Effects of pressure on the structure of metmyoglobin: Molecular dynamics predictions for pressure unfolding through a molten globule intermediate

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Abstract

We investigated the pathway for pressure unfolding of metmyoglobin using molecular dynamics (MD) for a range of pressures (0.1 MPa to 1.2 GPa) and a temperature of 300 K. We find that the unfolding of metmyoglobin proceeds via a two-step mechanism

native → molten globule intermediate → unfolded,

where the molten globule forms at 700 MPa. The simulation describes qualitatively the experimental behavior of metmyoglobin under pressure. We find that unfolding of the α -helices follows the sequence of migrating hydrogen bonds ($i, i + 4$) → ($i, i + 2$).

Keywords: molecular dynamics; molten globule; myoglobin; pressure; unfolding

The effect of pressure on chemical and biological systems can lead to improved understanding of the fundamental interactions and to improved processes. Thus, high pressure has been used in food sterilization, extraction procedures, bioconversion using barophilic microorganisms, and enzymology under supercritical conditions (Balny et al., 1992). In addition, microbiology under deep-sea high pressure conditions offers biotechnological opportunities and may provide insights into the origin of life. Microorganisms in the deep-sea live at high pressures (110 MPa at 10,660 m) at both low and high temperature and in darkness (Yayanos, 1995).

Experimental techniques such as optical spectroscopy, Raman scattering, and NMR have been used to observe pressure effects on proteins (Weber & Drickamer, 1983; Frauenfelder et al., 1990; Jaenicke, 1991; Silva & Weber, 1993; Gross & Jaenicke, 1994;

Jonas & Jonas, 1994). The pressure denaturation of monomeric proteins, the dissociation of oligomers, and the effects of pressure on macromolecular assemblages have provided insights into the microscopic mechanism of protein folding and the role of solvent in this process (Zipp & Kauzmann, 1973; Li et al., 1976; Chrysomallis et al., 1981; Weber & Drickamer, 1983; Silva et al., 1986; Silva & Weber, 1993; Weber, 1993; Dufour et al., 1994; Peng et al., 1994; Schulte et al., 1995; Silva et al., 1996). These effects are often reversible but can show different degrees of hysteresis.

Despite this progress, the detailed atomistic changes involved in pressure transformations have not been well characterized. To provide such information we carried out molecular dynamics (MD) simulations on metmyoglobin as a function of applied external pressure. We selected myoglobin since its folding mechanism and protein folding intermediates have been studied experimentally by many techniques. We find that the applied pressure perturbs the native structure of the protein, eventually destabilizing to denature the molecule. By examining the conformation of the intermediates from the MD, we determined the pathway for unfolding. The results are in agreement with the experiment.

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Simulation procedures

Dynamics

We carried out a series of MD simulations using Nosè–Hoover dynamics at constant temperature and pressure (NPT). We studied metmyoglobin (153 amino acid residues protein) for which experimental results for unfolding under pressure are available (Zipp & Kauzmann, 1973). The initial coordinates were taken from the X-ray crystal structure (PDB code 4mbn; crystallographic resolution of 2 Å) and minimized using periodic boundary conditions (PBC). The cell parameters were also minimized. Simulations were performed using the AMBER (Weiner et al., 1984) force field (FF) modified to include parameters for the heme group and a term to explicitly take hydrogen bonds (HBs) into account. The heme group parameters were selected from the Dreiding FF (Mayo et al., 1990), which gives a planar conformation for the heme group at 1 atm. This FF has been used in many studies of heme systems (Shelnutt et al., 1995). Charges for the heme were based on charge equilibration (Rappé & Goddard, 1991).

The MD simulations were performed for metmyoglobin (using PBC) at 300 K, using implicit solvent, a time step of 0.001 ps, a Nosè relaxation time of 0.01 ps, and various external hydrostatic pressures (10 values between 1 atm and 1.2 GPa). For each simulation, the pressure was increased from the previous value in steps of 25 MPa/10 ps, until the desired pressure was reached. At the desired pressure, 100 ps of dynamics was performed. The final conformation obtained at each pressure was used as the input to the simulation at the next higher pressure. The simulation box was allowed to change its shape but the cell angles were kept fixed at 90°. The dielectric constant was based on the corresponding values for water at the pressures and temperature of interest (300 K). The pressure dependence of the dielectric constant of water (at constant temperature) was estimated using the Bradley and Pitzer (1979) equation adjusted to fit experimental values. The dielectric constant was not taken as distance dependent.

Nonbond interactions

The nonbonded interactions were calculated using spline cutoffs ($R_{cut} = 9$ Å). The degree of protonation was based on pH 7. Thus, glutamic and aspartic acids were negatively charged, lysine and arginine were positively charged, and histidines were neutral.

Two sets of simulations were performed and analyzed. The first set [denoted as $\epsilon(T, p)$] used a dielectric constant appropriate for water at the same temperature and pressure of the simulations (based on extrapolation of the Bradley and Pitzer equation). These results are shown in Table 5 but not analyzed in detail here. The second set [denoted as $\epsilon^*(T, p/2)$] used the dielectric constant of water at the same temperature and half of the pressure used in the simulations. This leads to a dielectric constant smaller by approximately 7% from $\epsilon(T, p)$. Table 1 shows both ϵ and ϵ^* values. The ϵ^* results are analyzed herein.

Both simulations describe qualitatively the denaturation behavior of the protein under pressure. We define as “denatured” a state where the loss of secondary structure modifies the overall three-dimensional conformation structure since this would change the function of the protein. The main difference between the results with the two sets of dielectric constants (ϵ and ϵ^*) resides in the behavior of the solvent-accessible surfaces. With ϵ^* , the solvent accessibility of the aromatic residues (Table 3) increases significantly as the unfolding proceeds, as observed experimentally. With ϵ , the solvent accessibility of the aromatic residues changes little

Table 1. Dielectric constants used in the simulations^a

Pressure (MPa)	ϵ	ϵ^*
0.1	78	78
10	79	79
100	82	80
200	85	82
300	87	84
400	89	85
500	91	86
600	93	87
700	94	88
800	95	89
900	97	90
1,000	98	91
1,200	100	93
1,400	101	94

^aThe value ϵ is based on the Pitzer–Bradley formula. ϵ^* uses this relation but for $P/2$.

with unfolding. There are many possible explanations for this difference. The adjustment in the dielectric constant may reflect the need to account for the intrinsic dielectric constant of the protein [the estimated dielectric constant value of metmyoglobin in water at 300 K is 11 (Simonson & Brooks, 1996)]. It also could be that the Bradley–Pitzer estimate is not accurate at the extrapolated range above 500 MPa. A more recent formulation for the dielectric constant of water at temperatures from 238 to 873 K and at pressures up to 1,200 MPa has been reported (Fernandez et al., 1997). When compared to values from this formulation, our adjusted ϵ^* values are, at the most, 5% above them. The effect of ϵ or ϵ^* on the solvent accessibility values is more likely to be associated with simplifications in the simulations which use fixed charges (no polarization) and implicit solvent. Whatever the origin, the use of ϵ^* leads to good agreement with experimentally observed changes, and hence these simulations are the ones to examine for extracting atomistic interpretations. Therefore, we use ϵ^* in the simulations.

