Pressure Effect on the Conformational Fluctuation of Apomyoglobin in the Native State

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ABSTRACT: We have investigated the effect of pressure on fluctuations of the native state of sperm whale apomyoglobin (apoMb) by H/D exchange, fluorescence, and limited proteolysis. The results from intrinsic fluorescence showed that a large fraction of apoMb molecules is in the native conformation in the pressure range from 0.1 to 150 MPa at 293 K and pH 6.0. The H/D exchange of protons of the individual backbone amino acids in this pressure range was monitored by NMR. The rate of H/D exchange was enhanced at high pressure, with the protection factors for some residues decreasing by factors of more than 100 compared to the values at 0.1 MPa. The amplitude of the decrease of the protection factor varied among the individual amino acids on the same secondary structure unit. This result suggests that H/D exchange in apoMb is explained best by the penetration model, in which solvent penetrates into the protein matrix via small motions. The result from limited proteolysis under high pressure showed that a pressure increase does not induce local unfolding of the secondary structure units of apoMb. Conformational fluctuations much smaller than local unfolding evidently provide pathways for water to diffuse into the protein interior, and are enhanced by an increase of pressure.

Characterization of protein dynamics is important for understanding the physical properties and the functions of protein. The picture of protein dynamics has oscillated between two extreme views: one is a strongly fluctuating system, and the other is a rigid, aperiodic crystal (*1*). A variety of views for conformational states of apomyoglobin (apoMb) is a typical example for this statement. ApoMb, myoglobin without the heme, has 20% less helical structure (*2*), a slightly large radius of gyration (*3*), lower heat capacity, and less cooperative unfolding characteristics, as compared with those of holoMb. Results from mass spectroscopic study of apoMb (*4*) and conformational analysis of its single amino acid mutants (*5*) suggested that apoMb has many of the same characteristics of a molten globule, consisting of a set of two or more distinct but rapidly interconverting substates. On the other hand, a result from NMR has shown that the majority of the apoMb conformation adopts the structure similar to that of holoMb (*6*). The picture in favor depends strongly on the dominant experimental techniques and on the power of the theoretical tools. This is because proteins are complex systems and no single theory or model is sufficient at the present time to describe all aspects of protein structures and their functions (*1*).

To increase our knowledge about the dynamic properties of apoMb, we have applied high pressure to the protein solution in this study. The pressure effect on protein dynamics is informative for the investigation of the physical properties (*7, 8*) and the functions (*9*−*11*) of proteins, and has been studied by NMR (*12*−*14*), H/D exchange (*15*−*17*), phosphorescence (*10*), and MD simulation (*18, 19*). In the present study, we characterized the dynamics of apoMb by using a combination of fluorescence, H/D exchange, and limited proteolysis. H/D exchange in combination with NMR is a useful technique in monitoring protein conformational changes (*20*), and has been used for the structural characterization of the intermediate of apoMb (*2, 21*). Limited proteolysis can be used to probe the sites of high segmental mobility in protein (*22*), and has been used to detect the dynamic difference between holo- and apoMb (*23*).

EXPERIMENTAL PROCEDURES

Materials

Sperm whale Mb was purchased from Biozyme (Gwent, U.K.). Removal of the heme group was by the acid−acetone method (*24*). After the removal of the aggregates by G-75, apoMb was dialyzed against water. The pH value of the aqueous solutions of apoMb was adjusted to 6.0 by NaOH.

Methods

Fluorescence Spectroscopy. Fluorescence spectra at elevated pressures were obtained with a high-pressure cell, having three optical windows manufactured by Teramecs Co. (Kyoto, Japan), combined with a Shimadzu RF5000 spectrofluorometer (Kyoto, Japan). The concentration of apoMb was 5 μM. The reversibility of apoMb conformation after the pressure treatment has been confirmed by the CD spectroscopy (Jasco J-720, Tokyo, Japan).

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The average emission wavelength ($<\lambda>$) was calculated using the equation:

$$<\lambda> = \frac{\sum_{i=\lambda i}^{\lambda_N} (F_i \cdot \lambda_i)}{\sum_{i=\lambda i}^{\lambda_N} F_i}$$

where F is the fluorescence intensity and λ is the emission wavelength. Fitting analysis of the curve of the averaged wavelength versus pressure was done using the nonlinear least-squares method with Kaleida Graph.

