High-Resolution NMR Study of the Pressure-Induced Unfolding of Lysozyme†

Shantha D. Samarasinghe,‡ Douglas M. Campbell,‡ Ana Jonas,§ and Jiri Jonas*,‡

Department of Chemistry, School of Chemical Sciences, and Department of Biochemistry, College of Medicine, University of Illinois, Urbana, Illinois 61801

Received December 19, 1991; Revised Manuscript Received June 4, 1992

ABSTRACT: The pressure-induced reversible unfolding of lysozyme was investigated by high-resolution proton magnetic resonance spectroscopy by following the proton spectra of the following residues: His-15$^{\epsilon 1}$, Trp-28$^{\epsilon 3}$, Leu-17$^{\delta 2}$, Cys-64$^{\alpha}$, and Trp-108$^{\epsilon 3}$. The experiments were performed at pH 3.9 and 68.5 °C in the pressure range from 1 bar to 5 kbar both in the absence and presence of tri-*N*-acetylglucosamine (tri-NAG). From the pressure-induced changes of the equilibrium between the native and denaturated forms of lysozyme, the reaction volumes (ΔV) were calculated for each residue. Small but statistically significant differences in ΔV were found for residues located in different regions of the protein. For example, ΔV for the disulfide bonded Cys-64$^{\alpha}$ is smaller than the ΔV's found for the other residues. In particular, the effect of tri-NAG binding to lysozyme was a change of ΔV from -10.3 ± 0.6 cm^3/mol to -18.1 ± 1.7 cm^3/mol for the Trp-108$^{\epsilon 3}$ residue which is located close to the active site. It is important to note that the Cys-64$^{\alpha}$ residue also senses the binding of the substrate analog. The ability to detect statistically significant differences for ΔV of individual residues located in different regions of lysozyme represents the main result of these experiments.

Most studies dealing with the denaturation of proteins have been carried out at atmospheric pressure using temperature or the chemical composition of the medium as experimental variables. The interpretation of the results of such experiments is not straightforward since the change of temperature produces simultaneous changes both in volume and thermal energy, and their effects are difficult to separate. In contrast, using pressure as the experimental variable in studies of solutions of proteins allows one to perturb the environment of the protein in a continuous controlled way by changing only intermolecular distances (Weber & Drickamer, 1983).

It is well known that reversible denaturation (unfolding) of a protein in solution can be caused not only by an increase in temperature or by changes in the composition of the medium but also by application of high pressure (Zipp & Kauzmann, 1973; Weber & Drickamer, 1983). So far there have appeared only a few high-pressure studies dealing with the denaturation of proteins. In particular, Weber and co-workers (Li et al., 1976) used various fluorescence techniques to study the pressure denaturation of lysozyme and on the basis of their results proposed a plurality of pressure-denatured forms for this enzyme. Their experiments which used either intrinsic or extrinsic fluorescence probes followed the unfolding process in a nonspecific, global way, characteristic of the various experimental techniques used so far to follow pressure denaturation of proteins. In contrast, high-resolution NMR spectroscopy is a powerful technique for probing local changes in proteins. Until the present work, high-resolution NMR has not been used to follow the pressure-induced denaturation of a protein in solution. Since the process of denaturation is slow on the NMR time scale, the proton resonances from both native and denaturated states can be detected in the NMR spectrum.

Several studies reported in the literature provided the main motivation for the present work. First of all, the already mentioned high-pressure fluorescence study of lysozyme and lysozyme with bound tri-*N*-acetylglucosamine (tri-NAG) by Weber and co-workers (Li et al., 1976) represents the main reason why we have chosen to investigate lysozyme. Secondly, the pioneering study of Kundrot and Richards (1987) determined the crystal structure of hen egg white lysozyme at a hydrostatic pressure of 1 kbar and found a differential compressibility in the different regions of the protein. Third, Dobson and co-workers (Wedin et al., 1982) investigated the thermal denaturation of lysozyme using high-resolution NMR by following NMR signals for various residues located in different regions of the protein and concluded that unfolding can be represented by a cooperative, two-state process.

