyamines made possible the determination of the first four protonation constants of InsP_6. Since the third and the fourth stepwise protonation constants determined by means of microcalorimetry were equal, within the experimental errors, to the corresponding constants determined by potentiometry, we assumed that also the fifth to ninth stepwise constants were equal.

Molecular modeling calculations

For the molecular modeling approach to biologically relevant 1:1 and 2:1 amine-InsP_6 species, geometry optimization runs were performed. All the calculations were carried out in the gas phase by means of the methods described hereinafter as implemented in Gaussian 09.[45] The final structures obtained were all minima in the potential energy surface, being the nature of the stationary points verified through vibrational analysis. The initial geometries were built from the optimized InsP_6 geometries for the selected protonated species[15] and the optimized PM6 structure of the fully protonated amine. In each case, the polyamine cation was localized and directed to face the most negatively charged zone according to the electrostatic potential of the selected InsP_6 protonated species (Figure S1). Those inputs were then optimized by means of a restricted Density Functional Theory (DFT) method, using the B3LYP functional and the effective core potential LANL2DZ relativistic procedure.[46] The effect of the solvent was studied through a discrete model including between four and six water molecules, depending on the number and nature of the amine protonable groups.

Statistical analysis

A Multiple Linear Regression (MLR) method was used to relate the variations in the thermodynamic results (stability and enthalpic and entropic contributions) to the variations of linearly independent structural predictors (amine chain length and protonation states of the reactants). In this regard, the InsP_6-amine formation equilibria were input to The Unscrambler program[47] and the full cross validation alternative was selected. *p*-values (significance level) were employed to assess the significance of the observed variations. The statistically meaningful correlations ($p < 0.05$) were then used with explanatory purposes.

Results and discussion

Protonation of InsP_6 and polyamines

The obtained data for the protonation equilibrium constants for polyamines are shown in Table 1. Previous protonation constants of InsP_6 determined by means of potentiometric titrations under identical experimental conditions (37.0 °C in 0.15 M Me_4NCl) are also shown. Since very high protonation constants were reported for measurements performed in the absence of alkali metal ions as bulk electrolytes[48], in particular for the first protonation stages, we determined the InsP_6 protonation constants by means of calorimetric titrations under our experimental conditions. By this method, it was possible to determine the first four protonation constants, showing that, while the first and second constants are considerably higher, the third and the fourth ones are equal to the corresponding constants determined by potentiometry. For this reason, we assumed that also the successive stepwise protonation constants were coincident, giving rise to a set of InsP_6 protonation constants, included in Table 1, which justifies the calorimetric titrations over all the pH range (2-12).

Polyamine protonation constants determined in this work at 37.0 °C in 0.15 M Me_4NCl are highly consistent with available data.[35] For put, cad, spd and spm, various studies were previously carried out, even though none of them was performed under the same experimental conditions. Only for cad protonation, a previous study was carried out at 37 °C in 0.15 M $NaClO_4$, showing similar values (log K^H_1 = 9.99; log K^H_2 = 8.32[49]) with regard to our data. Almost all other previous protonation constants were measured at 20 or 25 °C. Since all protonation enthalpies of these polyamines are negative[35,50-57], the corresponding protonation constants are expected to be higher at 25 °C than at 37 °C. This fact is verified by all our data when compared to available protonation constants (for put, spm, spd) obtained with similar supporting electrolyte concentrations.[29,30,35,58]

Table 1 Overall protonation constants at 37.0 °C in 0.15 M Me_4NCl

	K^H_1	β^H_2	β^H_3	β^H_4	β^H_5	β^H_6	β^H_7	β^H_8	β^H_9	
InsP_6	10.8	21.3	31.63	40.42	47.32	53.04	56.14	58.0	59.9	Ref. [23]
InsP_6	11.68(6)[a]	22.38(3)	32.71(3)	41.50(4)	48.40	54.12	57.22	59.1	61.0	calorimetric
put	10.076(3)	19.156(4)								σ = 2.7
cad	9.90(1)	19.35(1)								σ = 2.9
agm	10.179(7)	19.32(7)								σ = 1.6
spd	10.369(8)	19.820(8)	27.89(1)							σ = 2.0
spm	9.95(1)	19.450(9)	27.84(1)	35.45(2)						σ = 2.2
Me_2hexaen	10.09(1)	19.45(2)	28.21(2)	35.93(3)	40.48(4)	43.59(4)	45.74(7)			σ = 1.0
3,3,3-tet	10.157(4)	19.689(4)	27.986(5)	34.993(8)						σ = 0.9
Me_2heptaen	9.69(2)	19.24(2)	27.98(3)	36.25(4)	42.67(7)	47.02(9)	50.21(1)	53.1(1)		σ = 1.9
Me_2octaen	9.65(2)	19.206(9)	27.99(2)	36.35(2)	43.82(2)	48.77(4)	52.55(5)	55.41(5)	58.05(5)	σ = 0.4

[a]Values given in parentheses are the 1σ statistical uncertainties on the last digit of the constant

A similar behaviour –that is, a decrease in protonation constant values– is found for Me_2hexaen, Me_2heptaen and Me_2ocataen in 0.15 M Me_4NCl at 37.0 °C with respect to previous values obtained at 25.0 °C in 0.15 M $NaClO_4$ solutions[32], in agreement with the exothermicity of the protonation processes involving these polyamines.[59] Species distribution diagrams *vs*. pH for the nine polyamines are presented in Figure S2.