H/D Exchange Monitored by COSY. The aqueous solution of apoMb at pH 6.0 was diluted into D_2O at pH 6.0 in a polypropylene tube to initiate the H/D exchange reaction. The pressure treatment of the solutions was performed after preincubation (10 min at 293 K) in which labile protons on the exposed side chains and some backbone amides were completely exchanged in order to reduce experimental errors. The pressure treatment was performed by compressing the solution in a polypropylene tube using a high-pressure vessel (Teramecs Co., Kyoto, Japan). After the incubation under high pressure, H/D exchange was quenched by adding the heme to reconstitute holoMb, bufferized by deuterated acetic acid with adjustment of the pH at 5.6, and was concentrated by an Amicon filter (YM-10) up to ca. 5 mM for COSY NMR measurement. The solution was equilibrated with CO and reduced with sodium dithionite. The COSY measurement of the reconstructed myoglobin was performed by a Brucker 500ARX spectrometer at 308 K. The exchange rate was monitored through a change in the volume of the individual cross-peak in the COSY spectra. The peak volumes of the cross-peaks of each backbone amide, after the H/D exchange reaction for 10 min at 273 K and 0.1 MPa, were used as a reference for the starting point of the reaction. The reversibility of apoMb conformation after the pressure treatment was confirmed by CD and fluorescence spectroscopy.

Limited Proteolysis. Limited proteolysis was performed by incubating apoMb solutions with protease in polypropylene tubes at pH 6.0 in an aqueous solution. Subtilisin was used because of its very broad substrate specificity. The enzyme:protein concentration ratio was 1:100 (by weight). The tube containing the proteins was put into a high-pressure vessel within 1 min after sample preparation. After incubation at an elevated pressure, the reaction was terminated by decreasing the pH by adding 1% aqueous trifluoroacetic acid. SDS−PAGE was performed using a slab gel with a concentration of 16.5% and a tricine buffer system. The N-terminal sequence analysis was performed on the peptide samples isolated by blotting from the gel utilizing an Applied Biosystems (Foster City, CA) protein sequencer (model 476A) equipped with an on-line analyzer (model 610A) of phenylthiohydantoin derivatives of amino acids.

RESULTS AND DISCUSSION

Intrinsic Fluorescence of ApoMb under High Pressure. Figure 1A shows the pressure effect on the intrinsic fluorescence of apoMb at pH 6.0 and 293 K in the pressure range from 0.1 to 400 MPa. A pressure increase caused a red-shift in the maximum wavelength of the emission

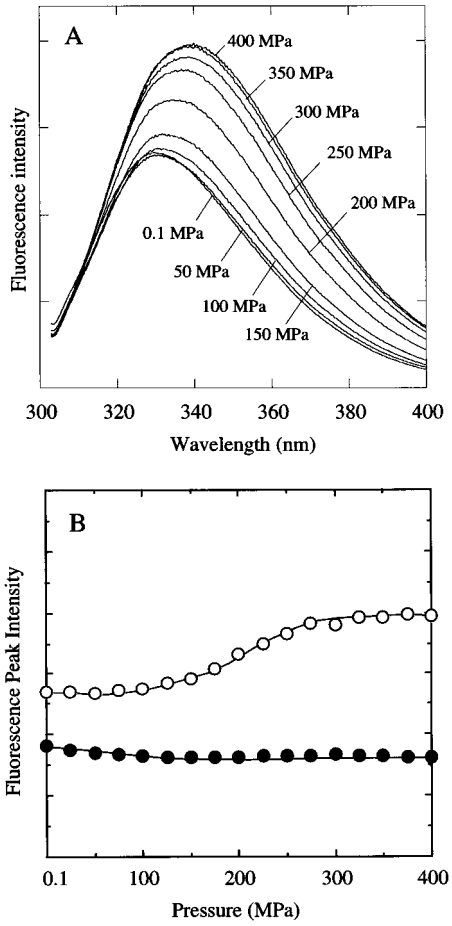

FIGURE 1: (A) Fluorescence spectrum of apoMb at pH 6.0 in the pressure range from 0.1 to 400 MPa. (B) Peak intensity of the fluorescence of apoMb in the pressure range from 0.1 to 400 MPa: (○) pH 6.0, (●) pH 2.2. Excited wavelength was 295 nm.

