

# Molecular dynamics simulations of HPr under hydrostatic pressure

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## 1 Abstract

The histidine-containing protein (HPr) plays an important role in the phosphotransferase system (PTS). The deformations induced on the protein structure at high hydrostatic pressure values (4, 50, 100, 150 and 200 MPa), were previously (Kalbitzer,

H., Görler, A., Li, H., Dubovskii, P., Hengstenberg, A., Kowolik, C., Yamada, H., and Akasaka, K. 2000. *Prot. Sci.* **9**: 693–703.) analyzed by NMR experiments: the non-linear variations of the amide chemical shifts at high pressure values were supposed to arise from induced shifts in the protein conformational equilibrium. Molecular dynamics simulations are here performed, to analyze the protein internal mobility at 0.1 MPa, and to relate the non-linear variations of chemical shifts observed at high pressure, to variations in conformational equilibrium. The global features of the protein structure are only slightly modified along the pressure. Nevertheless, the values of the Voronoi residues volumes show that the residues of  $\alpha$  helices are more compressed than those belonging to the  $\beta$  sheet. The  $\alpha$  helices are also displaying the largest internal mobility and deformation in the simulations. The non-linearity of the  $^1\text{H}$  chemical shifts, computed from the MD simulation snapshots, is in qualitative agreement with the non-linearity of the experimentally observed chemical shifts.

## 2 Keywords

Hydrostatic pressure; Nuclear Magnetic Resonance; molecular dynamics simulation; histidine-containing protein; chemical shift; Voronoi tessellation.

## 3 Abbreviations and symbols

NMR: Nuclear Magnetic Resonance

MD: molecular dynamics

HPr: histidine-containing protein

PTS: phosphotransferase system

EIN: Enzyme I

NOE: nuclear Overhauser effect

## 4 Introduction

The histidine-containing proteins (HPr)<sup>1</sup> plays an important role into the phosphotransferase system (PTS), which performs sugar transport in a variety of bacterial species. An histidine, located in the active site, is phosphorylated (His-15 in *Staphylococcus carnosus*) by the Enzyme I, and the phosphoryl group is then available for transfer to any sugar-specific IIA protein. The structures of HPr from several organisms were determined by NMR<sup>2-7</sup> and X-ray crystallography.<sup>8,9</sup> All these proteins have a similar tertiary structure<sup>10</sup> formed by a four-stranded anti-parallel  $\beta$ -pleated sheet and three right-handed structures (Fig. 1). In HPr from *Staphylococcus carnosus*, the anti-parallel  $\beta$ -pleated sheet consists of four  $\beta$  strands: A (residues 2-7), B (residues 34-37), C (residues 40-42) and D (residues 60-65). The right-handed structures are observed for residues 18-27 (helix H1), residues 47-53 (helix H2) and residues 71-85 (helix H3), with a  $3_{10}$  helix for positions 82-85. The active site loop (residues 8-17) linking the  $\beta$  strand A and H1 contains His 15. The regulatory region (residues 43-60) includes H2 and is parallel to the active site loop. The helices H1 and H3 are located on one side of the protein, whereas the  $\beta$  sheet is located on the other side, and the active site loop and the regulatory region are located on the top of the structure. The interaction surface of HPr with both Enzyme I (ENI) and IIA protein<sup>11</sup> is the same, and contains the helices H1 and H2, the active site loop and the C terminal part of H3.

The <sup>15</sup>N-labeled HPr from *Staphylococcus carnosus* was studied<sup>12</sup> by recording NMR <sup>15</sup>N-<sup>1</sup>H 2D HSQC experiments at hydrostatic pressure values of 0.1, 4, 50, 100, 150 and

200 MPa. As the NMR signal keeps the same dispersion at higher pressures than at 0.1 MPa, there is no protein denaturation during these experiments. Linear variations of  $^1\text{H}$  and  $^{15}\text{N}$  chemical shifts were observed for most of the resonances, but some resonances displayed large and non-linear variations. These resonances belong to residues locally clustered in the active site loop and in the regulatory helix H2: these residues are directly involved in the interaction with enzymes ENI and IIA.

Non-linearity of chemical shift variations under hydrostatic pressure, was observed for several proteins<sup>12-15</sup> and was proposed to be due to pressure-induced shifts in the conformational equilibrium of flexible regions. In the case of HPr, the internal mobility of the active site loop and of the regulatory helix H2, which is deduced from the non-linear chemical shift variations, may be required in order to adapt the surface to the interaction with other proteins.

The effect of hydrostatic pressure on proteins was already studied by molecular dynamics simulations,<sup>16</sup> performed on BPTI,<sup>17-19</sup> on protein-nucleic acid assemblies,<sup>20,21</sup> on myoglobin,<sup>22</sup> on superoxide dismutase<sup>23</sup> and on lysozyme.<sup>24</sup> Molecular dynamics (MD) simulations of the HPr from *Staphylococcus carnosus* at several hydrostatic pressure values, are here presented. These simulations are dealing with the non-linear variation of the chemical shifts with the hydrostatic pressure, and were thus analyzed in order to answer to the following questions: (i) is pressure inducing shifts in the protein conformational equilibrium ? (ii) can the non-linear variations of chemical shifts be explained only from the deformation of the protein structure ?

## 5 Materials and Methods

The starting conformation for the simulations was the first conformer of the NMR structure of HPr.<sup>4</sup> The molecular dynamics and molecular mechanics calculation were performed with the parm98 parameter set<sup>25</sup> and the TIP3P model for water.<sup>26</sup> The program AMBER 6.0<sup>27</sup> was used for the simulations performed with the Berendsen thermostat, and the program ORAC 4.0<sup>28</sup> for the simulations performed with the Nose-Hoover thermostat.

