Irreversible Unfolding of Myoglobin in an Aqueous Solution by Supercritical Carbon Dioxide

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The conformational changes in myoglobin, treated by microbubbling of supercritical carbon dioxide (SC-CO_2), were investigated by measuring the circular dichroism spectra in the ultraviolet range and compared with those in other proteins (ovoalbumin, bovine serum albumin, and β-lactoglobulin). Irreversible unfoldings were observed after the microbubbling of SC-CO_2 at 35 °C and 30 MPa for 30 min. The degree of unfolding depended on the number of intramolecular S−S bonds. α-Helix contents of myoglobin decreased with increasing density of SC-CO_2. Unfoldings of myoglobin induced by heating, pH-lowering, and the addition of a denaturant were reversible. The irreversible unfolding of myoglobin was also observed by the bubbling of gaseous CO_2 under atmospheric pressure, but heating was required.

Keywords: *Supercritical CO_2; microbubbles; unfolding; myoglobin*

INTRODUCTION

Supercritical carbon dioxide (SC-CO_2) has been used extensively for extractions (Vega et al., 1996), chromatography (Ibanez et al., 1994), and modifying chemical reactions (Jessop et al., 1994) because of its moderate operational conditions (low critical temperature and pressure). Carbon dioxide is also nontoxic, nonflammable, inexpensive, and readily available.

Enzyme preparations could undergo little conformational change under SC-CO_2 conditions, and retain their activity (Randolph et al., 1988). α-Amylase, glucose oxidase, lipase, and catalase also retained their activities after SC-CO_2 treatment at 35 °C and 200 atm for 1 h (Taniguchi et al., 1987). On the other hand, pectinesterase in orange juice could be partially inactivated by SC-CO_2 at 40−60 °C and 31 MPa (Balaban et al., 1991). Chen et al. (1992) investigated the inactivation of polyphenol oxidases from lobster, brown shrimp, and potato by subcritical CO_2. Inactivation of oxidoreductases containing metals by SC-CO_2 was reported by Endo et al. (1995). In our previous papers (Ishikawa et al., 1995a,b, 1996a,b), the microbubble SC-CO_2 treatment was proposed as a novel method for enzyme inactivation. Some enzymes which could be responsible for degrading food quality were significantly inactivated at 25 MPa and 35 °C for 30 min (Ishikawa et al., 1995a). Pectinesterase in orange juice was inactivated at 25 MPa and 35 °C for 30 min (Ishikawa et al., 1996a). "Sake" treated with microbubbles of SC-CO_2 had preserved its sensory quality close to that of namazake (non-heat-treated sake), while the heat-treated sake had lost its freshness (Ishikawa et al., 1995b).

It was found that enzymes in aqueous solutions were liable to be inactivated by treatment with SC-CO_2. The interaction between SC-CO_2 and enzyme (protein), therefore, has become of interest in recent years. The conformational changes of enzymes treated by high-pressure (subcritical) CO_2 were studied by Chen et al. (1992). The changes in secondary structure of polyphenol oxidases were shown by circular dichroism (CD) spectral analysis. However, little was described of what would be responsible for the changes. The conformational changes of glucoamylase and acid protease by microbubbling of SC-CO_2 were investigated in a previous paper (Ishikawa et al., 1996b). A close relationship was observed between the loss of activity and the decomposition of the α-helix structure.

The SC-CO_2 effects on the conformation of protein have been poorly understood. The object of the present investigation is to clarify the differences in the unfolding of myoglobin by microbubbling of SC-CO_2 and other treatments.

MATERIALS AND METHODS

Protein Solutions. Myoglobin from horse muscle was purchased from Nacalai Tesque Inc., Kyoto, Japan. Ovoalbumin (OVA) from turkey egg, bovine serum albumin (BSA), and β-lactoglobulin from bovine milk were purchased from Sigma Chemical Co., St. Louis, MO. Each protein was dissolved in 50 mM sodium phosphate buffer (pH 7.0) to a concentration of 50 μg/mL.

Apparatus and Procedure for Microbubble SC-CO_2 Treatment. The SC-CO_2 treatment was carried out using a Milton Roy X-10 system (Riviera Beach, FL). This system consists of a 120 mL stainless steel vessel, which has a cylindrical filter (10 μm pore size) made of sintered stainless steel for microbubbling of SC-CO_2 (Ishikawa et al., 1995a). A thermocouple was placed inside the treatment vessel to control temperature. The system had a back-pressure regulator valve that was used to control pressure.

