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# High pressure reveals structural determinants for globin hexacoordination: Neuroglobin and myoglobin cases

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ABSTRACT

**The influence of pressure on the equilibrium between five-(5c) and six-coordination (6c) forms in neuroglobin (Ngb) and myoglobin (Mb) has been examined by means of molecular dynamics (MD) simulations at normal and high pressure. The results show that the main effect of high pressure is to reduce the protein mobility without altering the structure in a significant manner. Moreover, our data suggest that the equilibrium between 5c and 6c states in globins is largely controlled by the structure and dynamics of the C-D region. Finally, in agreement with the available experimental data, the free energy profiles obtained from steered MD for both proteins indicate that high pressure enhances hexacoordination. In Ngb, the shift in equilibrium is mainly related to an increase in the 6c→5c transition barrier, whereas in Mb such a shift is primarily due to a destabilization of the 5c state.**

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**Key words: molecular dynamics; globins hexacoordination; essential dynamics.**

## INTRODUCTION

Hemoglobins form a widely distributed group of proteins and are responsible of a variety of biological functions in all kingdoms of life.[1–3] Many of these functions involve the coordination of small ligands to the heme group.[4] Among the six coordination positions of the Fe atom, the equatorial ones are occupied by nitrogen atoms of the heme unit. With regard to the axial sites, the proximal position usually coordinates His or Cys, and in many cases the distal one is occupied by exogenous ligands. The distal cavity of many of the globins belonging to this group contains a histidine residue (HisE7), which contributes to stabilize the heme-$O_2$ complex through hydrogen bonding.[5] However, in several heme proteins, ligand binding is impeded by direct coordination of HisE7 to the Fe atom, thus forming a hexacoordinated (6c) globin, as noted in neuroglobin (Ngb),[6] cytoglobin,[7] rice hemoglobin,[8] and tomato hemoglobin,[9] among others. On the other hand, myoglobin (Mb) and tetrameric human hemoglobin are relevant examples of pentacoordinated (5c) heme proteins, where the heme is ready to bind exogenous ligands.

Although heme proteins are generally classified as 5c or 6c, it may be postulated that a subtly regulated equilibrium between 5c and 6c states exists for most cases. This equilibrium allows 6c globins such as Ngb and cytoglobin to bind exogenous ligands when they are transiently found in the 5c state. This behavior results in proteins displaying moderate to high $O_2$ affinities, as noted in the fact that the $O_2$ affinity of Ngb is similar to that of Mb ($P_{50} \approx 2$ torr).[10] Moreover, it can be envisaged that fine tuning of the 5c↔6c equilibrium would provide a mechanism to modulate $O_2$ affinity. Thus, the more favored the 5c state, the higher the affinity, which in turn could depend on the residues located in the distal cavity. This is exemplified in Ngb, where Cys oxidation and disulfide bridge formation displaces the equilibrium towards the 5c state, resulting in a 10-fold increase in $O_2$ affinity.[11–13] In this scenario, knowledge of the molecular basis that underlies the regulation of the 5c↔6c equilibrium is crucial for deciphering the relationship between structure and function in globins.

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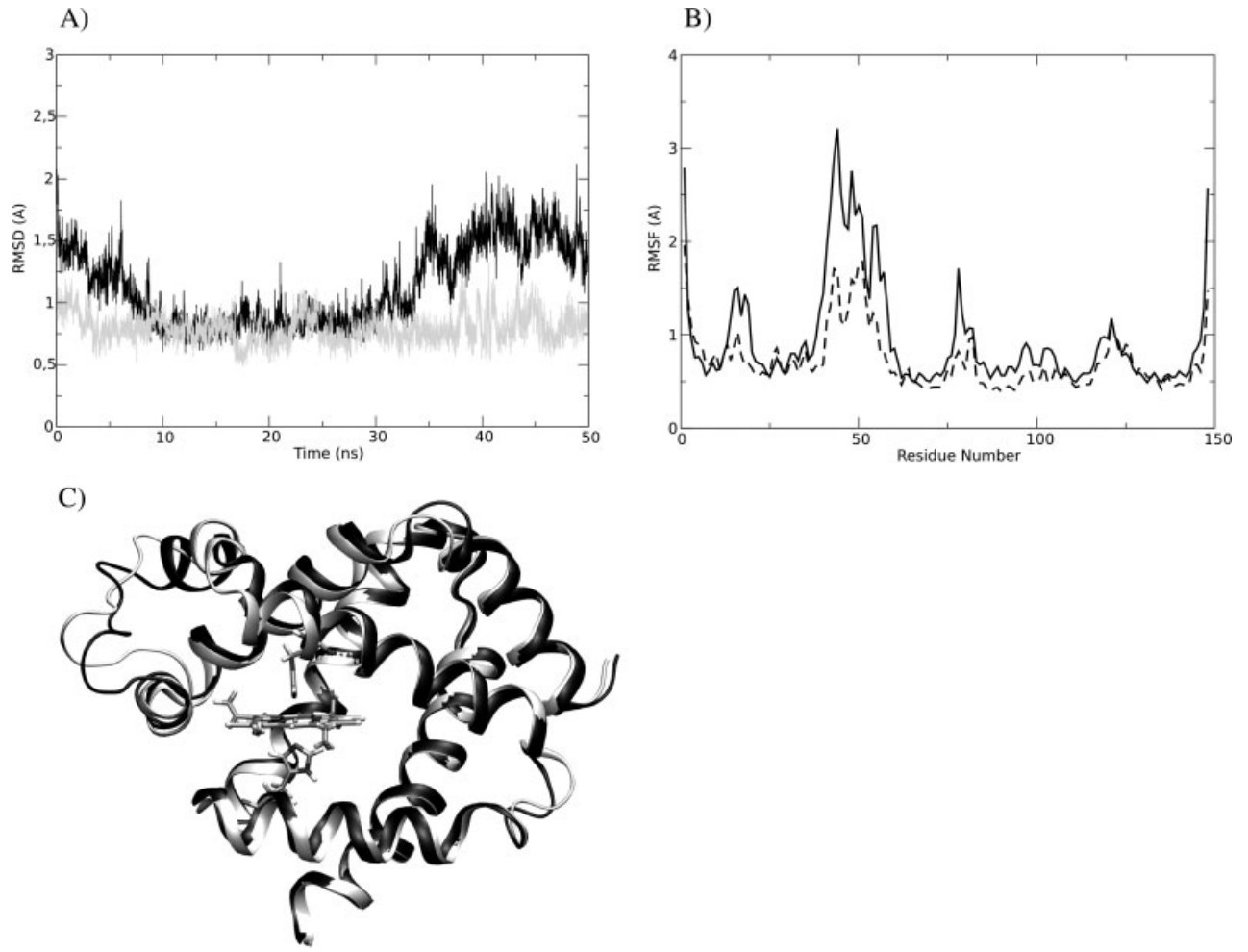