The system for the molecular dynamics (MD) simulations was built in the following way. The protein structures were hydrated by a box of water molecules in a pseudo-equilibrated configuration, and a cutoff of 9 Å was used along the three axes to discard any water molecule if it is farther than the cutoff from any solute molecule. The box dimensions were 59.5 x 49.4 x 50.3 Å<sup>3</sup> and the number of water molecules 3966. The sidechains of the residues Arg 17, Lys 28, 40, 41, 45, 57, 83 and Glu 1, 11, 36, 60, 70, 84, and the N and C terminal ends were charged. Eight Na<sup>+</sup> and one Cl<sup>-</sup> counter-ions were used to neutralize these charges, and to produce a ionic concentration of 10 mM, analogous to those used during the NMR experiments.<sup>12</sup> A cutoff of 9 Å was used for Lennard-Jones interactions, and long-range electrostatics interactions were calculated using the Particle Mesh Ewald protocol.<sup>29</sup>

The molecular dynamics (MD) simulations at different hydrostatic pressures were initiated with several rounds of semi-restrained and eventually unrestrained minimizations of the entire system followed by heating, equilibration and unrestrained (T, P,

N) ensemble MD.<sup>30</sup> Harmonic restraints of 100 kcal/mol  $\text{\AA}^2$  were placed on the protein atom and ion positions during the first round of 500 steps minimization, and were relaxed on the ions more quickly than on the protein atoms over the course of one subsequent 500-step minimization, and of three subsequent 250-step minimizations. The final round of 500 steps of minimization involved all atoms of the system.

Heating and system equilibration were performed as follows: 10 ps of heating up to 300 K at constant volume were done while restraining the protein atom and the ion positions with a restraint of 25 kcal/mol  $\text{\AA}^2$ ; 5 ps of MD at constant volume and 4 MD rounds of 2.5 ps at constant pressure, during which the restraints on the ions were reduced more quickly than on the protein atoms; a last MD round of 10 ps with a restraint of 1.5 kcal/mol  $\text{\AA}^2$  on the protein atoms and no restraints on the ions. This equilibration protocol was applied after each pressure jump.

Most of the isothermal-isobaric unrestrained simulations was performed using the Berendsen algorithm<sup>31</sup> for temperature bath coupling and a 2 fs time-step. All covalent bonds involving hydrogen atoms were kept rigid using SHAKE.<sup>32</sup>

Additional MD simulations (hpr-0.1n and hpr-200n) at 0.1 and 200 MPa, were run using the program ORAC 4.0<sup>28</sup> with a Nose-Hoover thermostat<sup>33</sup> for monitoring temperature and pressure, and an extended Lagrangian scheme for the integration of motion equations. A multiple-time-step scheme was used for the integration of the motion equations, with timestep values in the 0.5-12 fs range, according to the type (intramolecular or non-bonded) of considered interactions. The Nose-Hoover thermostat was characterized by an harmonic frequency of 30  $\text{cm}^{-1}$ . The equilibration of the

system was performed using a 240 ps simulation.

The MD simulations were run at a temperature of 298 K. The simulation lengths and hydrostatic pressure values are given in Table 1. The atom coordinates were saved each ps. Increasing pressure values of 0.1, 4, 50, 100, 150, 200 and 2000 MPa, were used. Each simulation at a given pressure started from the last snapshot recorded at the previous pressure. An additional backward MD simulation (hpr-0.1b) was run at 0.1 MPa starting from the last snapshot recorded at 200 MPa.

The analyzes were mainly performed with the program ptraj.<sup>27</sup> The program VMD<sup>34</sup> was used for the visualization of the trajectories. The analyzes were performed on the protein conformers sampled during the 0.5-2 ns (respectively 0.5-1 ns) interval of the 2 ns (respectively 1 ns) simulations. For all analyzes but the Voronoi volume calculations, all protein conformers recorded during the simulation were used. For the Voronoi volume calculations, one conformer out of 10 was used.

The Voronoi tessellation of the protein was determined using the program ORAC 4.0 as previously described.<sup>23</sup> The Voronoi polyhedra were determined on each protein residues, by using a cutoff value of 7 Å to define the neighbor atoms, and by taking into account only the heavy atoms.

Hydrogen chemical shifts were calculated from the protein simulation conformers using the program CS\_proton and the parameters determined in the literature.<sup>35</sup> Hydrogen and nitrogen <sup>15</sup>N chemical shifts were also calculated using SHIFTX.<sup>36</sup> Random coil chemical shift values<sup>37</sup> were subtracted from the theoretical and experimental chemical shifts before comparing them.

## 6 Results

### 6.1 Molecular dynamics simulation at 0.1 MPa

The reliability of the simulation was checked by comparing the conformations sampled during the trajectory with the NMR structure of HPr. The secondary structures of HPr were analyzed by calculating, for the 49 hydrogen bonds characteristic of the secondary structures, the mean NH...CO distances and the mean NHO angles during the trajectory along with their standard deviations. The differences between the mean angle and distances calculated along the trajectory and those measured in the HPr structure, were compared. For the distances, only 7 differences out of 49 are larger than 0.5 Å and among them, 4 are located in the helix H3 (residues 70, 74, 85, 86). Only 5 angle differences over 49 are larger than 20°, and among them, 3 are located in the helix H3 (residues 75, 76, 82).