Results

We analyze the results in terms of (1) secondary structure assignment [using Procheck (Laskowski et al., 1993)]; (2) the superposition to the initial structure; (3) solvent accessibility surface calculations; and (4) HB assignment for each final conformation obtained from the trajectories.

Secondary structure assignment

Figure 1 shows the secondary structure assignment for metmyoglobin as assigned in the PDB file (4mbn) and as assigned by Procheck for (1) the original X-ray structure; (2) the minimized structure; and (3) structures from MD simulations at various pressures.

The Procheck assignment differs in some positions from the PDB assignment (Takano, 1977) used to assign the standard eight

	[A] [B] [C] [D] [E]
PDB	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
PDB-check	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
Min	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
1atm	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
10MPa	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
100MPa	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
200MPa	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
400MPa	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
600MPa	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
700MPa	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
800MPa	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
1.0GPa	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
1.2GPa	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD RF KHLKTEAEMKASED
	1 60
	E [F] [G]
PDB	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
PDB-check	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
Min	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
1atm	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
10MPa	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
100MPa	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
200MPa	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
400MPa	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
600MPa	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
700MPa	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
800MPa	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
1.0GPa	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
1.2GPa	LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP
	61 H H 120
	[H]
PDB	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
PDB-check	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
Min	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
1atm	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
10MPa	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
100MPa	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
200MPa	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
400MPa	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
600MPa	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
700MPa	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
800MPa	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
1.0GPa	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
1.2GPa	GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
	121 153

XXX = Helix; X = Aromatic residue; Min = Minimized structure.

H = His residues involved in binding and release of the HEME group.

PDB = myoglobin helix limits based on the pdb 4mbn file assignment (Takano, 1977).

PDB-check = myoglobin helix limits based on PROCHECK (Laskowski et al., 1993) assignment.

Fig. 1. Secondary structure assignment for metmyoglobin as a function of pressure.

helical regions of myoglobin: (1) (Ser3–Glu18), denoted A1–A16; (2) (Asp20–Ser35), denoted B1–B16; (3) (His36–Lys42), denoted C1–C7; (4) (Thr51–Ala57), denoted D1–D7; (5) (Ser58–Lys77), denoted E1–E20; (6) (Leu86–Thr95), denoted F1–F10; (7) (Pro100–Arg118), denoted G1–G19; (8) (Gly124–Leu149), denoted H1–H26. Procheck does not assign as helical residues the residues Ser3 (conventionally called position A1), Glu18 (A16), Asp20 (B1), Ser35 (B16), His36 (C1), Lys42 (C7), Thr51 (D1), Ser58 (E1), Lys77 (E20), Pro100 (G1), and Gly124 (H1), all of which begin or end helices. Also, residues Glu83, Ala84, and Glu85 are assigned as part of helix F, although they are not conventionally considered

part of that helix. In addition, regions Asp44–Phe46 and Pro120–Asp122 are considered helical regions by Procheck. These helical regions correspond to both alpha helices and 3_{10} helices.

To be consistent in our analysis, the limits for the myoglobin helices A–H used in our secondary structure comparisons are the ones assigned for the minimized structure. Therefore, there are 116 residues initially in helix conformations for metmyoglobin, distributed in eight helical regions as follows: (A) Glu6–Val17; (B) Val21–Ser35; (C) Pro37–Phe46; (D) Glu52–Ala57; (E) Glu59–Lys77; (F) Glu83–Thr95; (G) Ile101–Arg118; and (H) Ala125–Lys147. Note that the region Asp44–Lys47 is considered here as

Table 2. Percentage of residues that remain in helical conformation relative to those in the minimized structure^a

P (MPa)	A 12	B 15	C 10	D 6	E 20	F 13	G 18	H 22	PTN 116	% Total 153
Min ^b	100	100	100	100	100	100	100	100	100	76
0.1	100	88	60	67	75	69	89	87	82	62
10	58	80	30	100	60	77	94	87	75	57
100	75	67	0	67	85	77	94	96	77	58
200	92	80	60	67	85	77	72	74	78	59
400	83	60	40	67	80	31	83	61	65	50
600	58	60	60	67	70	0	72	61	58	44
700	67	20	40	0	60	0	44	91	48	37
800	8	53	30	67	55	0	72	39	42	32
1,000	33	53	70	0	0	0	44	39	31	23
1,200	0	40	40	0	35	0	44	26	27	20

^aA-H indicates the helix. PTN indicates the sum over all helices. Total indicates the percentage related to the total length of the protein (153 amino acids). The number of residues in each helix is listed in row 2.

^bMinimized structure.

part of helix C despite its discontinuity at Phe43. Table 2 shows the percentages of residues remaining in the helix conformation during the simulations (compared to the initial minimized structure).

Solvent exposure

Table 3 summarizes several results from the MD simulations. Defining the structural change (δC_α) as the root-mean-square deviation (RMSD) between the C_α positions at a given pressure with the initial (minimized) structure, we see that δC_α increases with pressure but remains below 2.0 Å from 1 atm to 100 MPa (1,000 atm). The solvent accessibility of the aromatic residues (Phe, Tyr,

Table 3. The predicted RMSD of the C_α coordinates from the minimized structure (denoted δC_α) and the predicted residue solvent accessibility as a function of pressure (from NPT MD at 300 K using ϵ^*)

P (MPa)	δC_α (Å)	Solvent accessibility		Helix (%)
		ARSA ^a	TRPAC ^b	
X-ray	1.04	146.21	17.88	
Min ^c	0.00	115.45	3.54	100
0.1	1.52	157.45	3.48	82
10	1.84	151.14	0.98	75
100	1.89	169.42	3.22	77
200	2.34	130.07	0.80	78
400	2.53	134.74	1.89	65
600	2.88	169.25	0.00	58
700	3.39	293.25	6.37	48
800	3.89	332.17	13.93	42
1,000	4.29	266.93	52.21	31
1,200	4.99	469.18	46.75	27

^aARSA = Aromatic residues (Phe, Tyr, and Trp) solvent accessibility (Å²).