**Figure 1**

MD trajectory analysis of 6c Ngb. (**A**) Time-dependence of the RMSD (Å) for 6c Ngb at 1 bar (black) and 3 kbar (gray). (**B**) RMSF per residue along the MD trajectories sampled at 1 bar (solid line) and 3 kbar (dashed line). (**C**) Superimposed average structures calculated for the 10–30 (black) and 35–50 (gray) ns periods. Only one heme is depicted in sticks for the sake of clarity.

An experimental approach to understand the 5c↔6c transition consists of using pressure to displace the equilibrium between those states. Recent experimental results have shown that the 6c state is enhanced under high pressure.[14,15] In particular, this effect has been noted for Ngb, Mb, and tomato hemoglobin at pressures higher than 3 kbar. Nevertheless, the structural and dynamical features associated with this process are not well understood.

In this work, we have performed molecular dynamics (MD) simulations of Ngb and Mb at normal (1 bar) and high (3 kbar) pressure conditions to gain insight into the 5c↔6c equilibrium at the molecular level. We have analyzed the structural and dynamical characteristics of both proteins under those pressure conditions, as well as the free energy profiles for the 5c↔6c transition. Our results show that pressure changes the protein dynamics and specially reduces the protein mobility in the CD region (i.e., C and D helices and the CD loop), which in turn affects the equilibrium between 5c and 6c states.

## RESULTS AND DISCUSSION

To begin with the analysis of the equilibrium between 5c and 6c states in Ngb and Mb, we performed 50 ns MD simulations of each protein under different coordination and pressure conditions. A total of eight MD trajectories were run corresponding to 6c Ngb under normal (6c-Ngb-1b) and high (6c-Ngb-3kb) pressure, 5c Ngb under normal (5c-Ngb-1b) and high (5c-Ngb-3kb) pressure, and the corresponding four simulations for Mb (6c-Mb-1b, 6c-Mb-3kb, 5c-Mb-1b, 5c-Mb-3kb). The results are organized as follows. First, the general structural and dynamical features for Ngb and Mb at 1 bar and 3 kbar will be examined. Then, results from ED and

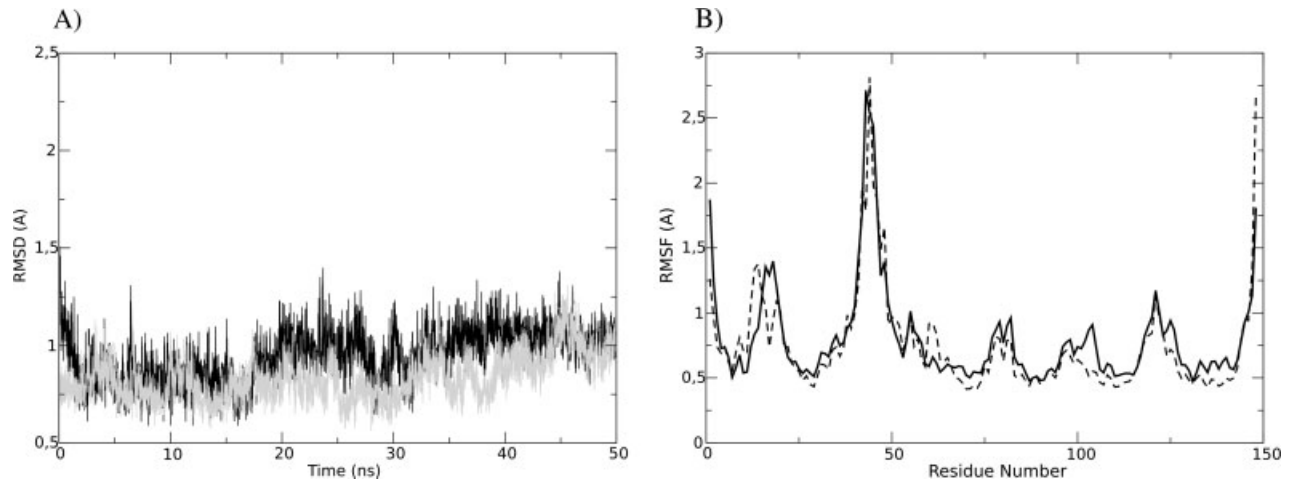


**Figure 2**

MD trajectory analysis of 6c Ngb. (**A**) Time-dependence of the RMSD (Å) for 5c Ngb at 1 bar (black) and 3 kbar (gray). (**B**) RMSF per residue along the MD trajectories sampled at 1 bar (solid line) and 3 kbar (dashed line).

conformational entropy calculations will be presented. Finally, the free energy profiles for the 5c↔6c transition will be discussed.