InsP_6 interaction with polyamines: potentiometric results

The equilibrium constants for the adduct formation furnished by the Hyperquad program are listed in Table 2. They refer to the general equation (charges are omitted):

$$p\ A + q\ L + r\ H \rightarrow [H_rA_pL_q]$$

where A, L and H are the neutral free form of each polyamine, the fully deprotonated form of InsP_6 (L^{12-}) and H^+ ions, respectively. As it can be seen in Figure S2, the lighter amines put, cad and agm predominate as the fully protonated species in pH values below 8, while spd, spm, and 3,3,3-tet having three and four basic groups, are fully protonated below 6. For heavier and more basic amines, partial protonation takes place over wider pH intervals. Since in the studied pH range, both amines and InsP_6 are present in different protonation states, the complex species should be better formulated as $[(H_nA)_p(H_{r-n}L)_q]$ where n is the number of protons associated to the polyamine. It has been shown that, in similar complexes, protonation is governed by the different basicity of the interacting species.[59-64] According to this criterion, we calculated the complex stability constants shown in Table S1.

Table 2 Interaction equilibrium constants determined by potentiometry at 37.0 °C in 0.15 M Me_4NCl for InsP_6 (L^{12-}) and polyamines for the processes p Amine + q L + r H → $[A_p(L)_qH_r]$ (charges are omitted for clarity).

pqr	1 1 4	1 1 5	1 1 6	117	118	119	1110	1111	219	2110	2111	2112	σ
put	46.29(3)	56.26(2)	64.68(4)	71.28(6)					93.3(2)	99.6(1)	103.3(2)		0.7
cad	46.51(5)	56.49(4)	64.69(9)	71.4(1)						99.6(3)	103.2(3)	105.8(3)	2.5
agm	46.05(5)	55.7(2)	64.41(6)	72.18(8)					94.9(1)	102.39(9)	108.4(1)	112.0(2)	1.1
spd	45.8(3)	56.81(3)	66.36(3)	74.17(3)	80.07(7)				98.27(5)	105.3(1)	110.8(3)	115.6(2)	1.5
spm	47.09(5)	57.46(2)	67.38(3)	76.37(2)	83.70(2)	89.27(2)	93.42(4)		99.84(5)	108.13(9)	116.36(5)	122.3(2)	0.5
3,3,3-tet		57.06(2)	67.03(1)	76.39(1)	83.97(3)	89.49(3)	93.71(5)			107.81(4)	115.88(3)	122.40(5)	1.0
Me_2hexaen		56.2(2)	66.81(3)	76.05(4)	83.31(5)	89.40(4)	94.09(7)	98.50(4)					1.3
Me_2heptaen		56.9(1)	67.35(3)	76.86(4)	84.61(5)	91.04(5)	96.17(7)	101.06(4)					1.7
Me_2octaen		57.47(9)	67.83(5)	77.67(5)	85.75(5)	92.47(5)	97.95(6)	103.00(3)					1.1

[a]Values given in parentheses are the 1σ statistical uncertainties in the last digit of the constant.

For all the studied systems, the complexes detected have 1:1 amine:InsP_6 molar ratio in different protonation states. Besides, also 2:1 amine:InsP_6 adducts are formed with the lighter polyamines. Figure 3 shows the comparative behaviour of some amines in the presence of InsP_6, showing the formation of the substantially stable adducts predominating in wide pH ranges. Most relevant species found in these studies are in line with previous reports including put, spm and spd.[30,31] InsP_6 displays higher association constants at 25 °C without background salt[31] or at 20 °C in 0.15 M Et_4NClO_4[30] than in our present case at 37.0 °C in Me_4NCl (Table 2). This fact could be related to the difference in the experimental conditions, in particular to the different temperature value. 2:1 amine:InsP_6 complexes are also detected for most systems, being especially relevant for spm. These species had also been previously reported for put, spd and spm under different experimental conditions.[31] It is worth mentioning that the inclusion of the 2:1 complexes is particularly critical in the potentiometric data fit under polyamine excess because their formation is favoured at high ligand to metal ratios.

The potentiometric results show that a strong association occurs between the anionic forms of InsP_6 and the protonated species of polyamines, due to the simultaneous presence in solution of ions with high and opposite charges. In agreement with electrostatic expectations and with a few exceptions, protonated forms of the amines associate more strongly in solution with more deprotonated (more negatively charged) forms of InsP_6 (Table S1). Similarly and with a few exceptions, the association with a given InsP_6 form becomes stronger as the positive charge on the polyamines increases (Table S1). According to the computational analysis performed on the most abundant adducts at physiological pH under equimolar conditions (depicted for example in Figures 3a, 3b), the significant stability of these InsP_6-polyamine complexes is also accounted for the formation of several intramolecular N-H···O and O-H···O hydrogen bonds between the interacting species, with the assistance of the solvent molecules (Figure 4). Whilst for the 1:1 species the polyamine cation is stabilized *syn* to the phosphate at C2, the second amine in the 2:1 complexes seems to be linked to the other side of the ring, facing the most negatively charged zones over the corresponding protonated InsP_6 form (Figure S1). From Figures 4e-g, it is manifest that ternary complexes for the shorter amines, or even binary adducts for the longest ones, would be energetically adverse, since that would involve an increment in the electrostatic and steric repulsion between the components. In fact, these species were not detected in the potentiometric studies.