In this study we used high-resolution NMR to follow the effect of pressure on the equilibrium constant for the native and denatured forms of lysozyme by observing the proton resonances of the following residues: His-15$^{\epsilon 1}$, Leu-17$^{\delta 2}$, Trp-28$^{\epsilon 3}$, Cys-64$^{\alpha}$, and Trp-108$^{\epsilon 3}$. Since these residues lie in the regions for which Kundrot and Richards (1987) determined different compressibilities, we were interested to find out whether these differences are also reflected in the determined reaction volumes (ΔV) for the individual residues. Dobson and co-workers (Wedin et al., 1982) proposed that thermal denaturation of lysozyme is a cooperative two-state process since all the observed resonances belonging to different residues gave equivalent ΔH and ΔS values for the unfolding process. Therefore, the determination of ΔV's for the same residues may contribute to resolving the fundamental question of how similar or different is pressure denaturation compared to thermal denaturation. We also attempt to determine whether pressure denaturation of lysozyme is a cooperative, two-state process in view of the proposal of a plurality of pressure-denaturated forms of lysozyme made by Weber and co-workers on the basis of their high-pressure fluorescence study (Li et al., 1976). Since it has been observed that binding of tri-*N*-acetylglucosamine (tri-NAG), a substrate analog of lysozyme, stabilizes the native form of lysozyme (Segawa & Sugihara, 1984), we have also studied the pressure denaturation of lysozyme with bound tri-NAG in order to determine the effect of tri-NAG binding on the unfolding process,

† This work was supported by the National Institutes of Health under Grant No. PHS 5 RO1 GM42452-03 and the National Science Foundation Grant No. NSF CHE 90-17649.

* To whom correspondence should be addressed.

‡ Department of Chemistry.

§ Department of Biochemistry.

0006-2960/92/0431-7773$03.00/0

attempting to detect possible differences in ΔV values obtained for residues located in or close to the active site.

The pressure effects on the high-resolution proton NMR spectra of D_2O solutions of hen egg white lysozyme at pH 3.9 and a temperature of 68.5 °C were measured in the pressure range from 1 bar to 5 kbar. Analogous experiments were performed for lysozyme with bound tri-NAG. The proton residues of interest in this study are representative of different structural regions of lysozyme with sufficiently resolved proton spectra for accurate intensity measurements (Dobson et al., 1984). His-15$^{\epsilon 1}$ has well-resolved native and denatured resonances, while the other residues have resolved native resonances only. His-15$^{\epsilon 1}$, Trp-28$^{\epsilon 3}$ and Trp-108$^{\epsilon 3}$, and Leu-17$^{\delta 2}$ are in α-helix structures, and Cys-64$^{\alpha}$ is in a β-sheet structure.

EXPERIMENTAL PROCEDURES

Lysozyme was purchased from ICN Biochemicals Inc., and tri-*N*-acetylglucosamine, deuterated acetic acid, and sodium acetate were obtained from Sigma Chemical Co. The purity of the lysozyme was checked by SDS–polyacrylamide gel electrophoresis on a high-density Phast Gel (Pharmacia LKB Biotechnology Inc). The overloaded gel did not reveal any protein impurities nor fragments upon staining with Coomassie Blue stain.

Enzyme solutions of 5 mM concentration were prepared in 0.02 M deuterated acetate buffer containing the deuterated acetic acid and sodium acetate in 99.9% D_2O, adjusted to an apparent pH of 3.9 at atmospheric pressure and room temperature. Under these conditions lysozyme is quite soluble at the high concentrations required for the NMR measurements (Wedin et al., 1982). However, it is well known that the pK_a of acetate buffer decreases with pressure, so that at the highest pressures used in this study (5 kbar) the effective pH of the solutions would be from 0.5 to 1 pH units lower than 3.9 (Zipp & Kauzmann, 1973). This change may facilitate the overall denaturation of the enzyme but is not expected to affect the relative ΔV volumes measured from the proton resonances for the individual amino acid residues of lysozyme, because four of the amino acid residues, Leu-17$^{\delta 2}$, Trp-28$^{\epsilon 3}$, Cys-64$^{\alpha}$ (disulfide linked), and Trp-108$^{\epsilon 3}$, are not ionizable, and His-15$^{\epsilon 1}$ is protonated at both pH values. Furthermore, the pK_a of the histidine residue is very insensitive to pressure changes (Zipp & Kauzmann, 1973). Prior to the NMR experiments, the lysozyme solutions were heated at 80 °C for 15 min in order to facilitate deuterium exchange of labile hydrogens and to simplify the NMR proton spectrum (Wedin et al., 1982), and they were centrifuged to remove precipitated protein. The activity of lysozyme was determined before and after compression, in order to assess the extent of irreversible denaturation, if any. The enzymatic activity was determined by monitoring the decrease in absorbance at 450 nm of dispersions of lyophilized *Micrococcus lysodiekticus* (Sigma Chemical Co.), as a function of time, after the addition of lysozyme. Assays (Jolles, 1969) were performed by withdrawing 3 μL of enzyme solution and diluting to 25 mL with 0.2 M acetate buffer. A 0.1-mL aliquot of this solution was added to 2.5 mL of the substrate dispersion (0.15 mg/mL). Absorbance was recorded for 30 min on an HP 8450A diode array spectrophotometer. The kinetic results showed that no significant changes occurred in enzyme activity during the NMR experiments at high pressures.