## MD simulations of neuroglobin

We begin our analysis by comparing 6c Ngb under 1 bar and 3 kbar pressure conditions. In both cases, the structure of the protein remains stable and no major global changes were observed during the simulations, as noted by the time dependence of the positional root-mean square deviation [RMSD; Fig. 1(A)]. The RMSD profile determined at 1 bar shows a slightly larger fluctuation, which mainly stems from changes in the CD region (see below). Under high pressure, the RMSD values are lower than those found at room pressure (mean values of 1.14 and 0.78 Å at 1 bar and 3 kbar, respectively). Inspection of the root-mean square fluctuation (RMSF) per residue [Fig. 1(B)] reveals that the fluctuations mainly affect the CD region (C-CD-D; Residues 40–60) and to a less extent the AB and EF loops (Residues 10–24 and 75–81, respectively), which agrees with the experimental β-factors.[16] It is also worth noting that the residue fluctuations are significantly reduced at high pressure conditions, specially in the CD region.

For 5c Ngb low RMSD values are obtained at both pressure conditions along the whole trajectories [mean values of 0.95 and 0.83 Å at 1 bar and 3 kbar, respectively; Fig. 2(A)]. The RMSF vs. residue plot shows that the most flexible fragments are the CD region and at less extent the AB fragment [Fig. 2(B)]. However, in contrast to the results found for 6c Ngb, the RMSF per residue determined for 5c Ngb are much less affected by pressure, even in the most flexible regions [see Figs. 1(B) and 2(B)].

To further understand the effect of pressure on the Ngb structure, we compared the average structures of Ngb in 5c and 6c states at both pressure conditions. Table I shows the RMSD between the average structures determined by considering the whole protein or exclusively the CD region (i.e., C and D helices and the connecting loop). The global fold of the structure is very similar (RMSD ranging from 0.64 and 0.70 Å) at low and high pressure, which indicates that pressure has little effect on the whole structural features of Ngb in both 6c and 5c states (Table I). Nevertheless, the difference in the CD region between the averaged structures at low and high pressure is notably higher for 5c Ngb, as noted in a RMSD of 1.43 Å, which compares with a RMSD of 0.81 Å for 6c Ngb. In addition, for all cases the structural change due to the transition between 5c and 6c states at both 1 bar and 3 kbar is significantly larger than the impact of the pressure (Table I).

Overall, the results show that the fold of the CD region in 6c Ngb is rather similar at both 1 bar and 3 kbar, even though the increase of the pressure largely reduces the configurational volume sampled by the CD

**Table I**

RMSD (Å) for the Backbone Atoms between Average Structures at Low and High Pressure

| | 1 bar vs. 3 kbar | | | |
|---|---|---|---|---|
| | 6c Ngb | 5c Ngb | 6c Mb | 5c Mb |
| Full protein | 0.64 | 0.70 | 0.67 | 0.82 |
| CD region | 0.81 | 1.43 | 0.30 | 0.52 |
| | 6c vs. 5c | | | |
| | 1 bar Ngb | 3 kbar Ngb | 1 bar Mb | 3 kbar Mb |
| Full protein | 1.71 | 1.70 | 1.66 | 1.78 |
| CD region | 4.15 | 3.82 | 1.39 | 1.50 |

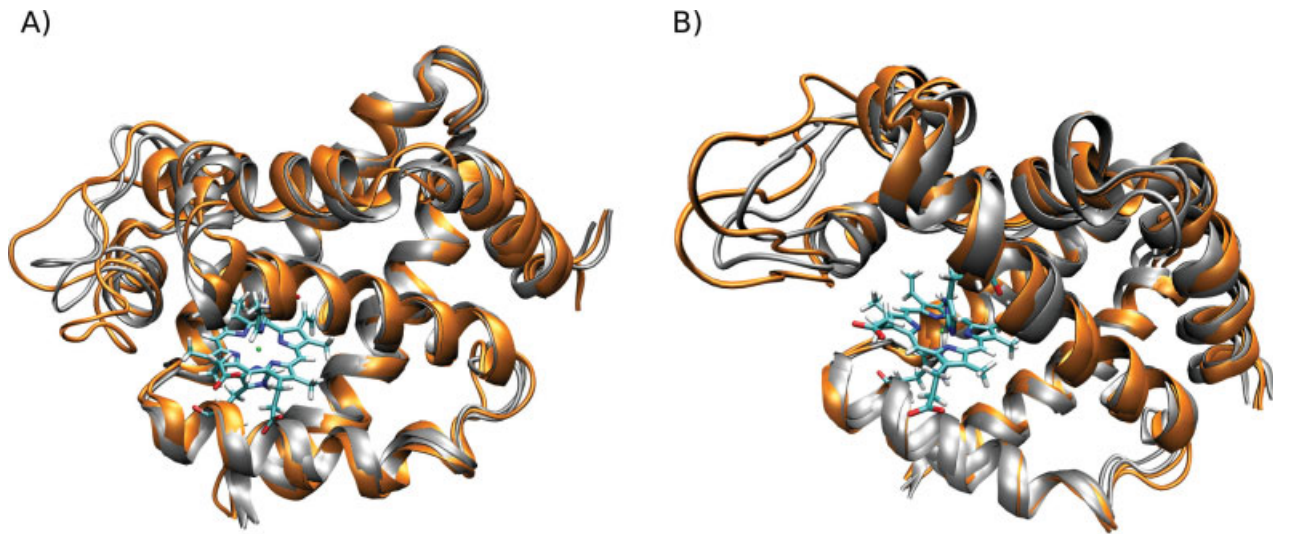


**Figure 3**

Representative snapshot of the largest fluctuations in the CD region for 6c (**A**) and 5c (**B**) states of Ngb. Snapshots at low and high pressure are shown in orange and grey, respectively.

loop [see Fig. 3(A)]. In contrast, there are larger differences between the structures adopted by the CD region in the 5c state at low and high pressures, even though the magnitude of the conformational fluctuations is not largely affected by pressure [see Fig. 3(B)].