Following this interaction model, the calculated structural parameters for the studied adducts (some selected examples are shown in Table S2, Figure 4) are in line with their stability (Table 2 and S1). Across the 1:1 or 2:1 species, it can be generally seen that the stability of the species tends

to raise as the number (n) of N-H···O and O-H···O hydrogen bonds increases, and also as well as proton-acceptor distances ($d_{H\cdots O}$) become shorter. However the scenario is complex, since each

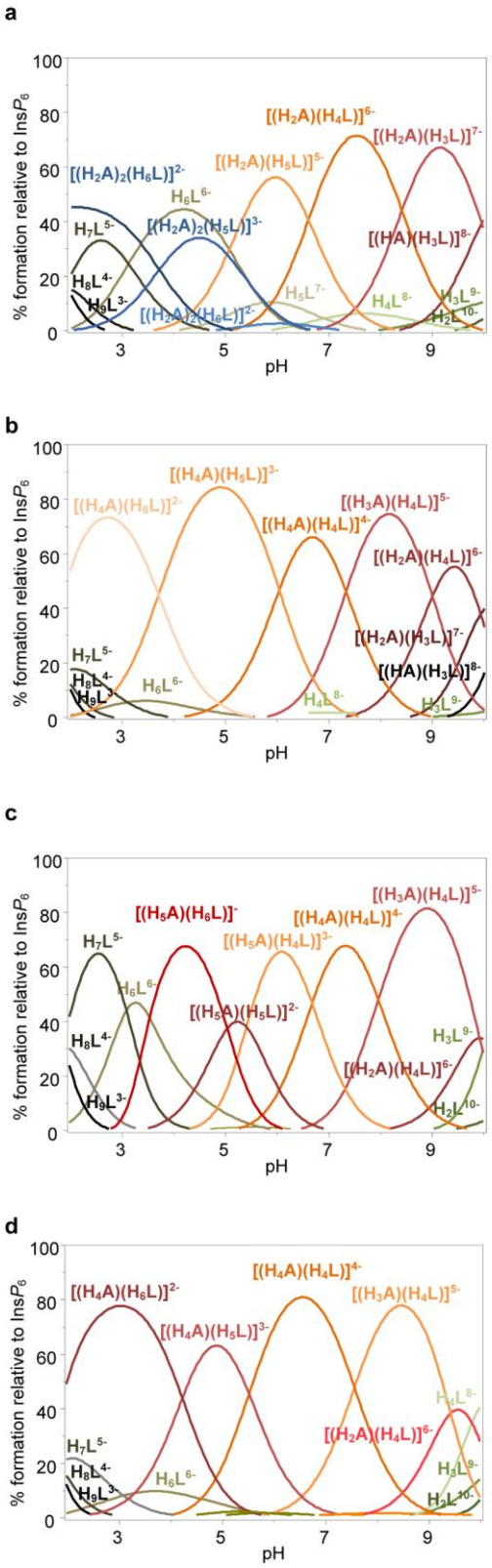

Fig. 3 Speciation of InsP_6 under simulated physiological conditions *vs.* pH at 37.0 °C in 0.15 M Me_4NCl. Conditions: $[InsP_6]_{total} = [amine]_{total} = 1$ mM. a) put, b) spm, c) Me_2octaen, d) 3,3,3-tet.