^bTRPAC = Tryptophan solvent accessibility (Å²).

^cMinimized structure.

and Trp, denoted as ARSA) and the tryptophan solvent accessibility (denoted as TRPAC) describe qualitatively the exposure of the buried aromatic side chains to the solvent. This is observed experimentally by the changes in the 270 to 290 nM region of the electronic absorption spectrum (Zipp & Kauzmann, 1973).

Figure 2 compares the pressure dependence of the predicted solvent accessibilities for the aromatic residues (ARSA) and for the tryptophan residues (TRPAC) with the predicted percentage of residues remaining in helix conformation.

The predicted aromatic solvent accessibilities show two well-defined regions, with a sharp transition between 600 and 700 MPa. The predicted tryptophan solvent accessibility also shows a transition that starts at 700 MPa, but it is more gradual and not complete until 1 GPa. The calculated percentage of residues remaining in the helix conformation decreases monotonically as the pressure increases, showing that the protein is gradually losing secondary structure elements as a result of the applied pressure. The 600 to 700 MPa transition region corresponds to the change from 58 to 48% in alpha-helix percentage.

The conserved sites of two globin family alignments are listed in Figure 3: (1) Bashford alignment, based on a set of 226 sequences (Bashford et al., 1987): denoted B-BCR for buried and B-SCR for surface residues; (2) Kapp alignment, based on a set of 700 sequences (Kapp et al., 1995): denoted as K-CR. These conserved sites are compared in Figure 3 to the helical residues of the calculated conformers under pressures of 600 MPa, 700 MPa, and 1.2 GPa. The conformers at 600 and 700 MPa were selected for

Fig. 2. Solvent accessibility (Å²) for aromatic residues (ARSA) and tryptophanes (TRPAC) as a function of pressure. Percentage of residues remaining in a helix conformation as a function of pressure (%AA).

[H]	[	A	]	[	B	]	[	C	]	[	D	
seq	VL	SEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFD	DRFKHLKTEAE									
Min		EWQLVLHVWAKV	VAGHGQDILIRLFKS	PETLEK	DRFK	EAE						
600MPa		QLVLHVW	DILIRLFKS	PETLEK		E						
700MPa		VLHVWAKV		LFK	PETL							
1.2GPa			DIL	LFK	ETLE							
K-CR		HVWAKV	HGQDILIRLFKSHPETLEKFD									
B-BCR		V VW V	IL LF P T F									
B-SCR		E Q H AK	R		E D							
FAPP		LVL W V	LI F									
[H]	D]	[	E	]	[	F	]	[	G			
seq	MK	ASEDLKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIKPIKYLEFISE										
Min	MKA	EDLKKHGVTVLTALGAILKK	EAELKPLAQSHAT	IKYLEFISE								
600MPa	MKA	DLKKHGVTVLTALG		LEFISE								
700MPa		DLKK VTVLTALG		LEFI								
1.2GPa		DLK VLTA		LEFI E								
K-CR		SEDLKKHGVTVLTALGAILK	LKPLAQSHA	YLEFISE								
B-BCR		L HG VL AL IL	LA	L I								
B-SCR		SED KK VT T A K	KP Q K P	E SE								
FAPP				K L I								
[H]	G	]	[	H	]							
seq	AI	IHLVLSRHPGDFGADAQGAMNKALELFRKDIAAKYKELGYQG										
Min	AI	IHLVLSR	ADAQGAMNKALELFRKDI AKYK									
600MPa	AI	IHLVH	QGAMNKALELFRKD									
700MPa	AI	IH	ADAQGAMNKALELFRKDIAAK									
1.2GPa	AI	I	ELFRKD									
K-CR	AI	IHLVHL	GAMNKALELFRKDI									
B-BCR	I	VL	M AL L D									
B-SCR		H	QG NK E									
FAPP	A	IHLV	K F IA									

Fig. 3. Comparison of helix assignments with experimental data. [H]-helix limits based on the pdb file 4mbn assignment (Takano, 1977); seq, myoglobin sequence; Min, metmyoglobin minimized structure; 600 MPa, 700 MPa, and 1.2 GPa secondary structure assignments at the respective pressures; K-CR = globin conserved residues (Kapp et al., 1995); B-BCR and B-SCR = globin buried and surface conserved residues (Bashford et al., 1987); FAPP = fast amide proton protection (Jennings & Wright, 1993).

comparison because of the transition observed in Figure 2 for solvent accessibility of aromatic residues. The conformer at 1.2 GPa was selected because it corresponds to the highest pressure of the simulation. Figure 3 also compares the residues in helical conformations at these pressures to residues that exhibit a Fast Amide Proton-exchange Protection (FAPP) during hydrogen pulse labeling experiments for refolding of apomyoglobin (Jennings & Wright, 1993). Table 4 collects these data by helix using the secondary structure assignment for the 700 MPa and 1.2 GPa conformers under pressure, the globin conserved residues, and the fast amide proton-exchange protected residues.

Observations from Figure 3 and Table 4 are:

1. Of the residues preserved in a helix conformation at a pressure of 700 MPa, 100% of helices B, C, and E are Kapp globin

conserved residues while helix A is 75% and helix H is 67% (helices D and F have no helical residues left).

2. By the Bashford alignment, all the helices remaining at 700 MPa have at least 37% of the preserved helix residues corresponding to buried conserved residues (B-BCR), except for helix H which has only 25%.
3. None of the residues in helices B and C that are preserved at 700 MPa correspond to Bashford surface conserved residues (B-SCR), whereas helices G, H, A, and E have from 13 to 56% of their preserved helical residues corresponding to B-SCR residues.
4. Of the 20 residues with protection from fast hydrogen exchange, only six [Leu9(A), Leu29(B), Ile30(B), Lys102(G),

Table 4. Percentage of residues predicted to be in metmyoglobin helices at 700 MPa that correspond to (a) globin conserved residues, (b) fast hydrogen-exchange protection rate residues (FAPP), and (c) hydrophobic residues in metmyoglobin helices under pressure

Helix x	Residues in helix at 700 MPa ^a (%) corresponding to				Hydrophobic residues ^b in helix (%)	
	Kapp ^c	Bashford ^d buried	Bashford ^d surface	FAPP ^c	700 MPa	1.2 GPa
A	75	50	38	80	63	0
B	100	67	0	33	67	67
C	100	50	0	0	50	25
D ^f	—	—	—	—	0	0
E	100	42	50	0	50	57
F ^f	—	—	—	—	0	0
G	100	37	13	63	75	75
H	67	24	24	100	48	33

^a% = Number of conserved residues in helix N at 700 MPa $\times$ 100/total number of residues in helix N at 700 MPa.