## MD simulations of myoglobin

The RMSD profiles obtained for 5c Mb at low and high pressures shows that the structure is stable along the whole trajectory [Fig. 4(A)]. The average structures obtained at both pressures are very similar (RMSD of 0.82 Å; Table I). When compared with Ngb, the CD region is notably less flexible [Fig. 4(B)]. Moreover, there is larger similarity between the CD conformations sampled at both pressures (RMSD of 0.52 Å; Table I). In addition, the rotation through the $\chi$ ($C\text{-}C_\alpha\text{-}C_\beta\text{-}C_\gamma$) dihedral angle of HisE7, which has been described as the His gate mechanism, has been observed at both pressure conditions.

Similar trends are observed for the trajectories obtained at low and high pressures for 6c Mb (Fig. 4 and Table I). Thus, the RMSD values are small and there is large similarity between the structures sampled at both pressures, even for the CD region (RMSD of 0.30 Å; Table I).

In summary, the preceding results point out that the average structures of Ngb and Mb are not greatly affected by pressure and that the main difference between the two proteins concerns the CD region. Thus, in both 5c and 6c states of Mb, the average structure of the CD region is slightly affected by pressure, and the conformational fluctuations of this fragment are small and remain mostly unaltered upon changes in pressure. In contrast, the structural and dynamical behavior of the same region in 5c and 6c states of Ngb show distinct trends at low and high pressures (see above). Moreover, the 5c↔6c transition has a much smaller effect on the average structure of the CD region in Mb (RMSD of 1.4–1.5 Å) than in Ngb (RMSD of 3.8–4.2 Å; see Table I). On the basis of these findings, it can be concluded that the CD region exhibits a much larger sensitivity to both pressure and coordination in Ngb than in Mb.

## Essential dynamics analysis

To further analyze the effect of pressure on the dynamics, the essential motions of Ngb and Mb at low and high pressure were determined by ED analysis. In all cases, the first five essential motions account for 53–68% of the overall protein motion in Ngb, whereas a lower percentage (38–49%) is explained in Mb (Table II).

The first essential motion observed for 6c Ngb at 1 bar accounts for around 50% of the positional fluctuation and corresponds almost exclusively to the motion of the CD region. At 3 kbar not only the amplitude of such motion is significantly reduced, accounting for 25% of the overall protein mobility, but also its nature is slightly different as it includes contributions from A and B helices and the EF loop. On the other hand, in 5c Ngb at low pressure both the first and the second modes, which display similar contributions, mainly involve the CD region and helix D with minor contributions of EF and AB loops. Nevertheless, at high pressure, the first essential mode (35% of the overall structural variance) is dominated by an up-and-down movement of the N-terminal extreme of helix E and a reorientational motion of the CD region. Interestingly, when helix E approaches