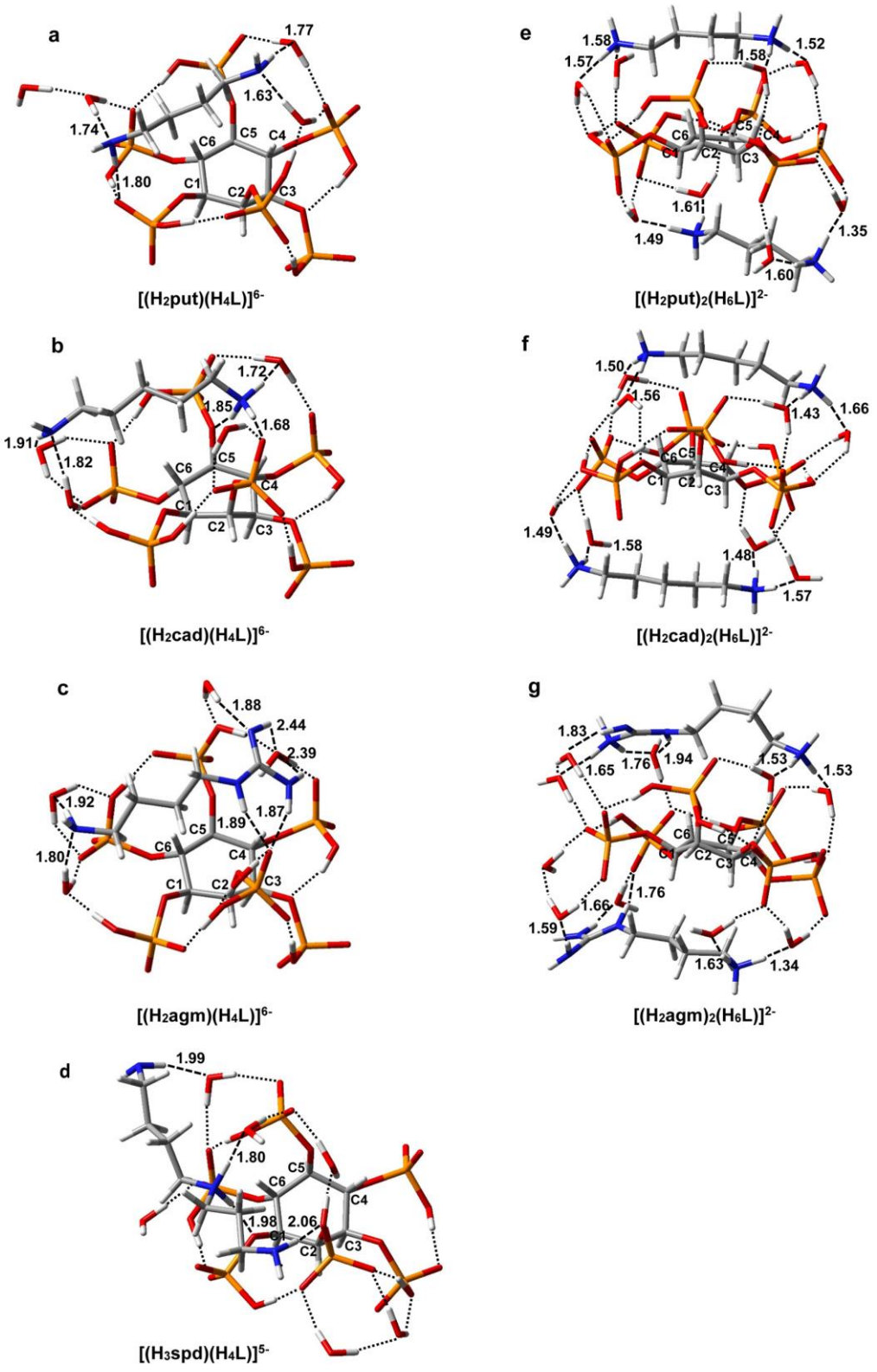

Fig. 4 RB3LYP/LANL2DZ geometries for some of the physiologically relevant 1:1 (a-d) and 2:1 (e-g) amine-InsP_6 species. The O-H···N and O-H···O hydrogen bonds are shown as dashed and dotted lines, respectively, with the associated distances for the former in Å. Colour code: C (grey), H (white), O (red), P (orange), N (blue).

structural factor exerts some effects that can partially counteract with the others. In detail, for $[(H_2A)(H_4L)]^{6-}$ adducts, the number of hydrogen bonds increases from put to agm, although they are progressively larger, explaining the slightly less stability for the agm complex (Figure 4). The $[(H_3spd)(H_4L)]^{5-}$ species is significantly more stable, but the comparison is not straightforward, as this adduct does not share the same stoichiometry. In this case there are more ionisable groups which can set up a higher number of H-bonds and different electrostatic interactions. Interestingly enough, data from Table S2 seem to indicate that compared to put, for spd the number and strength of N-H···O interactions do not explain the stability trend. In fact, the H-bonds seem to be weaker. However, the number of O-H···O bonds is substantially higher (an extra proton and more water molecules involved), and the terminal $–NH_2$ groups are exposed to the solvent (Figure 4), able to set up four new H-bonds with water molecules in the second hydration sphere (hydration stabilization). As it will be discussed in the following sections, this phenomenon can contribute to the reduction of the entropic factor as the length of the polyamine chain increases. For the $[(H_2A)_2(H_6L)]^{2-}$ adducts, the higher stability for agm can be rationalized by the increment in the number of N-H···O bonds and the strength of the O-H···O bonds (Figure 4 and Table S2).

Some exceptions to the electrostatic trend illustrate interesting structural aspects of the observed molecular recognition processes. A noticeable example is given by the association of the diprotonated species (H_2A^{2+}) of spd, spm, 3,3,3-tet, Me_2hexaen, Me_2heptaen and Me_2octaen with $H_3InsP_6^{9-}$ and $H_4InsP_6^{8-}$. Here, the less charged InsP_6 form interact more strongly (Table S1). The explanation for this trend lies in the particular thermodynamic and structural characteristics for the formation of these adducts. As it is clear from Figure 4, the theoretical data suggest that the most deprotonated forms of InsP_6 are basic enough to trigger an endothermic proton transfer from the amine to the inositol phosphate upon the adduct formation. The computational results suggest that these phenomenon becomes less significant as the InsP_6 gets protonated (see also the $[(H_2put)(H_4L)]^{6-}$ and $[(H_2put)(H_6L)]^{4-}$ optimized geometries in Figure S3), energetically favouring the most protonated complexes. This tendency is only observed for the larger amines in a partially protonated state, in which they are able to receive protons from InsP_6 as well. This adds another exothermic contribution (stabilizing effect), which is larger as the InsP_6 species is progressively protonated (less basic). These phenomena are also evident from the calorimetric results (see discussion below).

Analogously, in line with the electrostatic character of these association processes, the stability of the complexes formed by put, cad and agm with a given protonated form of InsP_6 raises as the positive charge on the polyamine increases (Table S1). This same trend is observed for all the other amines in low protonation states, while for higher protonation states (2 for spd, 3 for spm, 3

and 4 for Me_2hexaen, Me_2heptaen and Me_2octaen) complex stability decreases with increasing positive charge on the amines. This behaviour can be ascribed to the growing stiffening experienced by the polyamines upon protonation. The more charged the more rigid the species of these long polyamines become, being gradually less able to match the binding sites of InsP_6 anions. In line with this, the optimized structure for the $[(H_3spd)(H_4L)]^{5-}$ adduct (Figure 4d) shows that one of the terminal -NH_2 groups is far from the phosphate groups, interacting with the anion through the water molecules and being highly exposed to the solvent.

Another interesting feature, also in conflict with electrostatic expectations, is shown by Me_2hexaen, Me_2heptaen, and Me_2octaen, since, for a given protonation state of these longer polyamines and a given protonation state of InsP_6, complex stability increases with polyamine length (Table S1). Most likely, as the length and the number of nitrogen atoms of the polyamine increase, the protonated molecule becomes more flexible and more able to match the binding sites of the InsP_6 species, forming a larger number of hydrogen bond contacts by using both ammonium and amine groups. Along with Figure 4c, it is possible that the longer polyamines can be able to establish more O-H···O interactions, involving more water molecules, and also to enhance the hydration of the species by providing –NH and –NH_2 groups significantly exposed to the solvent.