The variation of protein secondary structures was determined, by analyzing the backbone H...O hydrogen bonds in the secondary structure elements. An hydrogen bond is considered to be established if the mean length of H...O is smaller than 2.5 Å. The comparison of the number of hydrogen bonds between the NMR structure and the trajectory recorded at 0.1 MPa reveals: (i) a slight decrease of the number of hydrogen bonds in the N terminal part of the  $\beta$  strand A, and in the C terminal part of the  $\beta$  strand B, (ii) the transient breaking of the hydrogen bonds in helix H2, which induces mean lengths larger than 2.5 Å and (iii) a slight increase of the number of hydrogen bonds in H1 and H3. The breaking of the H2 hydrogen bonds is not in

contradiction with the NMR measurements; as the nuclear Overhauser effects (NOE) are proportional to the inverse sixth power of the distances, hydrogen bonds can be disrupted in a transient way and the NOEs nevertheless observed.

The distances observed between hydrogens during the trajectory or in the NMR conformers were compared to interval distance restraints used to calculate the NMR structure. The mean value  $R$  of a given distance  $r$  along the trajectory or in the NMR structure, was calculated as:  $1/R^6 = \langle 1/r^6 \rangle$ , where  $\langle \rangle$  is the mean value, and  $r$  is the distance in each snapshot or NMR conformer. The distance restraint was considered as violated, if  $R$  is larger than  $U + 0.5 \text{ \AA}$  or smaller than  $L - 0.5 \text{ \AA}$ , where  $U$  (respectively  $L$ ) is the upper (respectively lower) bound of the NOE interval. According to this criterion, 79 NOE restraints are violated in the NMR structure, and 89 are violated during the 0.1 MPa MD simulation. The mean value of the upper violation is  $1.6 \text{ \AA}$  in the simulation, and  $1.3 \text{ \AA}$  in the NMR structure. The numbers of violated NOEs are small with respect to the total number of NOE restraints used to determine the structure (1108 restraints), and the upper violations are of the same order of value in the simulation and in the structure.

In the simulation performed on HPr from *Escherichia coli*,<sup>38</sup> only 7 NOE violations were observed, which is due to the following reasons: (i) the variation from upper or lower bound defining a NOE restraint violation was larger ( $1.0 \text{ \AA}$ ) in the *E. coli* simulation than the one used here ( $0.5 \text{ \AA}$ ), (ii) the authors determined the NOE violations on an averaged and minimized conformer obtained from the MD trajectory.

The fluctuations of atomic coordinates by residue (Fig. 2a) reveal some peaks of

larger fluctuations along the sequence, corresponding to the N terminal (residues 1-3) and C terminal (residues 83-88) parts, to the regulatory region (residues 47-53), to the active site loop (residues 9-14), to the region 38-41 of the  $\beta$  hairpin linking the  $\beta$  strands B and C, which are the closest  $\beta$  strands to H2. All  $\beta$  strands but C display small atomic fluctuations. These observations are in agreement with NMR  $^{15}\text{N}$  relaxation measurements,<sup>39</sup> which detected high internal mobility in the active site loop, the N and C terminal parts, and the  $\beta$ -strand C.

The MD simulation at 0.1 MPa retains most of the features of the HPr NMR structure. The observed internal mobility is in agreement with previous experimental observations.

## 6.2 Global changes induced by pressure

In all simulations, the root-mean-square deviation (RMSD) values of the atomic coordinates from the starting conformer reach a plateau after the first 300 ps of the simulation, and are smaller than 2.5 Å. In order to investigate the effect of the simulation length on the conformational change, the 2ns-simulation run at 200 MPa was continued up to 8 ns. The additional time interval displays a RMSD value of the protein structure with the starting point of about 2.7 Å. The different features of the protein structure (gyration radius, interaction with water, secondary structures, residue fluctuations) are not significantly different from those observed in the 0-2 ns interval. The total 14 ns-simulation run on HPr, from 0.1 MPa up to 200 MPa, are thus sufficient to analyze the conformational changes induced by pressure.

The changes induced on the protein structure by the hydrostatic pressure were monitored by analyzing the mean conformers of each MD trajectory. The RMSD values of atomic coordinates between the mean conformers, are in the ranges of 0.68-1.0 Å for all atoms and of 0.57-0.95 Å for heavy atoms: this is of the same order of value than the RMSD values between the conformers of the NMR structure.<sup>4</sup> The global changes induced on the protein structure by the hydrostatic pressure are on average of similar amplitude than the conformational variability in the NMR structure determined at 0.1 MPa. Mean RMSD values of 0.50, 0.64 and 0.63 Å are found for the 2-7, 20-41, 50-84 regions, which are the best defined regions in the NMR structure.<sup>4</sup> Larger mean RMSD values of 0.84 and 0.74 Å are found in the active site loop (residues 8-19) and in the regulatory region (residues 42-49), for which large RMSD values were observed in the NMR structure.<sup>4</sup> The less-well defined regions of the protein NMR structure, which are usually thought to be the more mobile ones, are thus undergoing the largest changes under hydrostatic pressure. A mean RMSD value of 0.68 Å is observed for the 85-88 region, at the C terminal part of H3. This helix, which already showed distortions at 0.1 MPa, is continuing to be deformed at higher pressures. The mean values of the hydrogens bonds of H2, which were larger than 2.5 Å at 0.1 MPa, are smaller than 2.5 Å for pressure values larger than 4 MPa. These smaller values are the sign of the increase of the proportion of formed hydrogen bonds, and are thus supporting the existence of a shift in the H2 conformational equilibrium.

Some  $\phi$  and  $\psi$  backbone angles of the protein, monitored at 0.1 and 200 MPa, are plotted in Fig. 3 for residues located in the active site loop (residues 7 and 10), in the

regulatory region (residues 50, 53 and 60) and in the helix 3 (residue 85). All display either changes in the  $\phi$  and  $\psi$  equilibrium, either changes of the  $\phi$  and  $\psi$  mean values, which are supporting a shift in the conformational equilibrium of the protein backbone.