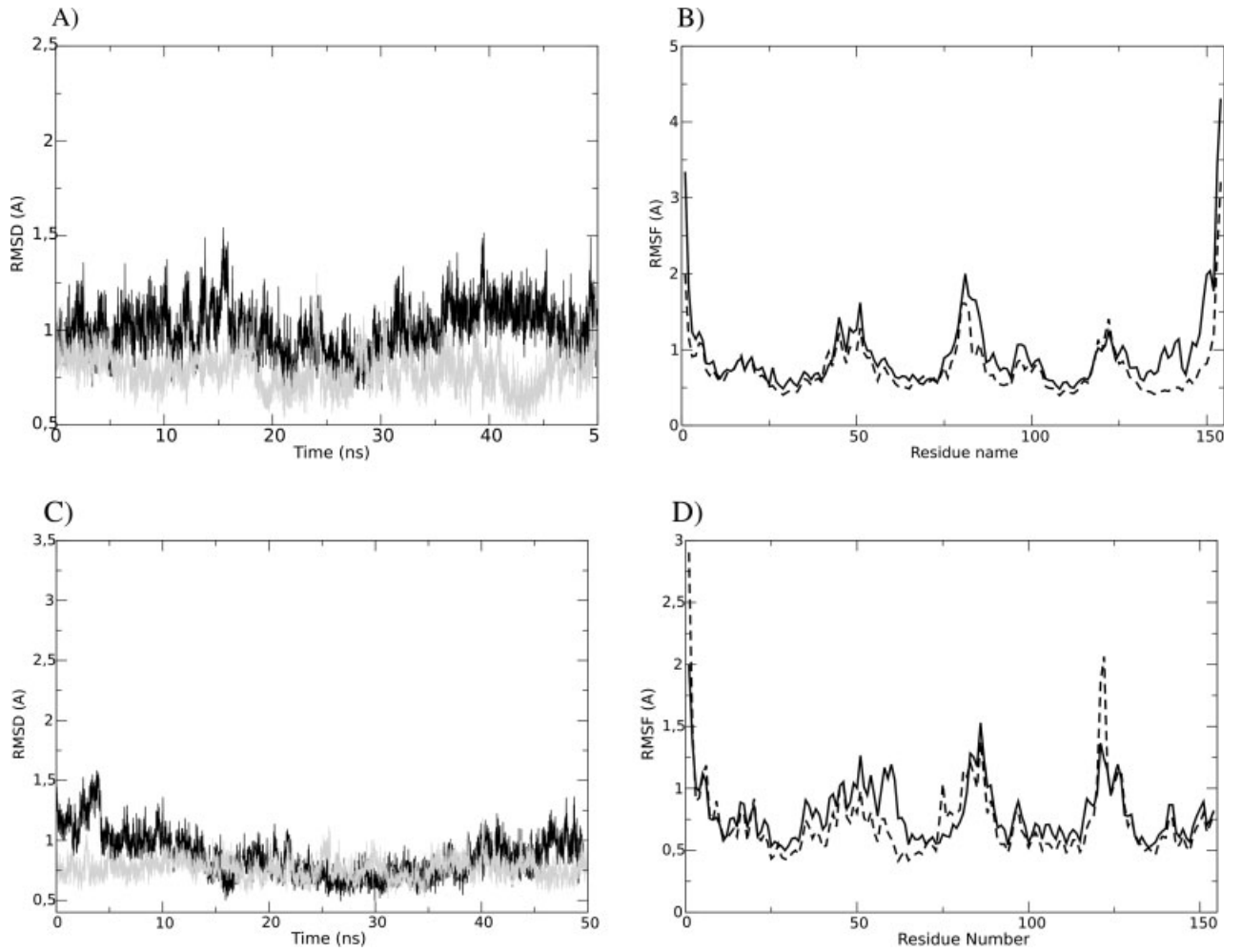


**Figure 4**

MD trajectory analysis of 5c and 6c Mb. (**A**) and (**C**): Time-dependence of the RMSD (Å) at 1 bar (black) and 3 kbar (gray) for 5c Mb and 6c Mb, respectively. (**B**) and (**D**): RMSF per residue along the MD trajectories sampled at 1 bar (solid line) and 3 kbar (dashed line) for 5c Mb and 6c Mb, respectively.

the heme group, the conformation of the CD region becomes closer to the one observed in the 6c average structure, indicating that this mode is probably involved in the 5c↔6c transition.

With regard to Mb, the first modes determined for the 6c state at both pressures have a similar contribution to the positional variance (20–25%). At 1 bar, it mainly involves motions of the EF loop, helix C, and at less extent the CD region, but is almost fully concentrated in the EF loop at 3 kbar. For the 5c state, the first mode (24%) mainly affects the motion of the EF loop (and some contributions of helix H and the CD region), it

**Table II**

Contribution of the First Five Essential Motions to the Overall Positional Fluctuation of Ngb and Mb at Low and High Pressures

| | Ngb | | | | Mb | | | |
|---|---|---|---|---|---|---|---|---|
| Mode | 6c 1 bar | 6c 3 kbar | 5c 1 bar | 5c 3 kbar | 6c 1 bar | 6c 3 kbar | 5c 1 bar | 5c 3 kbar |
| 1 | 0.50 | 0.25 | 0.21 | 0.35 | 0.20 | 0.25 | 0.24 | 0.11 |
| 2 | 0.06 | 0.15 | 0.20 | 0.08 | 0.10 | 0.09 | 0.09 | 0.09 |
| 3 | 0.05 | 0.06 | 0.05 | 0.04 | 0.06 | 0.05 | 0.07 | 0.07 |
| 4 | 0.04 | 0.05 | 0.04 | 0.04 | 0.05 | 0.04 | 0.05 | 0.06 |
| 5 | 0.03 | 0.03 | 0.03 | 0.03 | 0.05 | 0.03 | 0.04 | 0.05 |
| 1–5 | 0.68 | 0.54 | 0.53 | 0.55 | 0.46 | 0.47 | 0.49 | 0.38 |

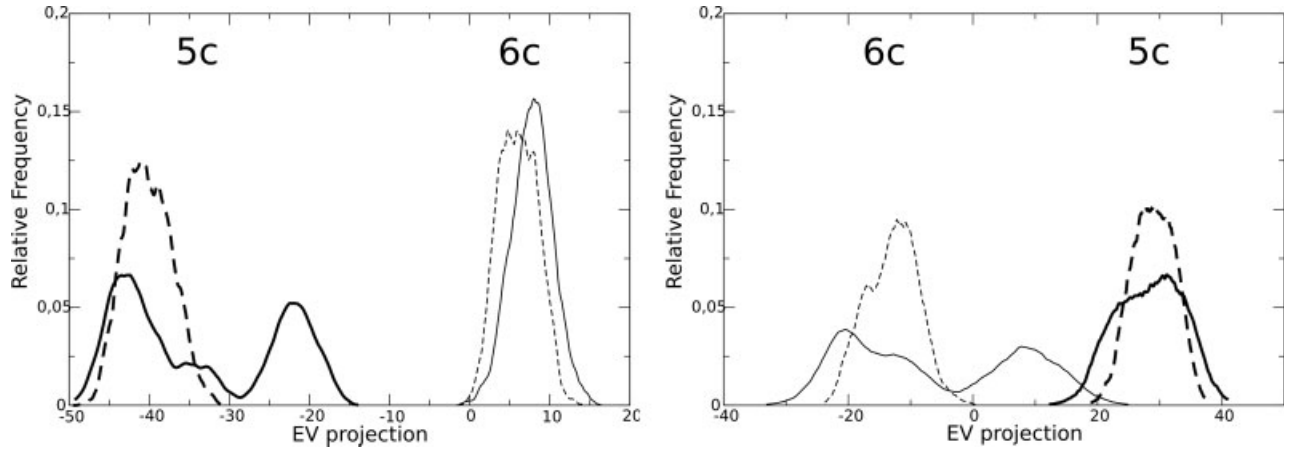


**Figure 5**

Mb (left) and Ngb (right) transition essential mode projection. Histogram of the projection of the transition mode calculated by combined essential dynamics. Results at 1 bar and 3 kbar are shown in solid and dashed lines (wider lines correspond to 5c trajectories).

being also the main structural element that contributes to the first motion (11%) at 3 kbar.