InsP_6 interaction with polyamines: biological remarks

As previously stated, the possibility that this interaction might influence the *in vivo* speciation of Ins*P*s is still unexplored. This is especially relevant due to the high concentration of biogenic amines, normally found *in vivo* in high excess relative to the Ins*P*s. According to our previous results[23], even at 50 μM available Fe(III), a negligible proportion of InsP_6 was predicted to be bound to iron, as well as very little iron would be bound to InsP_6 due to the high excess of Mg^{2+} present under cellular conditions. Taking into account the new values reported here for polyamine interaction with InsP_6, the situation can be further explored. To simulate the situation in the presence of biogenic polyamines in different concentrations, we can take [InsP_6] as 60 μM, which is in the upper limit of the levels reported for mammalian cells[27], assuming $[Mg^{2+}]_{available}$ to be 0.8 mM (calculated adding the total $[Mg^{2+}]$ that could be complexed by the InsP_6 present plus the free $[Mg^{2+}]$ present *in vivo*, estimated in 0.5 mM). The resulting simulation shows that even though $[Mg_5(H_2L)]$ is still present in solution, the polyamines compete with Mg^{2+} for InsP_6. This fact is especially relevant taking into account that the longer and more basic amines spm and spd are the most abundant in eukaryotic cells.[65] Taking for example spm concentration determined in bovine lymphocites (1.57 mM[66]) and the above mentioned conditions, all InsP_6 is predicted to be

associated to spm at physiological pH. But, since it has been estimated that due to the presence of phospholipids, nucleic acids and ATP, only 2.5% of spm should be free[66] to interact with InsP_6, we can recalculate the speciation according to this (*i.e.*, taking 0.04 mM spm). In this case, 33% of InsP_6 is still associated to magnesium ion. Spm, being the most basic of the biogenic polyamines, is expected to interact more strongly with the inositol phosphate species. In fact, spd for example retains only 5 % of InsP_6 under similar conditions but taking into account the reported value for free spd.[66]

InsP_6 protonation processes: calorimetric results

Table 3 shows the results of the calorimetric measurements performed for InsP_6 protonation processes at 37.0 °C in Me_4NCl 0.15 M. Analogous data were previously obtained at 25 °C and in a sodium containing media (from 0.1 to 1 M NaCl)[67]. Under those circumstances, even for the lowest sodium concentration conditions employed[67], InsP_6 is expected to be associated with sodium to a high extent, particularly for alkaline media, having a special influence on the first protonation processes. Protonation of negatively charged species in a solvent of high dielectric constant, like water, is accompanied by an important desolvation process, occurring upon charge neutralization of the interacting species. Such desolvation processes are expected to be endothermic and promoted by a largely favourable entropy change, while the formation of the anion-proton bond is expected to be exothermic and endoentropic.[59] All InsP_6 protonation stages, but the second and third ones, are endothermic, or almost athermic, and favoured by dominating entropic contributions (Table 3), thus indicating that they are mainly controlled by desolvation effects. But, in each protonation step, the incoming proton is bound to an InsP_6 anion of decreasing charge and the process is expected to become less exothermic (or more endothermic) for the species of lower negative charge. This occurrence has been previously observed[67] and it is also the case of our data, except for the second protonation stage.

Table 3 Protonation data for InsP_6 (L^{12-}) as obtained by calorimetry at 37.0 °C in 0.15 M Me_4NCl

	log *K*	Δ*G*° (kJ/mol)	Δ*H*° (kJ/mol)	*T*Δ*S*° (kJ/mol)
$L^{12-} + H^+ \rightarrow HL^{11-}$	11.68(6)[a]	-69.3(4)	9.90(8)	76.2(4)
$HL^{11-} + H^+ \rightarrow H_2L^{10-}$	10.70(5)	-63.5(3)	-30.08(8)	33.4 (3)
$H_2L^{10-} + H^+ \rightarrow H_3L^{9-}$	10.33(5)	-61.3(3)	-4.20(5)	57.1(3)
$H_3L^{9-} + H^+ \rightarrow H_4L^{8-}$	8.79(5)	-52.2(3)	0.70(7)	52.9(3)
$H_4L^{8-} + H^+ \rightarrow H_5L^{7-}$	6.90(6)	-41.0(4)	1.80(7)	42.8(4)
$H_5L^{7-} + H^+ \rightarrow H_6L^{6-}$	5.72(7)	-33.9(4)	5.34(5)	39.3(4)
$H_6L^{6-} + H^+ \rightarrow H_7L^{5-}$	3.10(9)	-18.4(5)	12.42(5)	30.8(5)
$H_7L^{5-} + H^+ \rightarrow H_8L^{4-}$	1.9(1)	-11.3(6)	19.55(7)	30.8(6)
$H_8L^{4-} + H^+ \rightarrow H_9L^{3-}$	1.9(1)	-11.3(6)	b	b

[a]Values in parentheses are standard deviation on the last significant figures, [b] not determined

The second and third InsP_6 protonation steps deserve a special attention. It has been theoretically and experimentally proved that a dramatic structural modification takes place with the protonation equilibrium $HL^{11-} + H^+ \rightarrow H_2L^{10-}$, corresponding to the transition from the 5a1e conformation of HL^{11-} to the 1a5e conformation of H_2L^{10-}.[15] The axial arrangement of phosphate groups allows a better minimization of the intramolecular electrostatic repulsion between the many negative charges of L^{12-} and HL^{11-} anions, while in the diprotonated H_2L^{10-} form the electrostatic repulsion has weakened enough to allow intramolecular hydrogen bonding to stabilize the equatorial conformation.[15] As shown by the data in Table 3, the transition to the more stable 1a5e conformation, occurring with the second protonation stage, is accompanied by a largely favourable enthalpic contribution, in contrast with the different behaviour of the other protonation stages. This negative $\Delta H°$ value in conjunction with a positive but lower $T\Delta S°$ contribution are consistent with the formation of stronger intramolecular H-bonds[15], capable of restricting the degrees of freedom of the phosphate groups. The third protonation step is still exothermic, although the enthalpic contribution is significantly close to zero. This can be explained by the fact that the 1a5e conformation of the highly charged H_2L^{10-} species is strongly stabilized upon protonation, since the electrostatic repulsion between the equatorial phosphate groups is largely relieved in the process.[15] It is interesting to note that the equilibrium constants associated to the second and third protonation stages do not show any peculiarity that gives evidence of such conformational change, due to evident enthalpy-entropy compensation.