fluorescence with an increase in its intensity. The fluorescence excited at 295 nm is due to the contribution of Trp7 and Trp14, which are located at the A helix in the N terminal region. Thus, the fluorescence changes in this figure reflect the change in the local environment around the A helix. The amplitude of the pressure-induced change of the fluorescence of apoMb at pH 2.2 was smaller than that at pH 6.0 (Figure 1B). This is because apoMb is completely unfolded at pH 2.2, and the environment of the tryptophan residue is not significantly influenced by a pressure increase. The average wavelength of the fluorescence of apoMb at pH 6.0 and 400 MPa was 13 nm lower than that at pH 2.2, indicating that apoMb is not fully unfolded in this condition. These observations indicate that the conformational transition from the native to the intermediate state of apoMb takes place in the pressure range between 150 and 200 MPa at pH 6.0.

Results consistent with ours have been reported by other groups studying pressure-induced denaturation of apoMb by fluorescence spectroscopy (*25*, *26*) and NMR (*27*, *28*). Vidugiris and Royer (*26*) have investigated the effects of pressure on horse heart apoMb in 10 mM bis-Tris buffer at pH 6.0 and 294 K, and have reported that a conformational transition occurs in the pressure range from 50 to 150 MPa. They analyzed the transition curve in the averaged wavelength of fluorescence versus pressure by fitting the data in terms of a two-state transition, and obtained a volume change from the native to the intermediate state of −70 mL/mol.

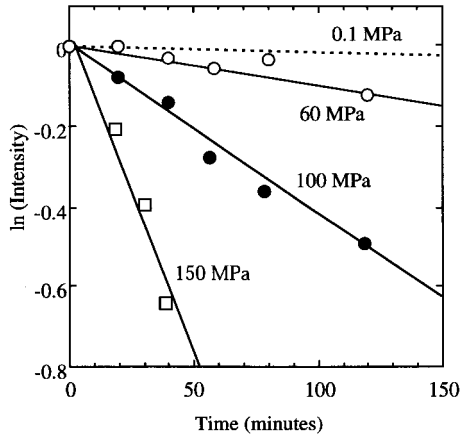

FIGURE 2: Representative pressure dependence of the kinetic behavior of H/D exchange of apoMb. Normalized intensities of Leu11 cross-peaks were plotted vs time. A broken line for 0.1 MPa was obtained from the protection factor estimated in the previous study (*2*).

Our result of the pressure-induced transition of sperm whale apoMb in pure aqueous solutions shown in Figure 1B indicates that a large fraction of the apoMb molecules is in the native state in the pressure range from 0.1 to 150 MPa, and the transition occurs in the pressure range from 150 to 300 MPa. We have obtained a volume change of −53 mL/mol using the same method as utilized by Vidugiris and Royer (*26*). The transition pressure and the volume change were slightly different from what Vidugiris and Royer obtained, which could be explained by the difference in apoMb species and the solution conditions.

H/D Exchange of ApoMb under High Pressure Monitored by NMR. H/D exchange in apoMb at an elevated pressure was monitored by NMR spectroscopy. After various exchange-out periods in the range from 20 min to 2 h (at elevated pressures), the exchange of the backbone amide proton was monitored by the volume change of the individual cross-peaks of NH and $C_\alpha H$ hydrogen in the COSY spectra. As shown in Figure 2, the H/D exchange rate was enhanced by an increase of pressure for every observed backbone amide proton of apoMb. The effect of pressure on the H/D exchange rate of each amino acid residue is related to the change in protection against the exchange expressed by the protection factor: $P = k_{rc}/k_{obs}$, where k_{obs} is the H/D exchange rate constant obtained experimentally and k_{rc} is the H/D exchange rate constant of each backbone amide proton in the unfolded state of apoMb calculated from the theoretically predicted rates of the random coil polypeptide (*29*), correcting for the value at high pressure (*30*). Seventeen amide protons showed cross-peaks of a sufficient intensity to allow determination of the exchange rate under high pressure. The *P* value of each amide proton was decreased by an increase of pressure. Table 1 shows the amplitudes of decrease in the *P* value under high pressure which were obtained by dividing the value at an elevated pressure by that at 0.1 MPa. We note here that the amplitudes of the relative decrease in the H/D exchange rate shown in Table 1 varied among the amide protons on the same helix.