For each experimental trial, 100 mL of protein solution was loaded into the treatment vessel, and then pressurized with

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CO_2. The feeding rate of CO_2 was set at 4.0 g/min. The feeding of CO_2 was continued during the treatment. The temperature and pressure were kept constant. The treatment conditions were similar to those of a previous paper (Ishikawa et al., 1996b), i.e., pressure varying from 8 to 30 MPa at 35 °C. At the end of the treatment, the vessel was slowly depressurized by releasing CO_2 over a period of about 5 min. After the treatment, each solution was subjected to CD analysis.

Procedure for Heat Treatment. Heat treatment was carried out by heating the cuvette in the appararus for CD spectral analysis. The heating temperature was set at 60−80 °C under atmospheric pressure. The CD spectra of heated protein were monitored in situ at the experimental temperature. After the treatment, each protein solution was cooled rapidly in ice water. After 6 h, the solution was subjected to CD analysis again.

Procedure for Heat Treatment with Bubbling of Gaseous CO_2. The heat treatment with CO_2 bubbling was carried out using a 200 mL glass vessel. The feeding of CO_2 into a solution was carried out using a filter (10 μm pore size) which was similar to that used for microbubbling of SC-CO_2. The protein solution (100 mL) was heated in a water bath while CO_2 was bubbled under atmospheric pressure and then cooled rapidly in ice−water. After 6 h, each protein solution was subjected to CD analysis.

Spectropolarimetric Analysis. The CD spectra of native and treated proteins were measured by scanning in a UV range of 200−250 nm using a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) and a Suprasil (Helma Cells, Jamaica, NJ) cuvette (1.0 cm light path) with a water jacket. Measurements of CD spectra were carried out at 20 °C except for in situ monitoring of the heated protein. The content of the α-helix structure was calculated by the formula proposed by Greenfield and Fasman (1969).

RESULTS AND DISCUSSION

Protein Unfolding by Microbubbling of SC-CO_2. Figure 1 shows CD spectra of proteins untreated (native) and treated by microbubbling of SC-CO_2 at 35 °C and 30 MPa for 30 min. The two typical negative extrema at 208 and 222 nm, which would be related to the α-helix structure, was observed in the native CD spectra of myoglobin, OVA, and BSA (Figure 1a−c). These CD spectra indicate that the proteins would be α-helix-rich. The α-helix contents in myoglobin, OVA, and BSA were estimated at 74%, 77%, and 85%, respectively. On the other hand, β-lactogloblin would be a β-structure-rich protein, and its α-helix content was estimated to be 15% (Figure 1d).

After the microbubble SC-CO_2 treatment, the decays of the negative ellipticity on the CD spectra were observed. The changes in the CD spectra of myoglobin, OVA, and BSA would be attributed to the decomposition of the α-helix structure. The change in the CD spectrum of β-lactogloblin would be mainly due to the β-structure decomposition. Among the α-helix-rich proteins, the most significant change in the CD spectra was observed for myoglobin. The negative ellipticity of the myoglobin CD spectrum was completely decayed after the SC-CO_2 treatment. The α-helix content of myoglobin was decreased to 12% (84% unfolding). A small change was observed for BSA, and the decrease of the α-helix content would be less than 10%. The difference in conformational changes of the proteins would be attributable to their intramolecular disulfide (S−S) bonds. Seventeen S−S bonds in the BSA molecule would tightly stabilize the conformational structure. There is no intramolecular S−S bond in myoglobin, and thus, the conformation would be liable to break down.