Table S3 shows the calorimetric results for the protonation processes of polyamines along with the corresponding entropy changes determined at 37.0 °C in Me_4NCl 0.15 M. The obtained data are in line with previously reported values.[33,50-57]

InsP_6 interaction with polyamines: calorimetric results

The enthalpy changes for the formation of InsP_6 polyamine complexes determined by means of isothermal titration calorimetry in 0.15 M Me_4NCl at 310.1 ± 0.1 K are listed in Tables 4 and 5 for put and Me_2hexaen, respectively, and in Tables S4 - S9 for the other amines, along with the log K, $\Delta G°$ and the derived entropic contributions. All these data were analysed with a multiple linear regression method to find statistically meaningful correlations between the experimental results and three linearly independent structural determinants of the molecular recognition: the number of atoms in the main chain of the polyamine, and the number of protons of the amine and InsP_6 interacting species.

Table 4 Thermodynamic data for the formation of InsP_6 (L^{12-}) complexes with putrescine (A). 0.10 M Me_4NCl, 37.0 °C

	log *K*	Δ*G*° (kJ/mol)	Δ*H*° (kJ/mol)	*T*Δ*S*° (kJ/mol)
$HA^{+} + H_3L^{9-} \rightarrow [(HA)(H_3L)]^{8-}$	3.50(4)[a]	-20.8(2)	-15.5(6)	5.3(6)
$H_2A^{2+} + H_3L^{9-} \rightarrow [(H_2A)(H_3L)]^{7-}$	4.39(4)	-26.1(2)	23.0(5)	49.1(5)
$H_2A^{2+} + H_4L^{8-} \rightarrow [(H_2A)(H_4L)]^{6-}$	4.02(6)	-23.9(4)	42.3(4)	66.2(6)
$H_2A^{2+} + H_5L^{7-} \rightarrow [(H_2A)(H_5L)]^{5-}$	3.72(8)	-22.1(5)	26.2(4)	48.3(6)

[a]Values in parentheses are standard deviation on the last significant figures

Table 5 Thermodynamic data for the formation of InsP_6 (L^{12-}) complexes with Me_2hexaen (A). 0.10 M Me_4NCl, 37.0 °C

	log *K*	Δ*G*° (kJ/mol)	Δ*H*° (kJ/mol)	*T*Δ*S*° (kJ/mol)
$H_2A^{2+} + H_3L^{9-} \rightarrow [(H_2A)(H_3L)]^{7-}$	4.0(2) [a]	-24(1)	-69.7(5)	-46(1)
$H_2A^{2+} + H_4L^{8-} \rightarrow [(H_2A)(H_4L)]^{6-}$	5.86(6)	-34.8(4)	-71.5(3)	-36.7(5)
$H_3A^{3+} + H_4L^{8-} \rightarrow [(H_3A)(H_4L)]^{5-}$	6.34(7)	-37.6(4)	-65.5(3)	-27.9(5)
$H_4A^{4+} + H_4L^{8-} \rightarrow [(H_4A)(H_4L)]^{4-}$	5.88(8)	-34.9(5)	-35.9(4)	-1.0(6)
$H_4A^{4+} + H_5L^{7-} \rightarrow [(H_4A)(H_5L)]^{3-}$	5.07(7)	-30.1(4)	-41.8(4)	-11.7(6)
$H_4A^{4+} + H_6L^{6-} \rightarrow [(H_4A)(H_6L)]^{2-}$	4.0(1)	-23.7(6)	-52.5(4)	-28.8(7)
$H_5A^{5+} + H_6L^{6-} \rightarrow [(H_5A)(H_6L)]^{-}$	3.90(8)	-23.1(5)	-23.8(4)	-0.7(6)

[a]Values in parentheses are standard deviation on the last significant figures

The stability of the different adducts (log *K*) is highly correlated with the number of atoms in the main chain of the polyamine ($p = 5 \times 10^{-5}$), as expected from the increase in the number of nitrogen atoms and the number of hydrogen bond contacts. In total agreement with an electrostatic model of recognition, the more protonated the InsP_6 form (less charged) is, the lower the stability of the complexes ($p = 0.05$). The protonation state of the amine is not positively correlated with log *K* ($p = 0.31$), since as it was previously discussed the longest polyamines do not follow this behaviour.

Another interesting feature of these thermodynamic data is the fact that, increasing the length and the number of nitrogen atoms of the polyamines, leads to an increasing number of exothermic complexation equilibria. This correlation is statistically verified ($p = 2 \times 10^{-8}$). Indeed, for the shorter diamines put, cad and agm, all complexation equilibria are endothermic with the exception of the equilibrium involving their monoprotonated forms ($HA^{+} + H_3L^{9-} \rightarrow [(HA)(H_3L)]^{8-}$), in the case of spd only the reaction of its diprotonated form with H_3L^{9-} and H_4L^{8-} are exothermic, while for the remaining longer polyamines all complexation equilibria are exothermic with a sole exception for spm.