The protein gyration radius was calculated along the trajectories, recorded at different pressures. The gyration radius  $R$  of the protein is defined as the root-mean-square distance of the atoms to their centroid:

$$R = \sqrt{\sum_{i=1}^N [x_i - X_i]^2 / N} \quad (1)$$

where  $x_i$  is the position vector of the atom  $i$ ,  $N$  is the number of atoms, and:

$$X_i = \frac{1}{N} \sum_{i=1}^N x_i \quad (2)$$

The gyration radius displays variations in the range 11.8-12.3 Å, which is a small percentage (4.1 %) of its mean value. This percentage is slightly larger than the one (0.9 %) observed in a previous MD simulation of BPTI at high hydrostatic pressure.<sup>18</sup>

In order to analyze the mechanical effect of the pressure on the protein tertiary structure, the bending angles of the  $\alpha$ -helices H1 and H3 axes were calculated in the following way. The vectors N(i)-C'(i+1), where C' is the carbon of the carbonyl group, were recorded along the trajectory, and the angles between successive vectors along the helix were determined. In a straight helix, all vectors are aligned, and the angles between them equal to 0: thus, the mean value of the angles, calculated on the helix

residues and on the trajectory, is giving the bending angle of the helix axis. No bending was calculated for the helix H2, as this helix short length makes difficult to get significant numbers for the mean angle values. The bending angle values calculated for the helices H1 (respectively H3) are increasing from  $11.0^\circ$  (respectively  $12.7^\circ$ ) to  $13.2^\circ$  (respectively  $14.4^\circ$ ) when the pressure changes from 4 to 50 MPa, which indicates a tendency of both helices to be more bended at high hydrostatic pressure. But, for larger pressures, the H1 bending angle is continuing to increase up to  $13.9^\circ$  at 200 MPa, whereas the H3 bending angle is decreasing to  $12.4^\circ$  at 200 MPa. This behavior of H3 may be due to the C-terminal position of the helix in the protein: the C terminal end of the helix is not covalently bound to the protein structure.

In order to further analyze the changes in HPr tertiary structure, the distances between the axes of secondary structure elements were determined. Points belonging to an helix axis were calculated as the middle points of the segments  $(N(i), N(i+2))$ ,  $(C\alpha(i), C\alpha(i+2))$  and  $(C'(i), C'(i+2))$ , where N,  $C\alpha$  and  $C'$  are the backbone heavy atoms. The axis of a  $\beta$  strand was the line crossing the heavy atoms of the strand backbone. The distance between two axes was then determined as the minimum value of all possible distances between the points belonging to these axes. The only distance between axes exhibiting significant variations along the hydrostatic pressure, is the distance between H3 and  $\beta$  strand A, which varies from  $8.12 \pm 0.31 \text{ \AA}$  at 0.1 MPa to  $7.77 \pm 0.20 \text{ \AA}$  at 200 MPa.

The parameters describing the global protein structure form do not vary much under the application of the hydrostatic pressure. The largest deformations are observed in

helices H2 and H3, and in the active site loop, and they are concerning shifts in the protein conformational equilibrium. There is no denaturation of the protein under hydrostatic pressure, which is in agreement with the dispersion of the NMR chemical shifts observed experimentally.<sup>12</sup> Moreover, the study of lysozyme under hydrostatic pressure, revealed that the chemical shifts change linearly with pressure in the protein denatured state.<sup>40</sup> Thus, the non-linear variation of chemical shifts in HPr is another argument supporting the native state of the protein, at high hydrostatic pressure.

Nevertheless, the simulation conditions, as the simulation timescale, the use of explicit solvent and of a dielectric constant of 1.0, are not favorising the protein unfolding. Indeed, the myoglobin unfolding under pressure was simulated<sup>22</sup> with implicit solvent and a dielectric constant values in the 78-101 range.

### 6.3 Variations of mobility

The majority of the protein residues undergoes a decrease of coordinate fluctuations along the application of hydrostatic pressure. Nevertheless, few residues located in the active site loop (residues 7-11, 15), in the regulatory region (residues 57-59) and in the C terminal part (residues 86-88) exhibit larger coordinate fluctuations at 200 MPa than at 0.1 MPa. These three regions being close to each other in the protein structure, their similar behavior is not surprising. This pressure effect on the residue fluctuations can be related to the influence of the temperature on the nitrogen pressure coefficients.<sup>12</sup> In both cases, residues near the active site loop are concerned.

The variation of internal mobility along the pressure was also monitored by cal-

culating the mean value and the standard deviation of the coordinate fluctuations at pressures in the 0.1-200 MPa range (Fig. 2b). Large variations of coordinate fluctuations are observed in the helices H2 and in the C terminal part of H3. The smallest variations of coordinate fluctuations are observed for residues located in the  $\beta$  strands A, B and D. The  $\beta$  strand C exhibits larger variations of residue coordinate fluctuation than the other  $\beta$  strands. This  $\beta$  strand is close to helix H2, and may be affected by the variations of internal mobility in this helix. The regions exhibiting large variations of coordinate fluctuations are the same as those displaying large RMSD values measured between protein mean conformers (section “Global changes induced by pressure”) and large atomic fluctuations at 0.1 MPa (Fig. 2a).

The regions exhibiting greater conformational flexibility at high pressure seem to be in contradiction with the remarks in the literature,<sup>41</sup> that the volume fluctuations are invariant along pressure, in the case of linear variations of the chemical shifts. But, in HPr, the non-linear variations of chemical shifts, is the cause of a variation in volume fluctuations.