^b% = Number of hydrophobic residues in helix N at pressure $P \times$ 100/total number of residues in helix N at pressure P .

^cKapp conserved residues (Kapp et al., 1995).

^dBashford buried and surface conserved residues (Bashford et al., 1987).

^e% = Number of FAPP residues in helix N at 700 MPa $\times$ 100/total number of fast amide proton protected residues in helix N for the minimized structure (Jennings & Wright, 1993).

^fNone observed.

Val114(H), and Leu115(H)] are not present in helix conformations at 700 MPa.

5. The percentage of hydrophobic residues compared to the total number of residues in each helix ranges from 33 to 75% for both 700 MPa and 1.2 GPa, indicating that some nonhydrophobic residues remain in the helix conformations at high pressure.
6. More than 50% of the residues preserved at 700 MPa are hydrophobic.

In Figure 4 we show the residues preserved in a helix conformation in more than two conformers for pressures higher than 700 MPa shaded from the helical residues observed in the minimized structure. Those are compared to the consensual set of the globin conserved residues [obtained by comparison of two globin alignments (Bashford et al., 1987; Kapp et al., 1995) and selecting their common residues]. Figure 4 shows that the considered residues have a strong tendency to remain in the helix conformation at high pressure.

Hydrogen bonds

Figure 5 shows the number of main-chain/main-chain HBs of different types as a function of pressure. The change in the total number of main-chain/main-chain HBs is small (from 132 at 0.1 MPa to 96 at 1.2 GPa). Thus, even at 1.2 GPa this number is still 72% of the initial number of main-chain/main-chain HBs.

All Conformers		
A	6 EWQLVLHVWAKV	17
B	21 VAGHGQDILIRLFKS	35
C	37 PETLEKFDRF	46
D	52 EAEMKA	57
E	59 EDLKKHGVTVLTALGAILK	77
F	83 EAELKPLAQSHAT	95
G	101 IKYLEFISEAIIHVLHSR	118
H	125 ADAQGAMNKALELFRKDIAAKYK	147

X = Globin Conserved Site.

X̄ = Site Preserved During Compression.

X̄ = Globin Conserved Site Preserved During Compression.

Fig. 4. Globin conserved sites preserved during compression.

The HBs are considered an $(i, i + 4)$ alpha-helical HBs when the $\text{CO} \cdots \text{HN}$ distance is $< 2.5 \text{ \AA}$ (equilibrium is $1.6 \text{ \AA}$). The number of $(i, i + 4)$ HB decreases monotonically as the pressure increases from 81 at 0.1 MPa to 21 at 1.2 GPa. This reflects a loss in helical structures but the decrease by 60 is much greater than the decrease of 36 in the total number of main-chain HBs. The reason for this difference resides in the $(i, i + 3)$ HBs (corresponding to a 3_{10} helix when three or more consecutive residues present it) and the

Fig. 5. Number of main-chain/main-chain hydrogen bonds O (residue i) $\rightarrow$ H-N (residue $i + X$) as a function of pressure.

$(i, i + 2)$ HBs (corresponding to a C_7 configuration). The number of $(i, i + 3)$ HBs fluctuates by only ± 7 bonds (23%) from its value at 1 atm (0.1 MPa) during the entire range of compression. The compensating factor to the decrease in alpha-helix HB is the uniform increase in $(i, i + 2)$ HB, which goes from 12 at 0.1 MPa to 38 at 1.2 GPa. Indeed the big change (11 to 20) occurs between 400 and 600 MPa, with continued large changes to 1.0 GPa. The conformers at 1.0 and 1.2 GPa have four of their HBs in an anti-parallel bridge.

Figure 6 shows a schematic view of the structures under pressure from 1 atm to 1.2 GPa. Here we see that the globular form of the protein is preserved even at sufficiently high pressures when the secondary structure elements are almost absent. The volume of the protein at 1 atm is 4.6% higher ($28,182 \text{ \AA}^3$ compared to $26,951 \text{ \AA}^3$) than the volume of the compressed but folded state at 600 MPa (where 58% of the residues originally in helix are still in helix conformation). Figure 7 shows more detail for the structure at 1 atm and 700 MPa.

Fig. 6. Schematic view of metmyoglobin under pressure from 1 atm to 1.2 GPa.

Fig. 7. Schematic view of metmyoglobin: (A) 1 atm and (B) 700 MPa (molten globule).

Discussion

Review of the literature

The effects of pressure and temperature in the stability of metmyoglobin have been studied at a series of pH values by following the changes in absorption of aromatic residues and the heme group (Zipp & Kauzmann, 1973). It has been found that at "any pH, from 4 to 13, a range of pressures exist in which the protein undergoes the changes

denatured $\rightarrow$ native $\rightarrow$ denatured state

when the protein is heated from 0 to 80 °C at constant pressure." The denaturation process is reversible in most cases. The onset pressure for denaturation depends on the pH and temperature. At pH 7 and 303 K, the protein is observed to be denatured at pressures above 600 MPa.

The Raman spectra of myoglobin at high pressure (Schulte et al., 1995) indicate no radical perturbation of the heme structure at pressures below 200 MPa. Nevertheless, the shift in the iron-proximal histidine vibration with pressure indicates conformational changes in the protein. Since the proximal histidine is linked to the iron and part of the protein backbone, the position of the helix F is believed to change as well.

A three-phase model for the refolding process of myoglobin in the presence of the heme group has been proposed based on a kinetic study monitored by five optical probe stopped-flow methods (Chiba et al., 1994):

1. In the first phase, a precursor of a heme pocket is formed (intermediate 1) and the hemin-dicyanide is captured, leading to intermediate 2. Forty percent of secondary structure is constructed in intermediate 2. Both intermediates are assumed to be molten globules. Intermediate 1 is speculated to be the I form of apomyoglobin, in which the A, G, and H helices are folded.
2. The second phase is the assembly of secondary and tertiary structures, especially around the hemindicyanide.
3. The third phase is the final structural rearrangement.