It was previously shown for phosphate and pyrophosphate complexes with Me_2hexaen and similar amines that different enthalpic contributions can accompany the formation of -N-$H^{+}\cdots^{-}$O-P (1) and -N···H-O- (2) hydrogen bonds.[59] Since hydrogen bonding determines a partial proton

transfer from donor to acceptor species, type (1) bonds are expected to contain an endothermic term due to partial deprotonation of an amine group and an almost athermic or endothermic term for partial protonation of H_3L^{9-} and more protonated InsP_6 species. On the other hand, type (2) bonds are expected to give an exothermic contribution for partial protonation of the amine group and an almost athermic or exothermic contribution for partial deprotonation of a phosphate group of the above InsP_6 species. All in all, type (1) and type (2) hydrogen bonds are expected to afford endothermic and exothermic charge transfer contributions, respectively, among other thermal effects. Accordingly, all complexation equilibria involving partially protonated forms of the studied polyamines have a markedly exothermic character, while all equilibria involving the completely protonated forms of the polyamines are endothermic, with a couple of exceptions for H_4spm^{4+} (almost athermic reactions). This is in line with the possibility for the interacting species to form mainly type (2) and type (1) hydrogen bonds, respectively. Indeed, when all data is considered, there is a statistically relevant positive correlation between the protonation degree of the polyamine and the ΔH values ($p = 0.033$). Regarding the protonation state of InsP_6, no correlation is found ($p = 0.65$). This is due to two different phenomena that counteract their effect on the enthalpic contribution. According to the electrostatic model of interaction, the more protonated the InsP_6 form (less charged) the higher the ΔH should be. Conversely, the more protonated InsP_6 species are less basic, decreasing the tendency for the proton transfer from the amine to the Ins$P$ and increasing the propensity for some of the InsP_6 protons to be relocated over the amine moiety, lowering the ΔH values. Although we believe that all these results are a meaningful coincidence, we must remember that the enthalpy changes for such association processes are determined by a subtle combination of effects coming from breaking and formation of bonds involving the interacting partners and many solvent molecules.

Regarding the entropic contributions to the complexation processes, we can note that, in a general sense, exothermic reactions are endoentropic while endothermic reactions are exoentropic, in agreement with a common trend showing that strong binding interactions ($\Delta H° << 0$) correspond to entropy loss, while weaker binding interactions ($\Delta H° \geq 0$) produce favourable entropic contributions. Accordingly, complexation equilibria involving put, cad, agm and spd display a very good linear enthalpy-entropy correlation (black squares in Figure 5a), following the compensatory relationship $T\Delta S° = \alpha\Delta H° + I$, where $\alpha = 0.99(2)$ and $I = 24.0(8)$ (correlation coefficient $R = 0.997$). Spm approximately matches the relationship (red squares in Figure 5a) which leads, after inclusion of spm data, to $\alpha = 0.93(3)$ and $I = 27(1)$ ($R = 0.990$), while the longer polyamines Me_2hexaen, Me_2heptaen and Me_2octaen give rise to a larger dispersion of data (Figure 5b) that still accounts for a rough compensatory relationship with $\alpha = 0.84(9)$ and $I = 27(1)$ ($R = 0.902$). Similar

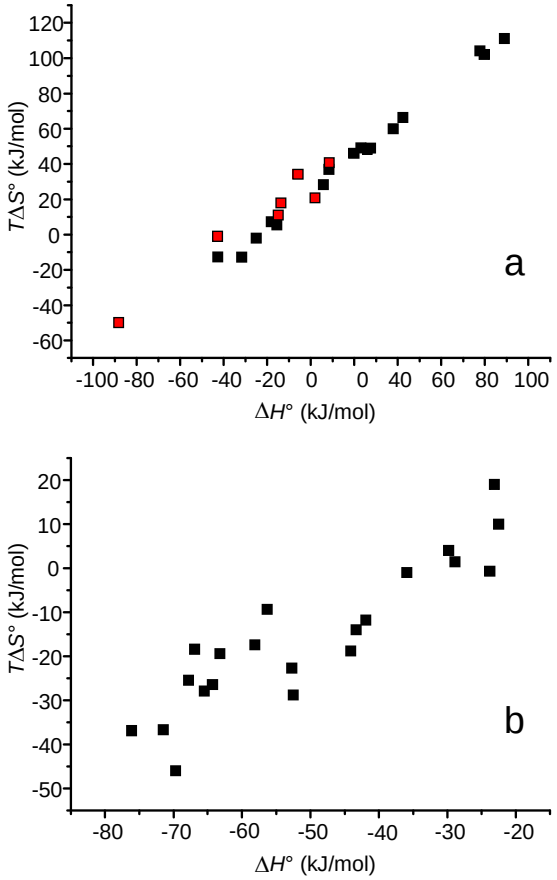

Fig. 5 $\Delta H°$ *vs.* $T\Delta S°$ correlations for InsP_6 complexes with a) put, cad, agm and spd (black squares) and spm (red squares), b) Me_2hexaen, Me_2heptaen and Me_2octaen.

compensation relationships generally hold for binding events involving weak interactions, like in the present case, and have been used to get estimations of the ligands conformational changes occurring upon complex formation, in agreement with the observation that the slope α of such linear correlations decreases with increasing ligand rigidity.[59,68-71] In our case, despite the fact that the linear fitting of $\Delta H°$ *vs.* $T\Delta S°$ values is not as good for longer polyamine as for shorter put, cad, agm and spd, we have a clear trend of decreasing α with increasing length and increasing number of nitrogen atoms of the polyamines, in agreement with a greater and increasing rigidity of longer polyamine upon protonation of their numerous protonation sites. This phenomenon explains the positive correlation between the amine protonation grade and the entropic change of the adduct formation (p = 0.018), since the amine initial state has gradually lower number of degrees of freedom as it gets protonated. This tendency is also influenced by the fact that the more protonated the amine (more charged), the higher will be the desolvation effect upon complexation, adding a further positive entropic contribution.