To reveal the real mechanism underlying this acceleration, we have investigated the pathway of H/D exchange in apoMb under high pressure. Two main pathways have been proposed for H/D exchange in protein (*31*). One is the high activation energy process, in which a proton is exchanged after the major cooperative unfolded state, and the other is the low activation energy process, in which the exchange is through the native structure. The fluorescence analysis in Figure 1B has shown that the major fraction of apoMb in the pressure range from 0.1 to 150 MPa is in the native state, suggesting that the low activation process is dominant in H/D exchange in the present experimental conditions. However, the high activation energy process cannot be completely excluded because the pathway via the minor fraction of the intermediate can be dominant when the exchange between the native and the intermediate state is fast enough. We have measured the kinetic rate of relaxation from the native to the intermediate state under high pressure by a pressure jump experiment. The changes in the intrinsic fluorescence intensity of apoMb were monitored as a function of time after the pressure jump. Figure 3 shows the time course of the fluorescence intensity change after the pressure jump of 50 MPa in the pressure range from 0.1 to 250 MPa. The increase in the fluorescence intensity indicates that the positive pressure jumps result in perturbation of the equilibrium

Table 1: Amplitudes of Decrease of the *P* Value of ApoMb at Various Pressures

		$P/P_{0.1Ma}{}^a \times 100$		
residue	helix position	60 MPa	100 MPa	150 MPa
Val 10	A8	0.9	0.4	0.1
Leu 11	A9	<9	<2	<0.4
Ile 28	B9	25	5	<0.025
Leu 29	B10	10	4	0.5
Ile 30	B11	5.7	0.85	0.14
Arg 31	B12	<0.02	<0.02	<0.02
Phe 33	B14	83	15	<0.02
Lys 34	B15	100	<0.025	<0.025
Val 66	E9	<0.03	<0.03	<0.03
Leu 69	E12	100	<0.02	<0.02
Thr 70	E13	<0.025	<0.025	<0.025
Leu 72	E15	<1	<1	<1
Gly 73	E16	<0.03	<0.03	<0.03
Ile 75	E18	50	15	5
Ile 112	G13	12	1.7	0.5
Val 114	G15	10	2.5	0.45
Leu 115	G16	6.7	1.7	0.17

[a] Values obtained in the previous study (*2*).

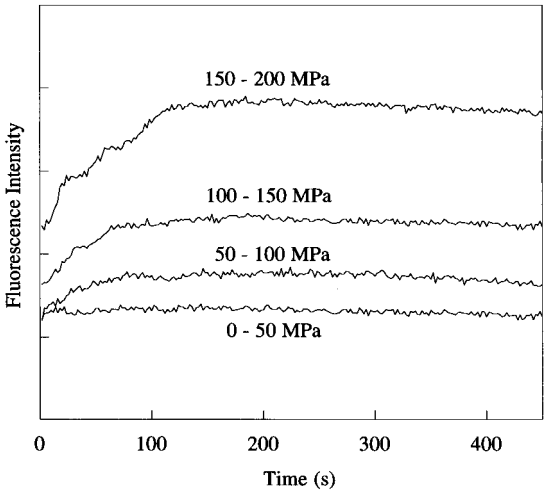

FIGURE 3: Relaxation profile for positive pressure jump obtained by monitoring the intensity of the intrinsic fluorescence of apoMb as a function of time after the pressure jump.