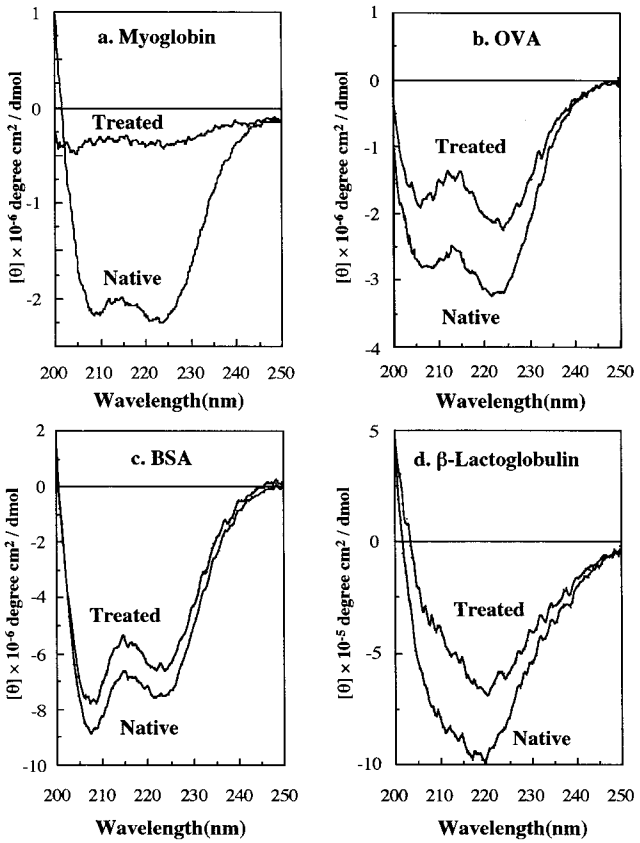

Figure 1. CD spectra of the proteins after microbubble SC-CO_2 treatment at 30 MPa and 35 °C for 30 min. The CD spectra were measured at pH 7.0 and 20 °C.

Figure 2 shows the CD spectra of myoglobin after microbubble SC-CO_2 treatment at 35 °C and 10−30 MPa for 30 min. The negative ellipticity decayed with an increase in the treatment pressure. Two negative extrema of the spectra were still observed after the treatment at 10 and 20 MPa, but disappeared with the treatment at 30 MPa. The α-helix content of myoglobin treated by microbubbling of SC-CO_2 at 35 °C and 8−30 MPa for 30 min was estimated as shown in Figure 3a. As the pressure increased from 8 to 30 MPa, the α-helix content decreased from 47% to 12%. Suzuki and Taniguchi (1972) described that the protein denaturation by pressurization only occurred above 100−300 MPa. Thus, the pressure effect below 30 MPa would not be the important factor for the conformational changes in myoglobin.

In a previous paper (Ishikawa et al., 1995a), enzyme inactivation profiles under microbubble SC-CO_2 treatment suggested that a high density of CO_2 would cause the significant decreases of enzyme activities. Figure 3b shows the effect of CO_2 density on the α-helix decomposition of myoglobin. The density of CO_2 is defined by pressure and temperature conditions. The significant decrease of the α-helix content was observed at high CO_2 density, i.e., above 0.8 g/cm^3. As CO_2 density becomes high, the rotational freedom in the CO_2 molecules decreases and hence the solvating power of CO_2 increases (Blitz et al., 1989). Under these circumstances, SC-CO_2 would modify the water−protein interaction, and then the protein conformation would be destabilized. Consequently, the disruption of hydrophobic bonds would occur and result in the breakage of hydrogen bonds in the α-helix structure.

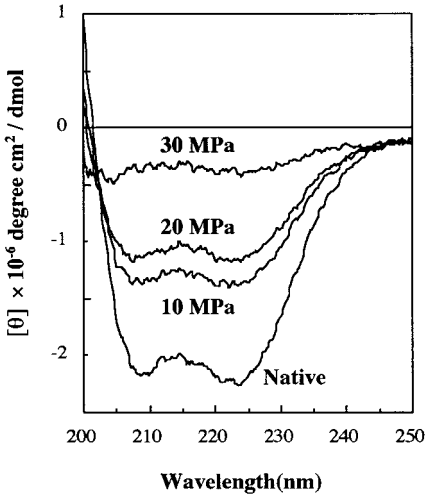

Figure 2. Effect of treatment pressure on the CD spectra of myoglobin treated by microbubbling of SC-CO_2 at 35 °C for 30 min. The CD measurement was carried out the same as in Figure 1.

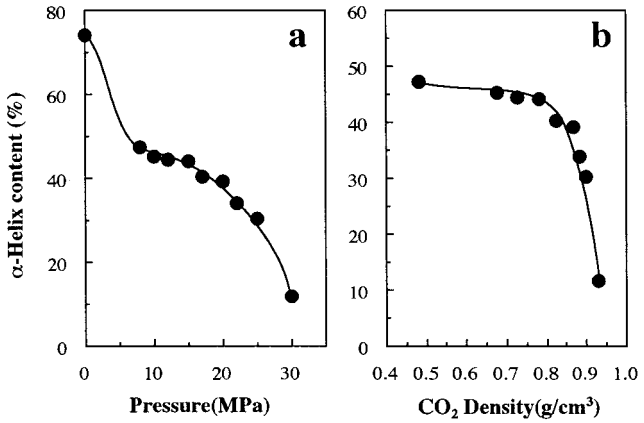

Figure 3. Effect of (a) treatment pressure and (b) CO_2 density on the α-helix content of myoglobin treated by microbubbling of SC-CO_2 at 35 °C for 30 min.