Computer simulation studies of the thermally induced unfolding of apomyoglobin using explicit water have been reported (Tirado-Rives & Jorgensen, 1993). In that work, the pH effect on apomyoglobin was considered by comparing simulations in which histidine residues were differently protonated. The resulting structures at 298 and 358 K were in good agreement with the experimental data available. Simulations at high pressure using explicit solvent have also been reported for BPTI (Kitchen et al., 1992), superoxide dismutase (Paci & Marchi, 1996), and HEW lysozyme (Hünenberger et al., 1995). None of them were able to observe unfolding in the time frame of the simulations (100, 120, and 210 ps, respectively), at 1.0 GPa of pressure and room temperature. However, unfolding was observed in the lysozyme simulation when the temperature was raised close to the thermal denaturation temperature.

Hirst and Brooks (1995) performed MD simulations on solvated isolated helices of myoglobin. Raymond et al. (1997) synthesized a series of peptides covering the entire sequence of sperm whale myoglobin and reported the conformation preferences of these peptides. These studies suggest that spontaneous secondary structure formation in local regions of the polypeptide plays an important role in the initiation of protein folding. Both studies indicate

that helix F is relatively unstable. They also suggested that binding to the heme group and packing interactions in the folded metmyoglobin are the main factors behind formation and stabilization of helix F. The order of stability for the helices derived from these studies is

$A, G, H > B > E > F$.

Because the peptides corresponding to helix A and region AB are not soluble in water, the high helical propensity observed experimentally in methanol solution for these peptides does not necessarily imply the existence of helical structures in water solution. Therefore, the question of whether the helix A region spontaneously forms a helix in water remains unanswered.

Although the three-dimensional structure of apomyoglobin has not been determined, there has been extensive experimental characterization of apomyoglobin and its folding pathway. Hydrogen-exchange pulse labeling and stopped-flow circular dichroism experiments (Jennings & Wright, 1993) suggest that the dominant refolding pathway of apomyoglobin is

unfolded $\rightarrow A\ G\ H \rightarrow A\ B\ G\ H$
 $\rightarrow A\ B\ C\ CD\ D\ E\ G\ H \rightarrow$ native,

where the early folding of the AGH intermediate is thought to be due to the intrinsic helix stability combined with interactions between helices. The earliest detectable intermediate formed during refolding corresponded to a molten globule containing secondary structure elements localized in the A, G, and H helices and part of the B helix. This intermediate has 35% helix content.

Fluorescence studies on native and acid compact forms of apomyoglobin under pressures ranging from 1 atm to 240 MPa have been reported (Bismuto et al., 1996a, 1996b). The tryptophanyl emission at 240 MPa and pH 7 suggests that the N-terminal region of the molecule retains elements of organized structure, although there are structural changes. The spectra at 240 MPa do not correspond to the unfolded molecule. The pressure induced structural changes of the acid compact form of apomyoglobin causes the exposure of the tryptophan residues, but does not affect the ability to bind ANS (1-anilino-8-naphthalenesulfonate).

Analysis of the simulation results: Changes in secondary structure with pressure

We find general agreement with the experiment, suggesting that NPT MD with implicit solvent provides an adequate description of the pressure induced unfolding of metmyoglobin.

The gradual loss of secondary structure elements as a function of the applied pressure is seen clearly in Figures 1, 5, and Table 2. Between 0.1 and 600 MPa, the percentage of the residues retaining a helix conformation is at least 58%. At 700 MPa, where a significant increase in the aromatic residues solvent accessibility is observed, the number of residues in the helix conformation drops to 56 (48%) from 116. At 800 MPa, 42% of the residues originally in helix remain in this conformation. This number decreases to 27% at 1.2 GPa. The conformer at 700 MPa (Fig. 7B) presents more than 60% of helices A, E, and H, and between 20 to 44% of helices B, C, and G. At 800 MPa, helices B, D, E, and G are more than 50% preserved, while helices C and H retain more than 30% of

their original helical residues. Although the total helical content decreases monotonically with pressure, the number of helical residues per helix (Table 2) fluctuates. Assuming that the percentage of helical residues preserved at high pressures is related to helix stability, we can derive a relative order of stability. Thus, helix F is clearly least stable. In addition, helices B, G, and H are by far the most stable over the entire pressure range. Helix H still retains 26% of its residues at 1.2 GPa, while helix E is fairly stable until 800 MPa. Helix A has some fluctuation between 700 MPa and 1 GPa, while helix D fluctuates between 600 and 800 MPa, and helix C seems to be less stable at low rather than at high pressures. Thus, we conclude that the stability is in the order

$$G > H > B > C, E > A > D > F.$$

The conformers at 700 and 800 MPa are globular structures with a high content of secondary structure and high solvent accessibility for aromatic residues. The tertiary structure is similar to the native state but not as rigid. All these characteristics agree with the description of a molten globule (Baldwin, 1991), suggesting that these denatured structures are molten globules.

Molten globule intermediates have been postulated as universal folding intermediates formed on the folding pathway of all proteins. Many aspects of folding pathways could be clarified if these intermediates can be well characterized. However, due to the rapid and generally highly cooperative nature of the protein folding process, the experimental characterization of folding intermediates is difficult.

The conformation of holomyoglobin is similar to that of apomyoglobin by several criteria (Hughson et al., 1990; Griko & Privalov, 1994; Eliezer & Wright, 1996). Therefore, one can compare data available for the apo form of myoglobin to our results. The general characteristics of the 700 and 800 MPa conformers are in agreement with the description of molten globule intermediates observed experimentally for apomyoglobin by many authors (Hughson et al., 1990; Baldwin, 1991; Jennings & Wright, 1993; Chiba et al., 1994), although some structural differences must be considered. In the pH unfolding and the pH refolding of apomyoglobin, only three of the major helices of myoglobin are found in the molten globule intermediates (helices A, G, and H), while the other two (B and E) are absent (Baldwin, 1991; Jennings & Wright, 1993; Chiba et al., 1994). In the present simulations, more than 55% of helix E is preserved for the 700 and 800 MPa structures (assumed to be equivalent to a molten globule). The differences between the pH unfolding pathway and the pressure unfolding pathway suggest multiple mechanisms for unfolding. It is possible that the observed different unfolding mechanism is a consequence of the structural differences between apomyoglobin and metmyoglobin; however, the structure of the pH intermediates observed during the refolding process in which the heme group is incorporated to the apoprotein (Chiba et al., 1994) indicates that this is unlikely.