Lysozyme solutions with tri-*N*-acetylglucosamine contained 0.015 M trisaccharide, a high enough concentration to ensure saturation of the enzyme with the ligand. Tri-*N*-acetylglucosamine is a potent competitive inhibitor of lysozyme rather than a substrate and is not expected to be hydrolyzed by the enzyme during the course of the NMR experiments (Powning & Irzykiewicz, 1966).

For the NMR experiments, the lysozyme solutions (3.5 mL) were transferred to a glass sample cell equipped with a Teflon piston, and the cell was placed into an especially designed, home-built high-pressure vessel made of a beryllium–copper alloy. The high-pressure vessel, which can be used at pressures up to 5 kbar, has been described previously (Jonas, 1987). This vessel fits into a wide bore 7.04-T Oxford superconducting magnet equipped with a GE GN-300 spectrometer system operating at 300-MHz proton frequency. The pressure is transmitted to the sample by CS_2, the pressurizing fluid, via the movable piston of the sample cell. A saddle coil made of enameled copper wire is used to transmit the rf signal. The pressure was measured with a Heise pressure gauge using a pressure setup which has been described elsewhere (Jonas, 1978). The temperature was controlled by circulating ethylene glycol/water through a copper tube wound around the pressure vessel and monitored via a copper/constantan thermocouple contained inside the vessel. The temperature was maintained at 68.5 ± 0.9 °C. The temperature of 68.5 °C and pH of 3.9 were selected to "poise" lysozyme near its main folding-unfolding transition (Wedin et al., 1982; Weber & Drickamer, 1983) and to allow significant pressure denaturation to occur by 5 kbar. The spectra were measured using one pulse technique. The sweep width was 5000 Hz; the 90° pulse length was 60 μs, and the number of acquisitions per spectrum was 200. The delay between the pulses was 10 s. The proton residues involved in this study are fully relaxed in less than 10 s since the highest spin lattice relaxation time observed for His-15$^{\epsilon 1}$ residue is 1.61 s (Dobson & Evans (1984). The FIDs were Fourier transformed, base line corrected, and phased for intensity measurements. At each pressure, the sample was equilibrated at least 30 min prior to measurements. In the analysis of spectra, the integrated areas under the peaks were measured by using the standard GEM software provided by General Electric. The total area under native and denatured His-15$^{\epsilon 1}$ peaks, which was taken as 100%, remains constant throughout the experiment. Since only the native peaks are well resolved for Leu-17$^{\delta 2}$, Trp-28$^{\epsilon 3}$, Cys-64$^{\alpha}$, and Trp-108$^{\epsilon 3}$, the concentration of the denatured form was determined by subtracting the area under the native peak from 100%. These measurements were proven to be very accurate, e.g., the area of the native peak for Trp-28$^{\epsilon 3}$ was 85.5 ± 3.8 at 1 bar and 51.9 ± 5.2 at 5 kbar, and the larger error at high pressures due to broadening was also indicated in Figure 2 by error bars.

The schematic structure of lysozyme was generated on the IRIS data station using standard software program QUANTA and the X-ray structure from the Brookhaven data base.

RESULTS

Figure 1 shows the ^{1}H NMR spectrum of lysozyme in the region from 10 to 5 ppm obtained at 68.5 °C and 3.0 kbar pressure. The spectrum resembles the spectra of native and partially denatured lysozyme obtained by Dobson and Evans (1984) at 68 and 77 °C and atmospheric pressure and illustrates the degree of resolution attainable with the pressure vessel. All chemical shifts are given in parts per million relative to the HDO peak. No internal standard was used as the accurate determination of the chemical shifts was not required. The resonance corresponding to His-15$^{\epsilon 1}$ is clearly observed