To further analyze the 5c↔6c transition the principal modes corresponding to the transition between 5c and 6c states was derived by combining the 5c and 6c trajectories (see Computational Methods), and then we determined its projection along the trajectory. The first essential mode of the 5c↔6c transition accounts for 45–60% of the overall structural variance between the 5c and 6c states of the protein. Interestingly, the transition mode in Ngb mainly involves a conformational rearrangement mostly limited to the CD region, whereas it has a more global nature in Mb, as it involves the motions of helices E and F and the concomitant fluctuation of the EF loop.

The projection of the first mode along the trajectory also shows significant differences between Ngb and Mb (see Fig. 5). In Ngb at 1 bar, there is a significant overlap between the distribution of the projection values obtained for the structures sampled for 6c and 5c Ngb, indicating that a significant fraction of the structures resemble both 6c and 5c states. Moreover, the two peaks observed for the 6c state at 1 bar denote the two conformational states mentioned above, which mainly affect the conformational space of the CD region. Noteworthy, when pressure is increased, the distribution of the projection values becomes sharper and there is no significant overlap between 6c and 5c states. In contrast to the preceding results, there is no overlap between the projection distribution of Mb at both pressures (see Fig. 5), which suggests the existence of a high barrier for the conversion between 6c and 5c states even at low pressure. For the 5c state, the distribution shows a bimodal profile, which, however, reflects a concerted movement of helix E towards helix F.

## Conformational entropy

To estimate the influence of pressure on the protein fluctuation, we calculated the conformational entropy using Schlitter and Andricioaei-Karplus methods. In these calculations, we only considered the backbone atoms and the N-terminal and C-terminal residues, which present large fluctuations and can interfere in the analysis (Ngb: Residues 1–5 and 142–148; Mb: Residues 1–10 and 145–154), were excluded. As expected, in all cases the increase of pressure leads to a decrease in the conformational entropy (see Table III), in agreement with the observed reduction in protein mobility. The highest variation is observed for 6c Ngb, which agrees with the larger effect played by pressure on the protein dynamics. On the other hand, the lowest variation is observed for the 6c Mb, consistently with the fact that at 1 bar it has low mobility.

## Free energy profiles for the 5c↔6c transition

Free energy profiles were determined to further explore the 5c↔6c transition in Ngb and Mb at low and high

**Table III**

Difference between Conformational Entropies Determined at 1 bar and 3 kbar Using Schlitter and Andricioaei-Karplus Methods

| | Schlitter | Andricioaei-Karplus |
|---|---|---|
| 6c-Ngb | 45.9 | 46.4 |
| 5c-Ngb | 33.8 | 32.3 |
| 6c-Mb | 8.1 | 7.8 |
| 5c-Mb | 31.9 | 33.5 |

The values (kcal/mol) correspond to $T\Delta S$ (at 300 K).

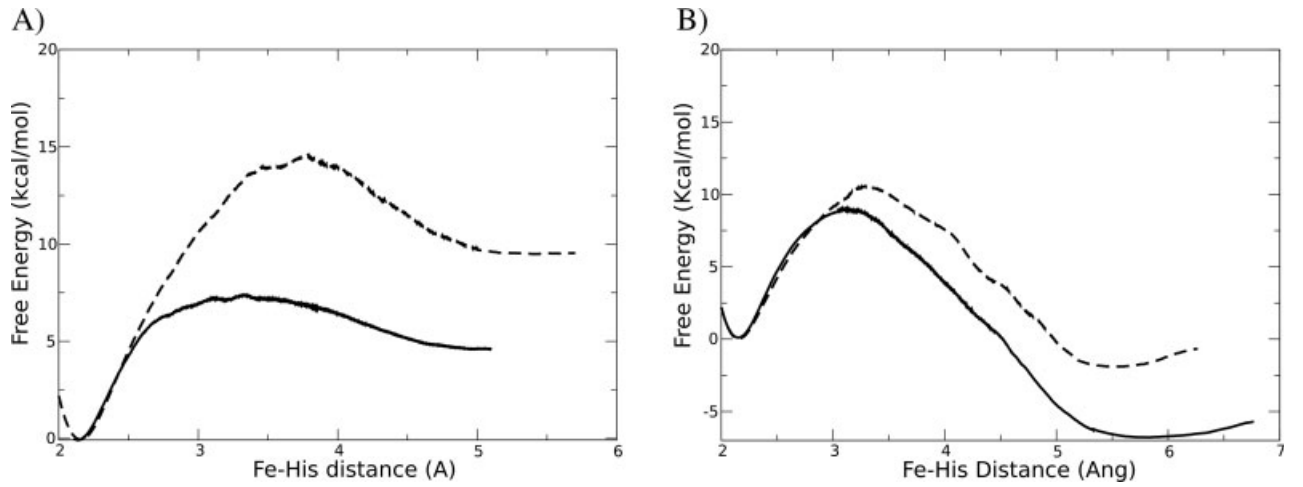


**Figure 6**

Free energy profile for the 5c↔6c transition at 1 bar (solid line) and 3 kbar (dashed line) in (**A**) Ngb and (**B**) Mb.

pressures. Data for Ngb at 1 bar from our previous work[13] was used for comparative purposes.

The barrier for the 5c↔6c transition in Ngb increased from 7 kcal/mol at 1 bar to 13 kcal/mol at 3 kbar (see Fig. 6), this effect being accompanied by a destabilization of 4 kcal/mol of the 5c state relative to the situation found at 1 bar. These findings agree with the available experimental data,[14] which indicates that the 6c state is favored by an increase in the pressure.