Additionally, the amine chain length exhibits a negative correlation with the $T\Delta S°$ values (p = 2 x 10^{-7}). The longer the polyamine, the more flexible it becomes, increasing significantly the loss of freedom upon complexation. Moreover, as it was discussed before, the longest polyamines are likely to expose some of their ionisable groups to the solvent after the adduct formation, decreasing the entropy of the hydration water molecules in the second hydration sphere. No statistically meaningful interrelation was observed for the entropic factor with the InsP_6 protonation state (p = 0.39). Again, two factors exert opposite effects. The more protonated InsP_6 species are less charged, lowering the number of water molecules displaced after the adduct formation (lower $T\Delta S°$). On the contrary, as the InsP_6 gets progressively protonated the anionic species become more rigid, and the complexation entropic change increases.

Also the parameter I, which can be regarded as a common (intrinsic) contribution to the stability of all complexes formed by the considered class of ligands ($\Delta G° = (1 - \alpha)\Delta H° - I$), furnishes interesting information on the complexation processes. In the case of the longer polyamines (spm, Me_2hexaen, Me_2heptaen and Me_2octaen), I value (27(1) kJ/mol) is somewhat greater that for shorter ones (24.0(8) kJ/mol). Spm, Me_2hexaen, Me_2heptaen and Me_2octaen contain a higher number of amine groups. Especially for the longest molecules, the amine groups are much closer to each other than in the shorter polyamines, being able to gather, upon protonation, greater positive charges, explaining the higher "intrinsic" contribution to complex stability.

We believe it is necessary to say a few cautionary words on the enthalpy changes listed in Tables 4, 5, S4–S9, in particular on consideration of the high values obtained for several complexation equilibria. These values were calculated from cumulative enthalpies of complex formation (Table S10), InsP_6 (Table S11) and polyamine (Table S12) protonation, furnished by the program HypΔH, on the assumption that the distribution of acidic protons between the interacting species in the complex takes place according to the basicity of the separated species. Although this assumption is largely verified for anion complexes involving protonated amines and phosphate-like anions[60-64], we do not have independent experimental evidences to confirm this role and we cannot exclude that a different one might apply to the present cases. For instance, the very high value $\Delta H°$ = -71.5 kJ/mol calculated for the equilibrium $H_2A^{2+} + H_4L^{8-} \rightarrow [(H_2A)(H_4L)]^{6-}$ (InsP_6:Me_2hexaen interaction, Table 5) reduces to $\Delta H°$ = -28.9 kJ/mol if a complex with the same stoichiometry but different proton distribution is assumed to form according to the equilibrium $H_3A^{3+} + H_3L^{9-} \rightarrow [(H_3A)(H_3L)]^{6-}$. Several similar cases can be found in Tables 4, 5, S4–S9, in particular for the longer polyamines. Furthermore, we cannot exclude that both complexes, with the same stoichiometry but different proton distribution, might coexist in solution, in particular for interacting species of similar basicity. Perhaps, the rather large dispersion of points in the enthalpy-entropy correlation shown in

Figure 5b might be due to the fact that some of the $\Delta H°$ and $T\Delta S°$ included in this linear correlation are not representative of the principal equilibria actually occurring in solution.

Concluding remarks

In this work we presented the acid-base and complexation characteristics of InsP_6 in the presence of significant concentrations of biogenic and synthetic polyamines. In this sense, we established the thermodynamic and structural parameters of the molecular recognition and calculated the chemical distribution of the most relevant species under simulated physiological conditions.

The potentiometric study showed a strong association between the anionic forms of InsP_6 and the protonated species of the polyamines. The complexes detected have 1:1 and 2:1 amine:InsP_6 stoichiometries, in different states of protonation, and are stable enough to compete with the major biological metal ions for the inositol phosphate. In general, protonated forms of the amines associate more strongly in solution with more deprotonated forms of InsP_6, in line with an electrostatic model of interaction. Some exceptions to this model illustrate interesting structural aspects of the observed molecular recognition processes: the most charged and rigid species seem to be less able to match the binding sites of InsP_6 anions. Besides, the complex stability increases with polyamine length because of the rise in the number of hydrogen bond contacts and the increased molecular flexibility.

Increasing the length and the number of nitrogen atoms of the polyamines causes an increment in the number of exothermic complexation equilibria. Strong binding interactions correspond to entropy loss, while weaker binding interactions produce favourable entropic contributions. The computational analysis shows that in the 1:1 species, the amines approach the 1a5e InsP_6 conformation *syn* to the phosphate at C2, setting up a network of -N-H···O- and -N···H-O- hydrogen bonds, mediated by the solvent molecules. The second amine in the 2:1 complexes seems to be linked to the other side of the ring. The calculated structural parameters are in line with the stability of the studied species: as the number and strength of the hydrogen bonds increases, the stability of the complexes tends to rise.

Experimental and theoretical data suggest a proton transfer from the amine to the inositol phosphate and vice versa within the adduct. The former, leading to an endothermic contribution, is important for the most protonated amines and deprotonated InsP_6 forms, while the latter, giving an exothermic contribution, is significant for the larger amines in a partially protonated state and highly protonated InsP_6 anions. The entropic changes occurring upon the adducts formation reveal

how the desolvation effects, the amine chain length and the growing stiffening experienced by the polyamines upon protonation affect the stability of the detected complexes.

The progressive determination of the thermodynamic, chemical and structural characteristics of all the species predicted to be present *in vivo* becomes necessary to have a full understanding of the complex chemical behaviour of these biomolecules.

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