Analysis of the simulation results: Changes in main-chain/main-chain HB with pressure

The conformers from 1 atm to 100 MPa show characteristics corresponding to the native structure. They have an RMS value of less than 2.0 Å from the native structure and a high content of preserved secondary structure (>77%). Figure 8 shows the ratio of

Fig. 8. Ratio of main-chain/main-chain hydrogen bonds $[O(i) \rightarrow H(i+4)]/[O(i) \rightarrow H(i+3)]$ as a function of pressure.

HBs $(i, i+4)/(i, i+3)$. The ratios between the HBs type $(i, i+4)$ and $(i, i+3)$ in the pressure range from 1 atm to 100 MPa are between 2.7 and 2.4. The conformers from 200 to 600 MPa are not as close to the native structure as the low pressure conformers, but have characteristics indicating that they are still native-like (suggesting that the protein retains its function and main properties). The secondary structure content in these conformers (relative to the initial structure) is higher than 55% and the solvent accessibility of the aromatic residues is low. The ratios of HBs $(i, i+4)/(i, i+3)$ are between 1.8 and 2.1. Above 1 GPa the protein is unfolded and clearly denatured as indicated by the lack of secondary structure and organization. The percentage of secondary structure preserved above 1 GPa is lower than 35%. The exposure of the aromatic residues is extremely high and the ratio of HBs $(i, i+4)/(i, i+3)$ falls below one. The molten globules at 700 and 800 MPa have percentages of preserved secondary structures between 42 and 48%. The exposure of the aromatic residues is higher than in the native-like conformers but much lower than in the denatured structures. The HB $(i, i+4)/(i, i+3)$ ratios for these conformers are, respectively, 1.3 and 1.2.

Taking into account the above considerations and comparing Figures 2 and 5, we define three regions in the pressure unfolding pathway of metmyoglobin:

1. The first region corresponds to the native and native-like states, which can be identified by a secondary structure content higher than 40% (relative to the total number of amino acid residues in the protein) and a $[O(i) \rightarrow H(i+4)]/[O(i) \rightarrow H(i+3)]$ main-chain/main-chain HB ratio ≥ 2 . This corresponds to the region from 1 atm to 600 MPa in our simulations.
2. The second region is the molten globule region, where the percentage of secondary structure is between 30 and 40% and the $[O(i) \rightarrow H(i+4)]/[O(i) \rightarrow H(i+3)]$ ratio is between 1.5 and 1. This appears between 700 and 800 MPa in our simulations.

3. Finally, the denatured region has less than 25% of the total number of residues in secondary structure and a $[O(i) \rightarrow H(i+4)]/[O(i) \rightarrow H(i+3)]$ ratio less than one. This occurs above 800 MPa in our simulations.

Some MD studies on isolated helices of myoglobin (Soman et al., 1991; Hirst & Brooks, 1995) identified the unfolding via $(i, i+3)$ HBs as one of the mechanisms of unfolding for the helices. The mechanism identified in our results is a $(i, i+4) \rightarrow (i, i+2)$ migration.

Comparison with other experiments

Besides the UV absorption of aromatic residues, another experimental technique for following the unfolding of metmyoglobin is tryptophan fluorescence. Our results (Table 3; Fig. 2) suggest that this technique would find denaturation at pressures higher than 800 MPa. Because the two tryptophanes are located in the N-terminal region on helix A, the high exposure of these residues is a good indication of structural changes in that region, but not a good indicator of the overall conformational state of the protein. Our simulation results are also in agreement with the fluorescence studies under pressure for apomyoglobin (Bismuto et al., 1996a, 1996b), which shows that the N-terminal region of the molecule is not unfolded at 240 MPa.

The globin family of proteins (of which myoglobin is a member) share a high degree of structural homology combined with a low degree of sequence homology (as low as 16% for distant related species). We find that more than 30% of the conserved helical sites common to the known globin proteins (Bashford et al., 1987; Kapp et al., 1995) are in secondary structure regions that are highly preserved in the pressure simulations. The observation that many of the globin-conserved residues remain in their native secondary structure conformation at high pressure for myoglobin suggests that these regions have a strong tendency to be in a helix conformation (Fig. 4). This is consistent with the common assumption that the conserved residues within a family of proteins are essential to determining the tertiary structure and the function of the proteins.

The conserved residues distribution in the structure of the molten globule intermediate at 700 MPa (Fig. 3) also provides information about which residues are important for the correct tertiary folding of the protein. In helices A, B, C, and G, buried conserved residues are present in higher percentage than surface conserved residues; in helix E they are less preserved; helices B and C have no surface conserved residues, and helix H has all buried and surface conserved residues preserved as helical residues in the molten globule. Thus, the molten globule helices preserve buried conserved residues in higher percentage than for surface conserved residues. This, plus the fact that more than one-half of the helical residues are hydrophobic, shows the presence of a tertiary structure core in the intermediate. This core is the preserved part of the original (completely folded) tertiary structure core.

Relation between hydrophobic effects and pressure unfolding

It is widely accepted that the native conformation of a protein in aqueous solution is stabilized mainly by hydrophobic interactions (Zipp & Kauzmann, 1973; Kauzmann, 1987). Thus, the denaturation process (which is characterized by the exposure of many nonpolar groups to the solvent) should resemble the simple process of transferring a nonpolar molecule from a nonpolar environment

(the protein core) into a polar one (water). This is consistent with some thermo-dynamic data on thermal unfolding. However, Kauzmann (1987) pointed out that this liquid-hydrocarbon model does not explain the behavior of the protein volume during pressure-induced unfolding. Some attempts have been made recently to explain this behavior (Payne et al., 1997; Hummer et al., 1998). These authors suggested that the pressure-induced unfolding is related to the pressure dependence of the hydrophobic interactions, which are considered the driving force for pressure unfolding. The overall picture of pressure denaturation from these studies is: (1) at high pressures, the water is inserted into the interior of the protein, because of the reduced cost of inserting a polar molecule into a non-polar aggregate (Hummer et al., 1998), and (2) the solvation of the hydrophobic groups buried in the native (folded) structure changes the compressibility of the solvated system, favoring denaturation.