## 6.4 Variations of volume

The variation of protein volume under hydrostatic pressure was analyzed through a Voronoi tessellation of the protein. The protein total volume is stable: the standard deviation values are always smaller than 0.7 % of the total volume, and the volumes vary in the 11400-12000 Å<sup>3</sup> range. This variation is similar to the variation calculated on superoxide dismutase.<sup>23</sup>

The residue Voronoi volumes are decreasing in the 0.5-8% range when the hydrostatic pressure increases from 0.1 to 200 MPa. As the variations of the volumes are smaller than their standard deviations, they are not significant for the considered range of hydrostatic pressures. In order to produce a significant volume variation, an additional MD trajectory (hpr-2000) was run at 2000 MPa. No effort was made to model the ice VII properties,<sup>42</sup> nor the water dielectric constant.<sup>22</sup> The purpose was to test the mechanical properties of the protein structure and not its physico-chemical properties, and we were not trying and not expecting to unfold HPr.

For the pressure values  $p$  from 4 up to 2000 MPa, the ratio of each residue volume at the pressure  $p$  and of its volume at 0.1 MPa was calculated. In the 4-200 MPa range, the ratio distributions are unimodal, centered on values decreasing from 1.00 for  $p = 4$  MPa (Fig. 4a), to 0.96 for  $p = 200$  MPa (Fig. 4c). The distribution widths are smaller than 0.1.

For the pressure value of 2000 MPa, the distribution of ratio values is bimodal (Fig. 4c) and shifted towards smaller ratio values. The mean ratio value is 0.84, and the distribution width is about 0.12. The less compressed 29 residues for which the ratio is larger than 0.86 are located in the helix H2 (residues 49-50), in the active site loop (residues 13, 15), in the  $\beta$  strands B, C (residues 32, 34, 37-36, 41) and D (residues 60, 62, 64, 67). The more compressed 25 residues for which the ratio is smaller than 0.83 are all but four (residues 1, 14, 38 and 64) located in helices H1, H3 and H2. Thus, in H2, the exposed residues 48, 51 and 52 exhibit ratios smaller than 0.83, and the buried residues 49 and 50 ratios larger than 0.86. Inversely, in the active site loop, the

exposed residues 13 and 15 have ratios larger than 0.86, whereas the buried residue 14 has a ratio smaller than 0.86. This difference of behavior probably comes from the differences in the secondary structure topologies.

From the observations quoted above, the  $\alpha$  helices and the loops seem more prone to compression than the  $\beta$  strands. The different behaviors of  $\alpha$  and  $\beta$  secondary structures follow the expansion in the 3D space of the hydrogen bond networks. Indeed, the network of the hydrogen bonds in  $\alpha$  helices is more locally spread in the space than the network of the hydrogen bonds in a  $\beta$  sheet. Thus, the  $\beta$  sheet is a larger object which is more difficult to compress than an  $\alpha$  helix. These results are in complete agreement with the observations made on the crystal structure of lysozyme at high hydrostatic pressure,<sup>43</sup> with the analysis of the pressure response of BPTI<sup>41</sup> and with the results obtained from MD simulations.<sup>44</sup>

## 6.5 Hydration of the protein

In each simulation snapshot, the hydrogen bond partners of a protein amide hydrogen were determined as the oxygen atoms closer than 1.4 Å to the hydrogen. These oxygens were then sorted into water or protein oxygens, and the relative residence times of protein and water oxygens were determined as percentages of the total simulation length. As the protein carbonyl and the water oxygens correspond to different chemical functions, their shielding effects on the <sup>15</sup>N and <sup>1</sup>H chemical shifts of the amide groups, are different.

The mean values and standard deviations of the residence times of the water

molecules were calculated for pressure values in the 0.1-200 MPa range (Fig. 5a). Residence times larger than 50% are obtained for residues located at the edges of the  $\beta$  sheet (residues 3, 5, 7, 41, 43, 44, 60), at the N terminal parts of  $\alpha$  helices (residues 16, 17, 48, 49, 51, 71), in loops (residues 9, 11, 14, 46, 68), or in a  $\beta$  hairpin (residues 38, 39). The residues located at the edges of the  $\beta$  sheet have usually a standard deviation of the water residence time smaller than 5%. As they are among the least compressed residues, it is not surprising that their hydration pattern is not much modified by the pressure.

The residues exhibiting standard deviations of the water residence time larger than 15% are located in the active site loop (residues 9, 10, 13, 14), in the helix H2 (residues 50, 51), in the regulatory region (residues 46, 56, 57), at the N terminal part of helix H3 (residues 68, 69), and in the  $\beta$  strand B (residue 35). These large variations of the residence times along the pressure can be due to the larger coordinate fluctuations at 200 MPa than at 0.1 MPa (section "Variations of mobility under hydrostatic pressure"), exhibited by the active site loop (residues 7-11, 15), by the regulatory region (residues 57-59) and by the C terminal part (residues 86-88).

In the active site loop and in the regulatory region, the residues undergo a large reorganization of their hydrogen bonds: the hydrogen bonds between Ile-9 and Thr-7, between Asp-10 and Ile-8 and between Gly-13 and Asp-10, are broken. Val-50 is establishing an hydrogen bond with Ser-46, and the hydrogen bond established by Lys-57 is switching from an equilibrium between Asp-10 and Glu-11 to an hydrogen bond with the sidechain oxygen of Asp-10. Shifts in the conformational equilibrium of the

active site loop and of the regulatory region are thus induced by pressure.