In the case of Mb, the 5c state is more stable at low pressure than the 6c state by about 6 kcal/mol. Under high pressure such a free energy difference is reduced to 1 kcal/mol, it being accompanied by a reduction in the barrier for the 5c↔6c transition. This finding is in agreement with the experimental data, which shows that 6c Mb is more likely observed at high pressure.[14] Interestingly, in these conditions the barrier is close to the well of the Morse potential used to describe the Fe-His bond, suggesting that the transition is almost entirely determined by the strength of the bond.

### Comparative analysis of the Ngb oxidized structure

We have previously shown that the disulfide bond between Cys CD7 and D5 rigidifies the CD region and favours the 6c↔5c transition by stabilizing the 5c state.[13] To examine the effect due to the formation of the S—S bond, simulations for the 5c and 6c states of Ngb in the oxidized form were run at both pressure conditions.

The RMSD between the average structures and the conformational entropy differences ($\Delta S$) between oxidized and reduced Ngb are presented in Table IV (as noted in the previous section, the entropy values were calculated only for the backbone atoms of Residues 6–141). As expected, the S—S bond has a significant effect on the structure of the CD region in the 5c and 6c states. However, the effect on the conformational entropy is notably smaller than the variation induced by pressure. In this context, it can be concluded that the disulfide bond mainly gives rise to a structural rearrangement primarily concentrated on the CD region, whereas changes in pressure have a broader structural impact.

## DISCUSSION

Several biochemical studies have shown that pressure has a significant effect on the behavior of proteins. In the case of globins, it has been shown that pressure can affect the degree of hexacoordination and thereby the ligand binding properties. In particular, experimental studies[14] on Mb and Ngb have shown that high pressure shifts the 5c↔6c equilibrium towards the 6c state in both cases. However, no molecular explanation has been given so far.

In agreement with the experimental observations, the free energy profiles of Ngb and Mb at both normal and

**Table IV**

RMSD (Å) between the Average Structures of Oxidized and Reduced Conformations of Ngb and Changes in Conformational Entropy at Different Pressure Conditions and Coordination States

| | RMSD | | TΔS | |
|---|---|---|---|---|
| | Full protein | CD region | Schlitter | Andricioaei-Karplus |
| 5c 1 bar | 1.45 | 3.44 | 14.9 | 14.9 |
| 6c 1 bar | 1.68 | 4.23 | 0.8 | −1.8 |
| 5c 3 kbar | 1.61 | 3.77 | 5.7 | 6.5 |
| 6c 3 kbar | 1.67 | 4.21 | 4.5 | 4.8 |

Values (kcal/mol) for the conformational entropy correspond to $T\Delta S$ (at 300 K).

high pressure conditions (see Fig. 6) show that MD simulations reflects the trends observed upon changes in pressure and therefore open a window to explore the molecular basis of this behavior. For Ngb under high pressure, the equilibrium is shifted towards the 6c state mainly due to an increase of the transition barrier. When compared with room pressure, the mobility of the 6c and 5c states is largely reduced (Table II) at high pressure, which in turn promotes a large decrease in the overlap between the configurational spaces sampled in the two states (see Fig. 5). These facts possibly contribute to the observed increase in the barrier. On the other hand, in Mb the increase of the pressure reduces slightly the barrier for the 5c↔6c transition due to the destabilization of the 5c state, which, however, continues to be more stable at high pressure (see Fig. 6). These findings agree with the marked reduction in the space explored by the 5c state (see Fig. 6) and the larger reduction in conformational entropy detected for 5c Mb when compared with 6c Mb (Table II).

The impact of the pressure on the protein structure is small and no significant changes are observed for the global structure along the dynamics. This is consistent with the large similarity found in the X-ray crystal structures of Mb at 1 bar and 2 kbar (RMSD of only 0.22 Å). Moreover, our results point out that the trajectories obtained using these structures as starting points are nearly indistinguishable. The pressure effects are, however, significant on the protein dynamics. At high pressure motions are restricted, as noted in the RMSD plots, the decrease in conformational entropy and the alteration of the essential motions at low and high pressure conditions.

From a more general viewpoint, it is interesting to note how our results can be related to previous studies on the pressure effects on protein structure and dynamics.[17–19] Many studies[20–23] have shown that under high pressure conditions the change in water compressibility significantly alters the solvation of the protein surface. For example, Ghosh *et al.*[21,22] showed that the barrier for separation of hydrophobic clusters increases monotonically with pressure and that the solvent-separated conformation is favored. Consistent with these observations and the observed pressure effects on Ngb 6c to 5c equilibrium are our previous results[13] and experimental measurements on Ngb mutants[24] that showed that several hydrophobic residues in the CD loop, namely as TyrCD3, PheCD8 and PheE4, are key elements in the 6c to 5c equilibrium. Also consistent with our data are neutron scattering[25] and theoretical[26] observations, which show that pressure does not alter significantly the equilibrium between protein conformations but significantly alters protein dynamics due to a change in water structure on the protein surface.

The above mentioned data, together with several works showing that under pressure water can penetrate into cavities and thus wet regions of proteins that are dry (see, Ref. 27, 28), point to the crucial role of water structure under high pressure conditions. However, given the complexities inherent to the study of the structure and thermodynamics of water on protein surfaces,[29,30] a more detailed analysis of these effects and the connection with pressure will be pursued in future works.