This proposed model that unfolding is caused mainly by the pressure dependence of hydrophobic interactions (Payne et al., 1997; Hummer et al., 1998) should not be observed in calculations such as ours with implicit solvent (since the interactions between water and nonpolar groups are not explicit). Nevertheless, our results properly describe the pressure unfolding of metmyoglobin. This suggests that the forces responsible for unfolding are represented in the simulations. In our implicit solvent model, the dielectric constant (representing the water) regulates the electrostatic interactions but does not affect the HB term. Our preliminary simulations (Table 5; unpubl. results) show that the changes in dielectric constant at high pressure modifies the solvent accessibility but does not alter the unfolding of the secondary structures (caused by HB migration processes). Thus, our simulations suggest that the driving force for pressure unfolding is HB migration (a main-chain effect), while the hydrophobic effects regulate the side-chain interactions that are responsible for the solvent exposure, volume, and tertiary packing.

Table 5. The RMSD from the minimized structure and residue solvent accessibility as a function of pressure [using $\epsilon(T,p)$]

<i>P</i> (MPa)	RMSD	Solvent accessibility	
		ARSA ^a	TRPAC ^b
X-ray	1.52	158.58	3.41
Min ^c	1.62	169.09	2.52
0.1	1.56	166.51	0.55
10	1.73	136.80	2.95
100	2.57	168.72	0.00
200	2.64	82.92	0.01
300	3.33	137.99	2.17
400	3.47	127.32	3.71
500	3.96	92.10	0.00
600	4.49	107.03	0.00
700	4.71	124.38	2.85
800	4.85	128.28	4.68
900	5.04	179.36	8.66
1,000	5.01	146.94	14.94
1,200	4.89	205.85	12.11
1,300	4.77	153.05	8.34

^aARSA = Aromatic residues (Phe, Tyr, and Trp) solvent accessibility ($\text{\AA}^2$).

^bTRPAC = Tryptophan solvent accessibility ($\text{\AA}^2$).

^cMinimized structure.

Assuming that the overall mechanism of unfolding resembles the folding mechanism, then our simulations of pressure unfolding suggest that the mechanism of protein folding is as follows:

1. *Chain to globule.* Under the influence of the hydrophobic forces, the unfolded polypeptide chain collapses into a globular form. This initial globule forms a primary HB network.
2. *HB migration.* The migration of the HBs leads to secondary structure formation. In this stage, molten globule intermediates can be formed.
3. *Packing fine structure.* The shaping of the protein into its native conformation is driven by hydrophobic and packing effects together with improvement of the HB network.

In this view, the first and second stages of folding are dominated by specific driving forces (hydrophobic interactions and HBs, respectively), while the third stage is intrinsically cooperative.

Conclusion

We find that NTP MD provides useful information about the pressure unfolding mechanism of metmyoglobin which correlates with the experiment. Our results indicate a two step unfolding mechanism:

native $\rightarrow$ molten globule $\rightarrow$ unfolded.

Our simulations suggest that the molten globule state of the protein can be characterized as having a percentage of secondary structure between 30 and 40% and a ratio $[O(i) \rightarrow H(i+4)]/[O(i) \rightarrow H(i+3)]$ of HBs of order 1.5 to 1. The transition to the molten globule occurs at 600 MPa, and the transition to the unfolded state occurs at pressures above ~ 900 MPa. Our results suggest that the preferred α -helix unfolding mechanism follows a $(i, i+4) \rightarrow (i, i+2)$ HB pathway. This suggests that the inverse process, $(i, i+2) \rightarrow (i, i+4)$, may be involved in the formation of helices. The results also suggest that migration of HBs plays a fundamental role in the folding/unfolding processes. These pressure unfolding results support the multiple mechanism hypothesis for unfolding and the idea that the intrinsic helical propensity of some regions in the protein is a fundamental factor in the folding mechanism. Molten globules have been proposed as universal intermediates in the folding pathway of proteins. Although there is some experimental evidence supporting this hypothesis (Baldwin, 1991; Jennings & Wright, 1993), it has not been clearly established experimentally that molten globules are involved in all folding processes. The existence of molten globule intermediates in the pressure unfolding pathway obtained in MD is an indication that these structures are natural intermediates in the folding/unfolding processes.

The order of relative stability of the metmyoglobin helices derived from our studies is

$G > H > B > C, E > A > D > F.$

The helices present in the molten globule intermediates at 700 and 800 MPa are ACEGH and BDEGH, respectively.

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References

- Baldwin RL. 1991. Experimental studies of pathways of protein folding. *Protein conformation, Ciba Foundation Symposium* 161:190–221.
- Balny C, Heremans K, Masson P. 1992. Haute pression et biologie: Une démarche d'avenir. *Biofutur* 112:37–40.
- Bashford D, Chotia C, Lesk AM. 1987. Determinants of a protein folding: Unique features of the globin amino acid sequences. *J Mol Biol* 196:199–216.
- Bismuto E, Irace G, Sirangelo I, Gratton E. 1996a. Pressure-induced perturbation of ans-apomyoglobin complex: Frequency-domain fluorescence studies on native and acidic compact states. *Protein Sci* 5:121–126.
- Bismuto E, Sirangelo I, Irace G, Gratton E. 1996b. Pressure-induced perturbation of apomyoglobin structure: Fluorescence studies on native and acidic compact forms. *Biochemistry* 35:1173–1178.
- Bradley DJ, Pitzer KS. 1979. Thermodynamics of electrolytes. Dielectric properties of water and Debye-Huckel parameters to 350°C and 1kbar. *J Phys Chem* 83:1599–1603.
- Chiba K, Ikai A, Kawamurakonishi Y, Kihara H. 1994. Kinetic study on myoglobin refolding monitored by 5 optical probe stopped-flow methods. *Proteins* 19:110–119.
- Chrystomallis GS, Torgerson PM, Drickamer HG, Weber G. 1981. Effect of hydrostatic pressure on lysozyme and chymotrypsinogen detected by fluorescence polarization. *Biochemistry* 20:3955–3959.
- Dufour E, Hoa GHB, Haertle T. 1994. High-pressure effects on β -lactoglobulin interactions with ligands studied by fluorescence. *Biochim Biophys Acta* 1206:166–172.
- Eliezer D, Wright PE. 1996. Is apomyoglobin a molten globule? Structural characterization by NMR. *J Mol Biol* 263:531–538.
- Fernandez DP, Goodwin ARH, Lemmon EW, Sengers JMHL, Williams RC. 1997. A formulation for the static permittivity of water and steam at temperatures from 238 K to 873 K at pressures up to 1200 MPa, including derivatives and Debye-Huckel coefficients. *J Phys Chem Ref Data* 26:1125–1166.
- Frauenfelder H, Alberding NA, Ansari A, Braunstein D, Cowen BR, Hong MK, Iben IET, Johnson JB, Luck S. 1990. Proteins and pressure. *J Phys Chem* 94:1024–1037.
- Griko YV, Privalov PL. 1994. Thermodynamic puzzle of apomyoglobin unfolding. *J Mol Biol* 235:1318–1325.
- Gross M, Jaenicke R. 1994. Proteins under pressure: The influence of high hydrostatic-pressure on structure, function and assembly of proteins and protein complexes. *Eur J Biochem* 221:617–630.
- Hirst JD, Brooks CL. 1995. Molecular-dynamics simulations of isolated helices of myoglobin. *Biochemistry* 34:7614–7621.
- Hughson FM, Wright PE, Baldwin RL. 1990. Structural characterization of a partly folded apomyoglobin intermediate. *Science* 249:1544–1548.
- Hummer G, Garde S, Garcia A, Paulaitis ME, Pratt LR. 1998. The pressure dependence of hydrophobic interactions is consistent with the observed pressure denaturation of proteins. *Proc Natl Acad Sci USA* 95:1552–1555.
- Hunenberger PE, Mark AE, van Gunsteren WF. 1995. Computational approaches to study protein unfolding: Hen egg white lysozyme as a case study. *Proteins* 21:196–213.
- Jaenicke R. 1991. Protein stability and molecular adaptation to extreme conditions. *Eur J Biochem* 202:715–728.
- Jennings PA, Wright PE. 1993. Formation of a molten globule intermediate early in the kinetic folding pathway of apomyoglobin. *Science* 262:892–896.
- Jonas J, Jonas A. 1994. High-pressure NMR-spectroscopy of proteins and membranes. *Annu Rev Biophys Biomol Struct* 23:287–318.
- Kapp OH, Moens L, Vanfleteren J, Trotman CNA, Suzuki T, Vinogradov SN. 1995. Alignment of 700 globin sequences: Extent of amino acid substitution and its correlation with variation in volume. *Protein Sci* 4:2179–2190.
- Kauzmann W. 1987. Thermodynamics of unfolding. *Nature* 325:763–764.
- Kitchen DB, Reed LH, Levy RM. 1992. Molecular dynamics simulation of solvated protein at high pressure. *Biochemistry* 31:10083–10093.