Unfolding of Myoglobin by pH Lowering. The α-helix structure would undergo a structural transition in acidic solution (Johnson, 1988). The dissolution of SC-CO_2 into an aqueous phase would produce carbonic acid, and thereby temporarily lower pH. The pH lowering effect by SC-CO_2 was reported by Balaban et al. (1991). The pH of orange juice decreased from 3.6 to 2.96 during the SC-CO_2 treatment at 31 MPa and 35 °C. The release of CO_2 after the treatment restored the pH value of the juice to its original value.

Figure 4 shows the changes of the CD spectra of myoglobin by lowering the pH from 7.0 to 2.0. Little change in the CD spectra was observed till pH 4.0. Important changes of the CD spectra were observed when the pH was adjusted to lower than 3.0. The CD spectra at pH 2.0 and 3.0 were typical spectra resulting from the random coil structure. However, the helix−coil transition was reversible and the CD spectrum was restored, showing a typical helix spectrum as in the native protein by raising the pH to 7.0. On the contrary, the changes in the CD spectra by the SC-CO_2 treatment were irreversible.

Unfolding of Myoglobin by Heating and Guanidine Hydrochloride (GuHCl). Protein unfolding is induced by heating or addition of a denaturant (urea, GuHCl, etc). Figure 5a shows the CD spectra of myoglobin heated at 60−80 °C for 30 min. Below 50 °C, any changes of the CD spectra were not observed (data not shown). The changes in the CD spectra increased with an increase in the temperature above 60 °C. The α-helix content decreased from 74% (native) to 29% (80 °C). Further changes in the CD spectra were not observed when the protein was heated at 80 °C for 120 min (data not shown). As shown in Figure 5b, the unfolding induced by heating was reversible, and the α-helix structure in myoglobin was refolded to 71% after cooling.

The CD spectra of myoglobin in GuHCl solution are shown in Figure 6. The spectra were measured at 215−250 nm because high GuHCl concentrations (>1 M) interfered with the measurement in the range of 200−215 nm. The CD spectra changed significantly with increasing concentration of GuHCl. The negative ellipticity of the CD spectrum decayed effectively in the concentration higher than 4 M GuHCl. Also at this concentration, the α-helix content of myoglobin was reduced to less than 5%. The restoration of the CD spectrum was observed after dialysis of the myoglobin overnight against 500 mL of 50 mM phosphate buffer (pH 7.0) at 5 °C. In this instance, the α-helix structure in myoglobin was refolded to 68%.

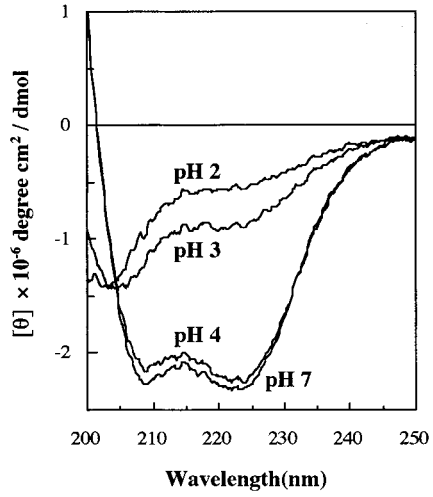

Figure 4. Effect of pH lowering on the CD spectra of myoglobin. The pH was adjusted by addition of HCl. The CD measurement was carried out at 20 °C.