toward the intermediate state. The time scale for relaxation in this pressure range was on the order of minutes, which is a similar magnitude to that of the pressure-induced unfolding relaxation of staphylococcal nuclease (*32*). The rate constants of the transition from the native to the intermediate state (k_{unfold}) in the pressure range from 50 to 200 MPa were less than 1.0 min^{-1}. On the other hand, the calculated value of the H/D exchange rate constant of the backbone amide protons of apoMb under high pressure (k_{rc}) ranged from 4.3 to 364.5 min^{-1}, and the averaged value at 100 MPa was 48.3 min^{-1}, which is greater than k_{unfold}. Therefore, when H/D exchange in apoMb is dominated by the high activation energy process at an elevated pressure, the total rate will be limited by the rate of the transition to the intermediate. However, if this is true, a drastic acceleration in the rate of exchange shown in Figure 2 is inconsistent with the fact that the fraction of the intermediate state at a low pressure is no more than several percentages of the total molecules, which was obtained from the transition curve shown in Figure 1B. Consequently, H/D exchange in apoMb at low pressures would be dominated by the low activation energy process, and pressure-induced enhancement of the rate of H/D exchange would be due to the changes within the native structure of apoMb.

More details concerning the H/D exchange mechanisms of apoMb under high pressure can be inferred from the result in Table 1. The low activation energy process is explained by the local unfolding model or the penetration model (*31*). In the former model, the segments on the secondary structure undergo local, reversible unfolding, involving the cooperative breathing of the hydrogen bonds in the segment, the disruption of the secondary structure, and the movement of the segment into bulk solvents where the exchange takes place. The decrease in the *P* value should have a similar magnitude on the same secondary structure unit for this model. It has been shown previously that the *P* values of the two central helices and the C-terminal helix of apocytochrome b_{562} (*16*) were decreased at high pressure by a similar factor. The total H/D exchange of this case is dominated by the pathway via the cooperative local unfolding of these three helices.

In the penetration model, on the other hand, solvent molecules diffuse into the protein matrix mediated by a small rapid fluctuation of interior atoms on the order of tenth to several angstroms. In this model, the amplitudes of the decrease in the *P* value do not necessarily correlate with the secondary structure unit, because there may be several steps required for the exchange of a particular site. This model seems to be valid for the case of the N-terminal helix of apocytochrome b_{562} (*16*) and the 43 amide protons of T4 lysozyme (*17*) in which NMR studies have shown that the amplitudes of the decrease in the *P* value by an increase in pressure did not correlate with the secondary structural unit in which the amino acid is located. Since the relative decrease in the H/D exchange rate of apoMb in Table 1 varied in amplitude among the amide protons on the same helix, H/D exchange in apoMb is explained best by the penetration model in which H/D exchange is mostly mediated by a small rapid fluctuation. Figure 4 shows the positions of amino acid residues on which the *P* value decreased by factors of more than 100 at 100 MPa. All of them except Val 10 are on the B and E helices around the heme pocket. This part of the molecule should be flexible because of the removal of the heme, and may allow water penetration to occur relatively easily under high pressure.

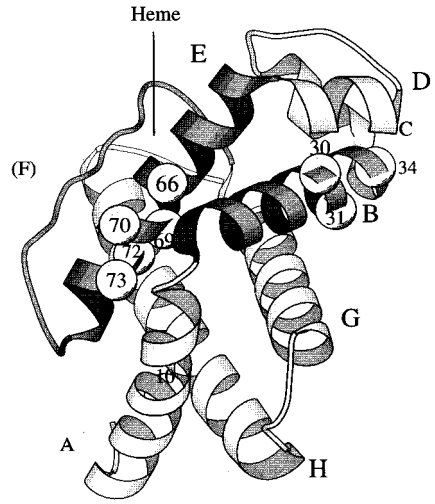

FIGURE 4: Cartoon model of the apoMb structure drawn using Molscript (*42*). The positions of amino acid residues in which the *P* value decreased by a factor of more than 100 at 100 MPa are indicated by spheres. The B and E helices are shown in gray.