The mean number of hydrogen bond partners and its standard deviation along the pressure variation were also calculated (Fig. 5b). The profile of the numbers of partners and of the water residence times are roughly similar: large numbers correspond to long times. But, some differences are observed for the standard deviation values: (i) residues for which small standard deviation values are observed for the residence times, and large ones for the number of partners, (ii) residues for which large standard deviation values are observed for the residence times, and small ones for the number of partners. The observation (i) means that the electronic environment of the amide hydrogen is constant, but the flexibility of the region is modified. The observation (ii) means that the electronic environment of the amide hydrogen is changing. The residues belonging to the class (i) are: 3, 5, 7, 11, 16, 38, 39, 41, 43, 54, 55, 60, 68, 71. They are located at the protein surface, and are thus particularly prone to variations of their flexibility through the application of pressure. The residues belonging to the class (ii) are located in the active site loop (residues 9, 10, 13, 14, 15), in the  $\beta$  sheet (residue 35), in the regulatory region (residues 46, 50, 51, 57) and at the N terminal part of H3 (residue 69). Change of electronic environment thus takes mainly place for residues located in the active site loop and in the regulatory region.

## 6.6 Relating the changes observed in MD simulations with the variation of experimental chemical shifts

The variations of  $^{15}\text{N}$  chemical shifts with pressure are smaller than 0.6 ppm in the  $\beta$  strands A, B and D. The  $\beta$  strand C, the closest strand to H2, displays the largest variations (0.78 for residue 41) among the  $\beta$  strands. The large conformational fluctuations induced by hydrostatic pressure in H2 produce certainly changes of the electronic environment, which are propagating on the neighboring secondary structures. Variations of the  $^{15}\text{N}$  chemical shifts in the 0.4-1.0 ppm range are observed in the active site loop (residues 8-16), in H1 (residues 16-29), in the regulatory region (residues 48-51, 55-59) and in H3 (residues 72-86). The larger variations observed in helices, and loops are in good agreement with the larger compression at high hydrostatic pressure of these regions. On the other hand, the small variations of chemical shifts observed in the majority of  $\beta$  strands agree with their limited compression at high pressure.

The large variations of chemical shifts observed in H3 and in H1, are mainly concerning residues which have sidechains directed towards the protein exterior. These large values can be due to the bending of helix axes observed at high hydrostatic pressure (section "Secondary structure stability"). Indeed, as the bending angle is positive for both helices and as they are in anti-parallel orientation, both are bending towards protein interior. This behavior has an influence on the secondary structure hydrogen bonds and thus on the chemical shift values.<sup>45</sup>

Using the programs CS\_proton,<sup>35</sup> and SHIFTX,<sup>36</sup> theoretical  $^1\text{HN}$  and  $^{15}\text{N}$  chem-

ical shifts were determined as the mean values of chemical shifts calculated on each snapshot of the MD simulation. The theoretical and experimental shifts with respect to random coil values were compared for all amide hydrogens involved into an hydrogen bond specific of secondary structure. A linear regression, performed for each hydrostatic pressure value, between the calculated and experimental shifts, gives correlation coefficients in the 0.70-0.75 range and slope values in the 0.33-0.39 range for the CS\_proton prediction. These ranges are of the same order of value than those obtained previously.<sup>46</sup> Concerning the chemical shifts calculated with SHIFTX, the correlation coefficients are in the 0.57-0.70 range for the HN nuclei, and in the 0.82-0.86 range for the <sup>15</sup>N nuclei. These values are only slightly smaller than those obtained in the Ref. 36: but, in this article, the method was tested on high-resolution X-ray crystallographic structures.

The non-linear variations of chemical shifts with respect to pressure were analyzed<sup>15</sup> by fitting the chemical shift variation along the pressure with the function:

$$\delta = \delta_0 + (p - p_0)\alpha + (p - p_0)^2\beta \quad (3)$$

$\alpha$  and  $\beta$  being the linear and non-linear coefficients of the chemical shift variation,  $\delta_0$  the chemical shift value at 0.1 MPa, and  $p_0 = 0.1$  MPa. The fitting was performed using a linear least-square to obtain two sets of parameters  $\alpha$  and  $\beta$  from the <sup>15</sup>N and <sup>1</sup>H experimental chemical shifts. The residues 30, 42 and 68 were not processed, as their chemical shift values were not measured at all pressures. Both sets of obtained  $\beta$

values (Figs. 6a and 6b) display large absolute values in the active site loop (residues 10-16). This observation can be related to the fluctuations in electronic environment and to the shift in conformational equilibrium observed in this loop (section “Hydration of the protein under hydrostatic pressure”).

In other protein regions, different patterns are observed for  $^{15}\text{N}$  and  $^1\text{H}$   $\beta$  values. The  $^{15}\text{N}$   $\beta$  values display isolated maxima in the  $\beta$  strands B and C (residues 33, 38), in the regulatory region (residues 56, 59) and in H3 (residues 77, 81). Large  $^1\text{H}$   $\beta$  values are observed in the N terminal parts of helices H1 (residues 21-28) and H3 (residues 71-78). The non-linearity of  $^1\text{H}$  chemical shifts in H1 and H3 may reflect the bending of the helices axes, and their larger compression under hydrostatic pressure.

As it was already observed in the literature,<sup>15</sup> the non-linearity of  $^1\text{H}$  chemical shift is thus related to changes in conformational equilibrium at high hydrostatic pressure. Indeed, regions prone to compression or internal mobility, display maxima of  $^1\text{H}$   $\beta$  values. One can also notice that the active site loop, which displays, at the same time, large variations of the electronic environment of amide groups and conformational fluctuations, is the region exhibiting the largest  $\beta$  values for  $^{15}\text{N}$  and  $^1\text{H}$  chemical shifts.