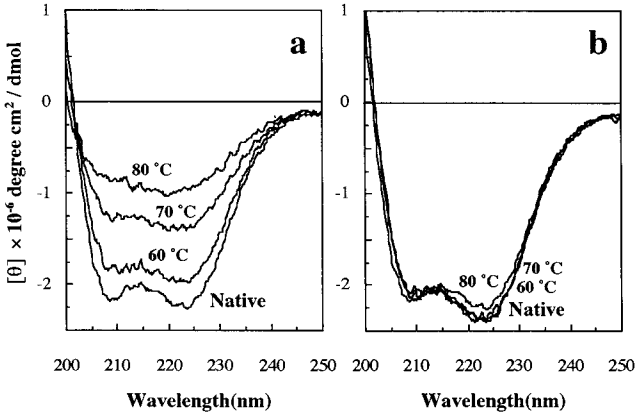

Figure 5. CD spectra of myoglobin (a) during heating and (b) after cooling. In situ CD monitoring was carried out using the cuvette with controlled temperature. The CD spectra (a) were measured at each temperature with the elapse of 30 min. The CD spectra (b) were measured at 20 °C after cooling.

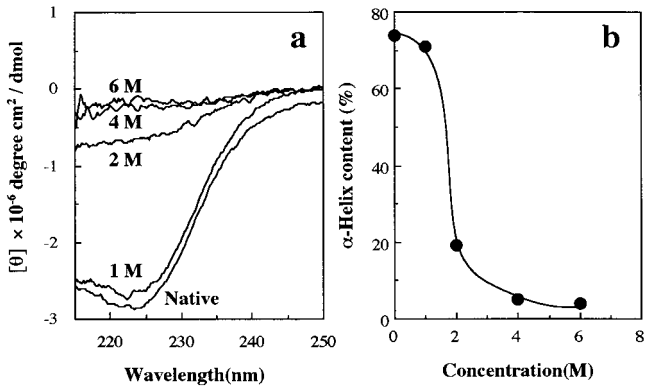

Figure 6. CD spectra of myoglobin in GuHCl solution. GuHCl was added to each protein solution at a concentration from 1 to 6 mol/L. The CD spectra were measured at 20 °C.

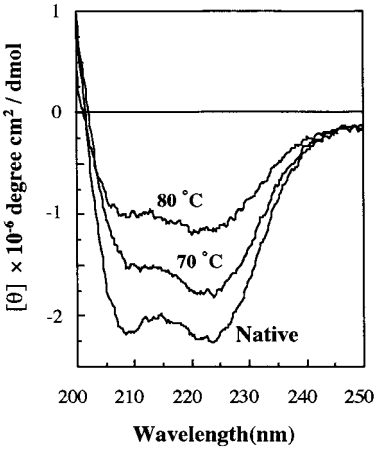

Figure 7. CD spectra of myoglobin by heating during CO_2 bubbling under atmospheric pressure. The heating temperature was set at 70 and 80 °C. Both CD spectra were measured at 20 °C.

Irreversible Unfolding of Myoglobin by Heating with Bubbling of CO_2. Protein unfolding was readily induced by pH lowering, heating, and the addition of a denaturant as described above. It is known that irreversible unfolding would occur as in the protein aggregates as a consequence of heating (Dalgleish, 1990) or high-pressure treatment (Dumay et al.,1994). In the present experiments, protein aggregation, which would be related to the intermolecular interaction, did not occur because of its low concentration (50 μg/mL). We considered that adsorption of CO_2 onto the protein molecules being unfolded would prevent the molecules from refolding. Adsorption of gaseous CO_2 onto lysozyme and egg albumin was described by Mitsuda et al. (1977). They suggested that the CO_2 binding sites in a protein molecule would be ϵ-amino, α-amino, and guanidium groups. Adsorption of CO_2 by proteins in the supercritical region was investigated by a gravimetric method under supercritical conditions (Nakamura et al., 1991). They reported that CO_2 was adsorbed onto proteins at about 10 wt % at 313 K and 7.4 MPa. Weder et al. (1992) investigated the binding of SC-CO_2 to arginine residues.

Figure 7 shows the CD spectrum of myoglobin cooled, which had been heated at 70 and 80 °C while CO_2 was bubbled at atmospheric pressure for 30 min. The CD spectra indicated that the unfolded conformational structure was retained even after the treatment. As mentioned above, the α-helix content of the myoglobin held at 80 °C was 29%; on the other hand, the α-helix content of myoglobin was restored only to 34% by CO_2 bubbling. It was suggested that CO_2 would be adsorbed onto the unfolded myoglobin molecules and disturb the refolding of the molecule.

The irreversible conformational changes in myoglobin was observed on both SC-CO_2 treatment and gaseous CO_2 bubbling. Bubbling of gaseous CO_2, however, required the heating above 70 °C for the irreversible folding. In contrast, SC-CO_2 treatment caused the irreversible unfolding of myoglobin without heating.

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