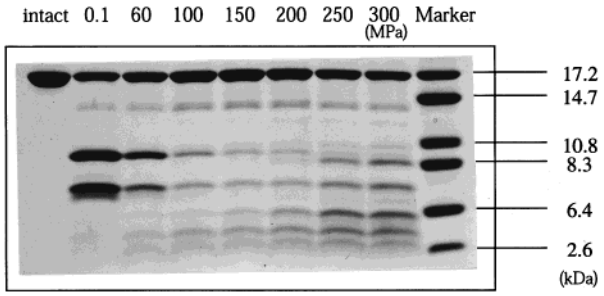

FIGURE 5: SDS−PAGE of peptide fragment from the limited proteolysis of apoMb with subtilisin for 30 min in the pressure range from 0.1 to 300 MPa.

Limited Proteolysis of ApoMb with Subtilisin under High Pressure. We have investigated the conformational state of apoMb by limited proteolysis for further insights into the dynamics under high pressure. The apoMb solution, in the presence of subtilisin, was incubated at an elevated pressure, and the pattern of protein degradation was monitored with SDS−PAGE. Figure 5 shows the SDS−PAGE analysis of the proteolysis of apoMb with subtilisin at various pressures from 0.1 to 300 MPa. The band for the intact apoMb became intense by an increase of pressure from 0.1 to 150 MPa, indicating that the rate of proteolysis with subtilisin is reduced. This is consistent with the previous result, which states that the protease activity of subtilisin was reduced by an increase of pressure (*33*). On the other hand, the SDS−PAGE pattern above 150 MPa shows that the rate of proteolysis is accelerated by an increase in pressure. The result from fluorescence has shown that a pressure-induced transition to the intermediate state occurs above 150 MPa, and that the acceleration of proteolysis originates from the increase of the conformational fluctuation of the substrate protein.

Figure 6 shows the time course for the digestion of apoMb by subtilisin. The pattern of proteolysis at 0.1 MPa showed two major fragments of approximate molecular masses of 7

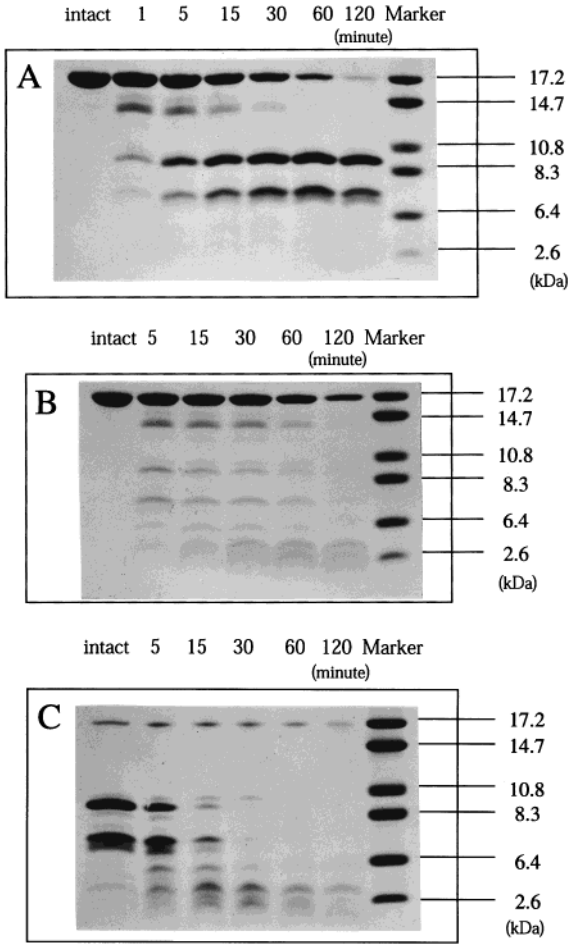

FIGURE 6: Time course of the SDS−PAGE pattern of peptide fragment from the limited proteolysis of apoMb with subtilisin. (A) 0.1 MPa. (B) 150 MPa. (C) Time course of the SDS−PAGE pattern of the limited proteolysis at 150 MPa for the peptide fragments produced by subtilisin treatment of apoMb at 0.1 MPa for 2 h.