Theoretical values of the  $\beta$  coefficients were calculated, as in Eq. 3, from the variation of the theoretical  $^1\text{H}$  and  $^{15}\text{N}$  chemical shifts along the pressure. For the  $^1\text{H}$  chemical shifts calculated with CS\_proton, the profile of theoretical  $\beta$  values (Fig. 6c) is different from the profile of experimental  $\beta$  values (Fig 6a). Maxima of theoretical  $\beta$  values are observed in the active site loop (residues 10-15), as for experimental val-

ues. But, larger maxima are observed in the helix H2 (residues 50-54) and in the C terminal part of helix H3 (residues 83-86) than in the active site loop. The profile of  $^1\text{H}$  theoretical  $\beta$  values, calculated from the results of SHIFTX, is in better agreement with experiment than the profile calculated from the CS\_proton results. On the other hand, the  $^{15}\text{N}$   $\beta$  profile calculated from the results of SHIFTX, disagrees with the  $^{15}\text{N}$  experimental profile in the regulatory domain: this may be due to the referencing of the  $^{15}\text{N}$  chemical shifts on the NMR spectra. But, all theoretical profiles are in qualitative agreement with the observations made in Ref. 12, where the non-linear variations of chemical shifts were described for residues located in the active site loop, in the regulatory region and in H3.

## 6.7 Hysteresis of the changes induced by hydrostatic pressure

The backward pressure jump from 200 to 0.1 MPa produces an increase of total energy of about 800 kcal/mol, which is about the opposite of the cumulative energy variation between pressure values of 0.1 and 200 MPa. The RMSD value of the protein atomic coordinates with respect to the initial conformation, is about 1.5 Å during the first ns of the simulation, and jumps from 1.5 Å up to 2.5 Å during the time interval 1.4-1.6 ns. Two average protein structures were calculated from the snapshots recorded in the 0-1 ns and in the 1.5-2 ns time intervals. The RMSD between the two average structures is 1.49 Å for the complete protein, whereas it is 1.87 Å for the H2 region (48-53). The increase of RMSD with respect to the initial conformation, observed in the second part of the trajectory, comes thus from a variation of the helix H2 conformation.

The NOE restraint violations were analyzed on the trajectory, as it was done for the first simulation at 0.1 MPa. 93 NOEs restraints are violated: 78 distances are larger than  $U+0.5 \text{ \AA}$  and 15 are smaller than  $L-0.5 \text{ \AA}$ . In the first trajectory recorded at 0.1 MPa, the number of upper violations was 74, and the number of lower violations was 15: these numbers are similar to those observed here.

The bending angles of the helices H1 and H3 were calculated as described previously, on conformers extracted from the intervals 0.5-1 ns and 1.5-2 ns (section "Secondary structure stability"). The bending angle of the helix H3 is remaining constant during both sub-intervals, whereas the bending angle of the helix H1 is decreasing from  $13.3 \pm 4.6^\circ$  to  $11.5 \pm 4.0^\circ$ .

The number of hydrogen bonds of the secondary structures was analyzed as described in section "Global changes induced by pressure". Almost all secondary structures display the same number of hydrogen bonds as during the trajectory recorded at 200 MPa. The two exceptions are: the helix H2, in which the hydrogen bonds are partly disrupted (mean lengths larger than  $2.5 \text{ \AA}$ ), and H3, which displays one more hydrogen bond than at 200 MPa.

A Voronoi tessellation of the protein structure was determined along the trajectory. The total protein volume is  $12014.4 \pm 88.4 \text{ \AA}^3$ . The difference of the protein volume between hpr-0.1 and hpr-0.1b is  $42.9 \text{ \AA}^3$ , and smaller than the volume standard deviations. The mean values of Voronoi volumes of each residue calculated in hpr-0.1 and hpr-0.1b, are in good agreement, as a linear regression between them gives a correlation coefficient of 1.00, a regression coefficient of 1.01 and a regression constant of  $-0.66 \text{ \AA}^3$ .

The variations of the protein structure after the reverse pressure jump from 200 to 0.1 MPa, concern the transient breaking of the H2 hydrogen bonds, the increase of the number of hydrogen bonds in H3 and the decrease of the bending of H1. These limited changes are reversing the protein structure deformations induced by pressure.

## 6.8 Comparison of the Berendsen and the Nose-Hoover thermostats

The trajectories recorded using the Berendsen and Nose-Hoover thermostats, at pressure values of 0.1 and 200 MPa, were compared two-by-two, at a given pressure value.

The comparison of NOE restraints violations, gyration radii, and secondary structure hydrogen bonds, shows no significant difference between both thermostats. For both thermostats, most of the residue fluctuations decrease from 0.1 up to 200 MPa, but some residues display an increase of fluctuations. These residues are located in the active site loop (residues 7-11, 15), in the regulatory region (residues 57-58) and in H3 (residues 86-88), for the Berendsen thermostat, and are located in H1 (residues 24, 28), in the  $\beta$  sheet (residues 43, 66, 68) and in H3 (residues 75, 83), for the Nose-Hoover thermostat.

The mean Voronoi volumes calculated for each protein residue with the Nose-Hoover and Berendsen thermostats, were compared at both pressure values of 0.1 and 200 MPa. The agreement between the volume values calculated using the two thermostat is very good: a linear regression between them gives correlation coefficients of 0.99 (0.1 MPa)

and 1.00 (200 MPa), regression coefficients of 0.99 (0.1 MPa) and 0.99 (200 MPa), and regression constants of  $1.56 \text{ \AA}^3$  (0.1 MPa) and  $1.39 \text{ \AA}^3$  (200 MPa).

Both thermostats are thus giving similar pictures of the protein MD simulations.

## 7 Discussion-Conclusion

The internal mobility and the behavior under hydrostatic pressure of HPr from *Staphylococcus carnosus* have been analyzed by MD simulations.

The MD simulation at 0.1 MPa is keeping the global features of the protein NMR structure. The analysis of the atomic fluctuations shows that the regulatory region, the N and C terminal parts and the active site loop are flexible, and provides a picture of internal mobility similar to the experimental observations.