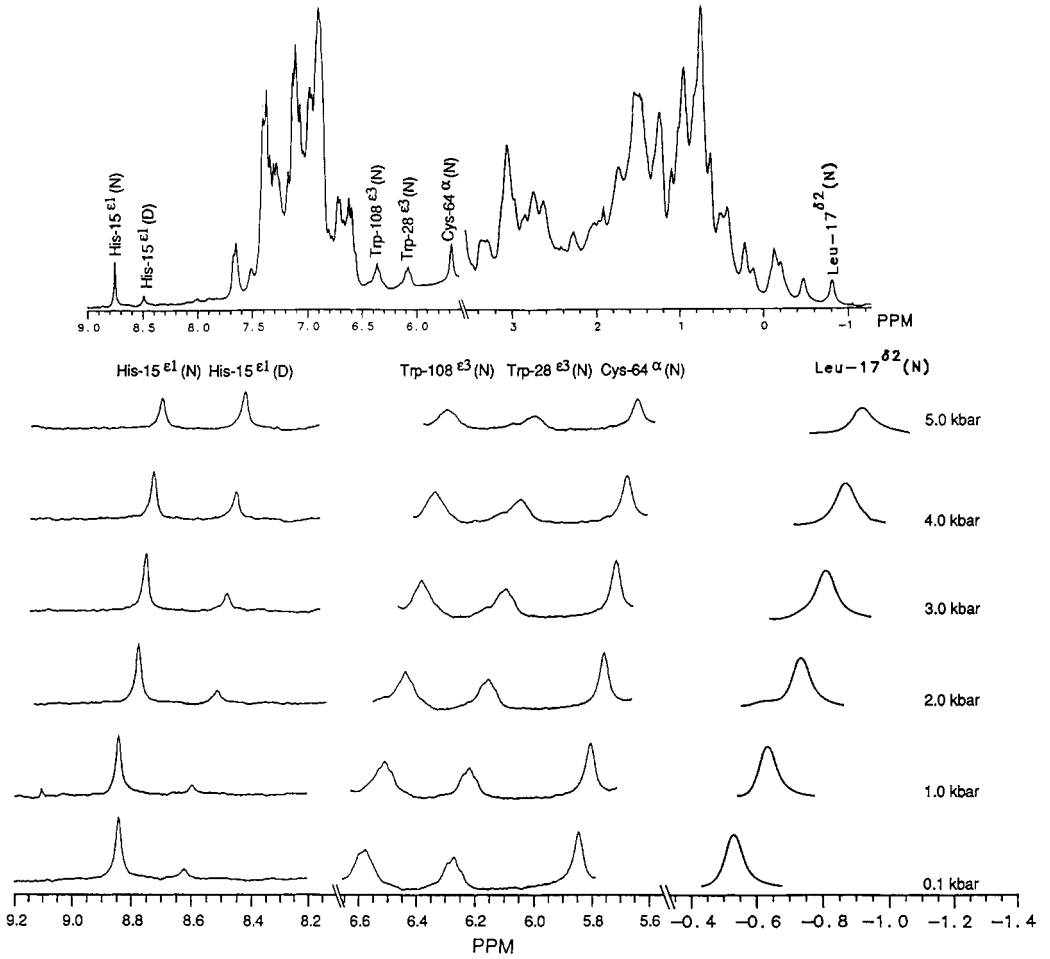

FIGURE 1: High-resolution proton NMR spectrum of lysozyme at pH 3.9 and 68.5 °C at 3.0 kbar. The pressure effects on NMR spectra of residues studied are also shown in the pressure range from 0.1 to 5 kbar.

at 8.75 ppm, the resonance for His-15$^{\epsilon 1}$ in the denatured state is at 8.47 ppm, and the resonances for Trp-108$^{\epsilon 3}$, Trp-28$^{\epsilon 3}$, and Cys-64$^{\alpha}$ in the native state are resolved at 6.38, 6.09, and 5.70 ppm, respectively, at 3 kbar. The native form resonance for Leu-17$^{\delta 2}$ was resolved at –0.80 ppm. These assignments are based on the work of Poulsen et al. (1980), Delepierre et al. (1982), and Redfield et al. (1982). Under native conditions, the integrated areas under the above resolved peaks are identical and represent the rf absorption of a single proton. The small spurious shoulder near the Trp-28$^{\epsilon 3}$ resonance appeared only in two of our seven independent experiments; however, we included these experiments in the calculations which follow. Any error introduced due to the spurious peak was included in the standard deviation indicated as error bars for each pressure. With increasing pressures, the overall spectrum approaches the characteristics of the spectrum for thermally denatured lysozyme shown by Dobson and Evans (1984), with the resolved native peaks decreasing in intensity. For the residues of interest, only the resonance of denatured His-15$^{\epsilon 1}$ is resolved and increases with pressure. The percentage of the denatured and native state for the His-15$^{\epsilon 1}$ residue was obtained from the integrated areas under the two peaks. For all the other residues, the percent of the native form was calculated from the integrated area under the respective native peak by reference to the area under both His-15$^{\epsilon 1}$ peaks taken as 100% (McDonald et al., 1971).