- Laskowski RA, MacArthur MW, Moss DS, Thornton JM. 1993. PROCHECK: A program to check the stereochemical quality of protein structures. *J Appl Cryst* 26:283–291.
- Li T, Hook JW, Drickamer HG, Weber G. 1976. Plurality of pressure-denatured forms in chymotrypsinogen and lysozyme. *Biochemistry* 15:5571–5580.
- Lide DR, ed. 1994. *CRC handbook of chemistry and physics*, 74th ed. CRC Press. pp 6-10; 6th ed. CRC Press. pp 56.
- Mayo SL, Olafson BD, Goddard WA III. 1990. DREIDING: A generic force field for molecular simulations. *J Phys Chem* 94:8897–8909.
- Paci E, Marchi M. 1996. Constant-pressure molecular dynamics techniques applied to complex molecular systems and solvated proteins. *J Phys Chem* 100:4314–4322.
- Payne VA, Matubayasi N, Murphy LR, Levy RM. 1997. Monte Carlo study of the effect of pressure on hydrophobic association. *J Phys Chem B* 101:2054–2060.
- Peng XD, Jonas J, Silva JL. 1994. High-pressure NMR study of the dissociation of ARC repressor. *Biochemistry* 33:8223–8329.
- Rappé AK, Goddard WA III. 1991. Charge equilibration for molecular dynamics simulations. *J Phys Chem* 95:3358–3363.
- Reymond MT, Merutka G, Dyson HJ, Wright PE. 1997. Folding propensities of peptide-fragments of myoglobin. *Protein Sci* 6:706–716.
- Schulte A, Buchter S, Galkin O, Williams C. 1995. Raman-spectroscopic observation of a conformational change at the heme protein linkage in myoglobin at high-pressure. *J Am Chem Soc* 117:10149–10150.
- Shelnutt JA, Song X, Hobbs JD, Medforth CJ, Smith KM, Miura M. 1995. Computer-design of porphyrins for application in catalysis and photoactive devices. *Am Chem Soc* 209(2):143–BTEC.
- Silva JL, Foguel D, Dapoian AT, Preverige PE. 1996. The use of hydrostatic pressure as a tool to study viruses and other macromolecular assemblages. *Curr Opin Struct Biol* 6:166–175.
- Silva JL, Miles EW, Weber G. 1986. Pressure dissociation and conformational drift of the β dimer of tryptophan synthase. *Biochemistry* 25:5780–5786.
- Silva JL, Weber G. 1993. Pressure stability of proteins. *Annu Rev Phys Chem* 44:89–113.
- Simonson T, Brooks CL. 1996. Charge screening and the dielectric-constant of proteins: Insights from molecular-dynamics. *J Am Chem Soc* 118:8452–8458.
- Smith PE, Pettitt BM. 1994. Modeling solvent in biomolecular systems. *J Phys Chem* 98:9700–9711.
- Soman KV, Karimi A, Case DA. 1991. Unfolding of an alpha-helix in water. *Biopolymers* 31:1351–1361.
- Takano T. 1977. Structure of myoglobin refined at 2.0 Å resolution: Crystallographic refinement of metmyoglobin from sperm whale. *J Mol Biol* 110:537–568.
- Tilton RF, Petsko GA. 1988. A structure of sperm whale myoglobin at nitrogen gas-pressure of 145 atmospheres. *Biochemistry* 27:6574–6582.
- Tirado-Rives J, Jorgensen WL. 1993. Molecular dynamics simulations of unfolding of apomyoglobin in water. *Biochemistry* 32:4175–4184.
- Weber G. 1993. Thermodynamics of the association and the pressure dissociation of oligomeric proteins. *J Phys Chem* 97:7108–7115.
- Weber G, Drickamer HG. 1983. The effect of high-pressure upon proteins and other biomolecules. *Q Rev Biophys* 16:89–112.
- Weiner SJ, Kollman PA, Case DA, Singh UC, Ghio C, Alagona G, Profeta S, Weiner P. 1984. A new force-field for molecular mechanical simulation of nucleic acids and proteins. *J Am Chem Soc* 106:765–784.
- Yayanos AA. 1995. Microbiology to 10,500 meters in the deep-sea. *Annu Rev Microbiol* 49:777–805.
- Zipp A, Kauzmann W. 1973. Pressure denaturation of metmyoglobin. *Biochemistry* 12:4217–4227.