The MD simulations at high hydrostatic pressure are in qualitative agreement with the NMR observations. The global fold of the protein is kept, the secondary structures are stable. The analysis of the residue volume variations under hydrostatic pressure, through a Voronoi tessellation, shows that the  $\alpha$  helices are generally more compressed than the  $\beta$  sheet. This larger compression is in agreement with the internal mobility observed in this region, and could play a role in the interaction of HPr with enzymes ENI and IIA. Indeed, the larger compression of this region reveals larger possibilities of protein deformation, to adapt to the conformational changes arising during the protein-protein interaction.

The hydrogen bonds established by protein backbone amide hydrogens with water

or protein oxygens were analyzed along the trajectories. The regions exhibiting the largest variability in the type of partners (water oxygen or protein carbonyl) are the active site loop and the regulatory region.

The non-linearity in the chemical shift variation was quantitatively analyzed on the experimental and computed chemical shifts. A better agreement between experimental and theoretical  $\beta$  values is found for the  $^1\text{H}$  chemical shifts calculated with SHIFTX,<sup>36</sup> than for those calculated with CS\_proton.

According to the purposes (i) and (ii) given in the introduction, the simulations presented here permitted (i) to determine the shifts in conformational equilibrium induced by pressure. These shifts concern mainly the  $\alpha$  helices and are partly reversible, as shown in the simulation hpr-0.1b. The point (ii) was answered through the comparison of the experimental and theoretical  $\beta$  values: for the calculations performed with SHIFTX, the theoretical and experimental  $\beta$  profiles are in good agreement for the  $^1\text{H}$  chemical shifts, but larger discrepancies are observed for the  $^{15}\text{N}$  chemical shifts. However, the ensemble of all theoretical profiles is in qualitative agreement with the observations made on the NMR spectra.<sup>12</sup>

An approach was proposed recently<sup>47,48</sup> to determine protein NMR structure at high hydrostatic pressure from the variations of experimental chemical shifts, and was applied to solve the NMR structures of lysozyme and BPTI. The deformations induced by pressure on the structures are limited, and the  $\alpha$  helices display more compression than the  $\beta$  strands. These deformations are thus quite similar to the observations made here in the HPr simulations. Furthermore, the calculations presented here are

permitting to test the efficiency of the chemical shift calculation to reproduce the variations of protein structure under pressure, in order to validate the efficiency of the method<sup>47,48</sup> proposed for the structure calculation at high pressure using the variations of chemical shift values. The results obtained here show that if non-linear variations of chemical shifts are observed, the choice of the method to calculate the chemical shifts is critical to be able to reproduce the non-linearity.

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## 10 Tables

Table 1

Molecular dynamics simulations run on HPr.

Simulation id	Thermostat	Hydrostatic pressure (MPa)	length (ns)
hpr-0.1	Berendsen	0.1	2
hpr-4	Berendsen	4	1
hpr-50	Berendsen	50	1
hpr-100	Berendsen	100	1
hpr-150	Berendsen	150	1
hpr-200	Berendsen	200	8
hpr-2000	Berendsen	2000	2
hpr-0.1b	Berendsen	0.1	2
hpr-0.1n	Nose-Hoover	0.1	2
hpr-200n	Nose-Hoover	200	2

## 11 Figure Captions

Figure 1

Tertiary structure of the histidine-containing protein (HPr) from *Staphylococcus carnosus*.<sup>4</sup> The  $\alpha$  helices are colored in red, the  $\beta$  sheet in green, and the loops in light blue. The figure was realized using Molscript v2.1<sup>49</sup> and Raster3D 2.7b.<sup>50</sup>

Figure 2

(a) Coordinate fluctuations ( $\text{\AA}$ ) by residues calculated on the trajectory recorded at 0.1 MPa. (b) Mean values and standard deviations of the coordinate fluctuations by residues calculated on the trajectories run in the 0.1-200 MPa range. The data are displayed according to the residue numbers.

Figure 3

Variation of  $\phi$  and  $\psi$  values during the MD trajectories recorded at 0.1 (magenta, red) and 200 MPa (blue, green). The angles  $\phi$  are plotted with magenta and blue lines, the angle  $\psi$  with red and green lines. The plots were realized for residues Thr 7 (a), Asp 10 (b), Val 50 (c), Leu 53 (d), Glu 60 (e) and Gly 85 (f). The angles are plotted along the simulation time, in ps.

Figure 4

Distributions of the ratios of residues Voronoi volumes, between 0.1 and 4 MPa (a: solid line), 0.1 and 50 MPa (a: dotted line), 0.1 and 100 MPa (b: solid line), 0.1 and 150 MPa (b: dotted line), 0.1 and 200 MPa (c: solid line), 0.1 and 2000 MPa (c: dotted line).

## Figure 5

Analysis of the hydration pattern of amide hydrogens. (a) Mean residence times of the water molecules, expressed in percentage of the total simulation length. (b) Mean numbers of hydrogen bond partners, including water oxygens and protein carbonyl oxygens. The values are plotted along to the protein residue number. The mean values of residence times and of the numbers of partners are calculated on trajectories recorded at hydrostatic pressures values in the 0.1-200 MPa range.

## Figure 6

Absolute values of the  $\beta$  coefficients ( $10^6$  ppm.MPa $^{-2}$ ) calculated from the variation of the experimental  $^1\text{H}$  (a) and  $^{15}\text{N}$  (b) chemical shifts, and from the variation of the theoretical chemical shifts calculated with CS\_proton ( $^1\text{H}$ , c) and SHIFTX ( $^1\text{H}$  d,  $^{15}\text{N}$  e). The data are plotted along the residue protein number.