Finally, our results complement our previous work on the 5c↔6c equilibrium in Ngb,[13] where formation of the disulfide bridge between CysCD7 and CysD5 altered the equilibrium between 6c and 5c states, and therefore the oxygen affinity. Present results also point out the crucial role of the CD region as the main determinant of the 5c↔6c equilibrium. Pressure effects on Ngb are more significant in the CD region, where restriction of the mobility increases the transition barrier. In Mb, the CD region appears to be more rigid and cannot adopt a favorable conformation for the 6c state, so that the transition between 5c and 6c states requires a more global conformational rearrangement.

## CONCLUSIONS

Our results from MD simulations of the 5c and 6c states of Ngb and Mb under normal and high pressure conditions show that the main effect of pressure is to reduce overall protein mobility, especially in the most flexible regions. In agreement with the experimental data, the transition free energy profiles show that increasing the pressure enhances hexacoordination. In Ngb, pressure mainly affects the mobility of the CD region and increases the barrier for the transition, with a higher impact on the 6c to 5c equilibrium. In Mb, the 5c↔6c transition involves a more global structural rearrangement, and pressure seems to destabilize the 5c state, shifting the equilibrium towards the 6c conformation. Overall, present results stress the key role played by the nature of the CD region in determining the coordination state of globins.

## COMPUTATIONAL METHODS

### Classical molecular dynamics

The initial structures of 6c Ngb were taken from murine and human X-ray structures (PDB entries 1Q1F[31] and 1OJ6,[16] respectively) as described previously by Nadra *et al.*[13] The 5c structure was obtained from the 6c one by slowly dissociating the distal histidine from the heme and then releasing the Fe-HisE7 bond constraint. In all these structures, cysteines CD7 and D5 are in the reduced state (i.e., without the S—S bond). The initial 5c structure of Mb was taken from the X-ray structure of sperm whale Mb (PDB code 1VXD[32]). The 6c structure was obtained by slowly approaching Nε2(HisE7) to the

Fe, and afterwards adding the Fe-HisE7 bond to the force field. For comparison purposes, another simulation was performed for 5c Mb, but starting from the high pressure structure of Mb (PDB code 1JP8[33]).

The starting structures were immersed in a preequilibrated octahedral box of TIP3P water molecules. The standard protonation state at physiological pH was assigned to ionizable residues. Special attention was paid to the protonation of histidines, which were assigned on the basis of the hydrogen-bond pattern with neighboring residues. For distal (HisE7) and proximal (HisF8) histidines, protonation was chosen to be in the Nδ position. Besides the protein, the final system contains 5260 water molecules (around 18,200 atoms) for Ngb, or 6290 water molecules (around 21,000 atoms) for Mb. All simulations were performed at 300 K and pressures of 1 bar and 3 kbar using Berendsen thermostat and barostat.[34] Periodic boundary conditions and Ewald sums (grid spacing of 1 Å) were used to treat long range electrostatic interactions. The SHAKE algorithm was used to keep bonds involving hydrogen atoms at their equilibrium length. A 1 fs time step for the integration of Newton's equations was used. The Amber99 force field[35] was used for all residues but the heme, whose parameters were developed and thoroughly tested by our group in previous works.[36,37] All simulations were performed with the PMEMD module of the AMBER9 package.[38]

Equilibration consisted of an energy minimization of the initial structures, followed by a slow heating up to the 300 K (4 steps of 50 ps at 150, 200, 250, and 300 K). To perform the simulations at 3 kbar, the pressure was slowly increased by performing 1 ns runs at 500, 1000, 1500, 2000, 2500, and 3000 bar. For each structure, 50 ns long MD production runs were performed. Frames were collected at 1 ps intervals, which were subsequently used to analyse the trajectories.

### Essential dynamics

To examine the dynamical properties of proteins and their influence on the 5c↔6c transition, essential dynamics (ED) analysis was used.[39] ED involves diagonalization of the covariance matrices of atomic positions along the trajectory, yielding the eigenvectors that define the essential motions of the protein. This analysis was performed only for the backbone atoms. Terminal residues were excluded to avoid masking of the essential motions of the protein core by the high flexibility of terminal regions. ED analysis of combined trajectories was also performed to gain insight into the 5c↔6c structural transition. Finally, projection of the MD trajectories onto selected essential motions was performed to analyze the configurational space explored along the MD run. This type of analysis is valuable to explore the structural and dynamical relationships in proteins.[13,37]

### Free energy profiles of the 5c↔6c transition

Free energy profiles were computed to obtain thermodynamic information of the 5c↔6c transition. The profiles were determined by means of constant velocity multiple steered MD (MSMD) simulations using Jarzynski's inequality,[40] which relates equilibrium free energy values with the irreversible work required to drive the system along a given reaction coordinate. Here the reaction coordinate was chosen as the Fe-Nε2(HisE7) distance. Calculations were performed using a force constant of 400 kcal/(mol Å) and a pulling velocity of 2.5 Å/ns. Two independent sets of 10 MSMD simulations were performed in each direction (forward:dissociation; backward:association) and the final profile was obtained by combining both sets of simulations. Error bars correspond to the standard deviation between the free energy values obtained for each independent set. Because the transition involves the formation/breaking of a bond (the Fe-His bond), a Morse potential was introduced to describe such a bond in the force field (parameters involve an equilibrium distance of 2.12 Å and an energy constant of 10 kcal/mol). This approach has been successfully used in ligand binding studies of heme proteins.[41]

### Conformational entropy

Conformational entropy calculations were performed by diagonalization of the Cartesian covariance matrix using Schlitter[42] and Andricioaei-Karplus[43] methods. Because the entropy depends on the length of the trajectory, entropies were determined for intervals ranging from 20 to 50 ns and the corresponding values were fitted using Eq. (1), where $S_\infty$ corresponds to the limit entropy at infinite simulation time.[44]

$$S(t) = S_\infty - \alpha/t^{2/3} \quad (1)$$

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