and 10 kDa (Figure 6A). Blotting and N-terminal protein sequencing of the fragment of 7 kDa gave amino acid sequences of Ala-Gln-Ser-His and Ala-Thr-Lys-His, which correspond to the sequences of 90−93 and 94−97 of myoglobin. The initial nicking of apoMb occurred at Leu89−Ala90 and His93−Ala94 peptide bonds at the F helix region, indicating that the local structure of the F helix region of apoMb is disordered. The digestion of apoMb by subtilisin at 150 MPa gave short fragments of 2−3 kDa, in addition to the two major fragments from the cleavage of the F helix (Figure 6B). Blotting and protein sequencing of these short fragments gave a mixture of the sequences. Comparison of all the possible combinations of the obtained sequences to the original sequence of myoglobin allows the identification of the sequences of these short fragments. We have found that the combined sequences of Tyr-Ala-Leu-Gly and Gly-Ala-Ile-Leu correspond to the sequences of 70−73 and 73−76 on the E helix, respectively. This result indicates that Leu69−Tyr70 and Leu72−Gly73 on the E helix were cleaved at 150 MPa. Additionally, the calculated molecular mass of the fragment from a double cleavage on the E and F helices is 2−3 kDa, which is the same as the short fragment shown in Figure 6B. These results may lead to a conclusion that local unfolding of the E helix occurs under high pressure. However, when the E and F helices are cleaved by a simultaneous process, the fragments from the cleavage only at the E helix (7.8 and 9.0 kDa) should also appear in addition to the fragment from the F helix cleavage. The absence of these bands in Figure 6B suggests that the F helix region of intact apoMb is cleaved first and a sequential cleavage of the fragments occurs in the proteolysis process of apoMb under high pressure.

To confirm this assumption, the fragments from the cleavage at the F helix produced by proteolysis at 0.1 MPa were treated with subtilisin under high pressure (Figure 6C). The time course of the pattern of SDS−PAGE showed rapid production of the short fragments (2−3 kDa) which are similar in size to those shown in Figure 6B. Thus, the short fragments in Figure 6B are produced by cleavage of the E helix sequentially after cleavage of the F helix region. This result suggests that a pressure increase from 0.1 to 150 MPa does not induce local unfolding of the E helix region of the intact apoMb. This is consistent with the result shown in Figure 5 that the rate of proteolysis was decreased by a pressure increase from 0.1 to 150 MPa, since the rate of the proteolysis should be enhanced by local unfolding. The present results from limited proteolysis support the idea that small conformational fluctuations of apoMb provide pathways for H/D exchange at low pressures.

CONCLUSION

The present results from a combined use of fluorescence, H/D exchange, and limited proteolysis indicate that the small fluctuations of apoMb, which mediate the diffusion of the solvent into the protein matrix, are enhanced by an increase of pressure. In the previous study, it has been shown that fluctuations of RNase T1 and phosphoglycerate kinase are enhanced by an increase of pressure (*10*), whereas those of apoazurin (*10*) and BPTI (*12*−*14*) were reduced by an increase of pressure. These results concerning the effect of pressure on fluctuation of protein are important for the study of the stability and folding process of proteins using high pressure (*34*−*37*), because fluctuation of proteins is related to its compressibility (*38*, *39*) which is a significant thermodynamic parameter in this field. Prehoda et al. have cast a caution that the assumption of compressibility change between folded and unfolded states as zero can screw the quantitative analysis of the pressure-induced conformational transition (*40*). Thus, knowledge concerning the pressure effect on compressibility of native and denatured proteins is necessary for the quantitative analysis of the pressure-induced conformational transition. The rate of H/D exchange in protein has been suggested to be correlated to its compressibility (*31*), and a detailed analysis of H/D exchange in proteins under high pressure would provide useful information for understanding the effect of pressure on compressibility of protein. However, it has been shown that the relation between volume fluctuation and compressibility is of limited applicability to highly hydrated states of protein because fluctuation may be partly determined by the process of water exchange between the protein interior and the bulk solvent (*41*). Therefore, quantitative information about compressibility of the denatured state of protein under high-pressure cannot be obtained from the H/D exchange experiment alone; other sophisticated methods are necessary.

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