The calculated percentages of the denatured form for each residue are shown in Figure 2 as a function of increasing pressure. For each residue, the results are given in the absence and in the presence on tri-*N*-acetylglucosamine binding to lysozyme. Each data point is the mean from seven independent experiments including the standard deviations in the form of error bars. Inspection of these results indicates that the maximum degree of denaturation attainable under the present experimental conditions is ~53%. The major differences, which are statistically significant because they exceed two standard deviations from the mean, are observed for the Cys-64$^{\alpha}$ and Trp-108$^{\epsilon 3}$ residues, in the presence and absence of tri-*N*-acetylglucosamine. The Cys-64$^{\alpha}$ in the absence of substrate analog appears to be somewhat more resistant to pressure denaturation than the other residues but with added tri-*N*-acetylglucosamine becomes more easily denatured at high pressures. Binding of tri-*N*-acetylglucosamine, on the other hand, increases the proportion of the native form of Trp-108$^{\epsilon 3}$ at low pressures.

The data given in Figure 2 can be plotted in linear form as ln K_{eq} versus pressure (Figure 3). The equilibrium constant for denaturation (K_{eq}) is calculated from the primary data as follows:

$$K_{eq} = \%\ \text{denatured form}/\%\ \text{native form}$$

The slopes of the lines are related to ΔV, the reaction volume, by (Weber & Drickamer, 1983)

$$p\Delta V = RT \ln K_{eq}$$

where p is pressure, R is the gas constant, and T is temperature in degrees Kelvin. Figure 3 shows representative linear plots for His-15$^{\epsilon 1}$, Cys-64$^{\alpha}$, and Trp-108$^{\epsilon 3}$ residues. From linear regression analysis we obtain y intercepts that give the K_{eq} values at atmospheric pressure. In the absence of tri-

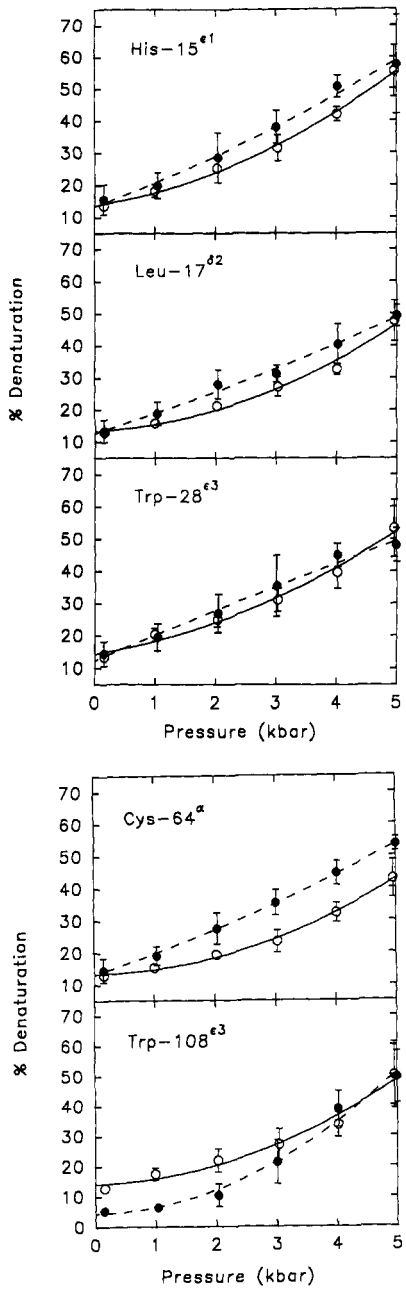

FIGURE 2: Calculated percentages of the denaturated form for each residue studied in lysozyme at pH 3.9 and 68.5 °C as a function of pressure. Full symbols describe lysozyme in the presence of tri-NAG, and open symbols denote lysozyme in the absence of tri-NAG. The experimental points represent the average of seven independent measurements. The larger error bars for high pressures reflect the effect of pressure broadening of the NMR peaks.

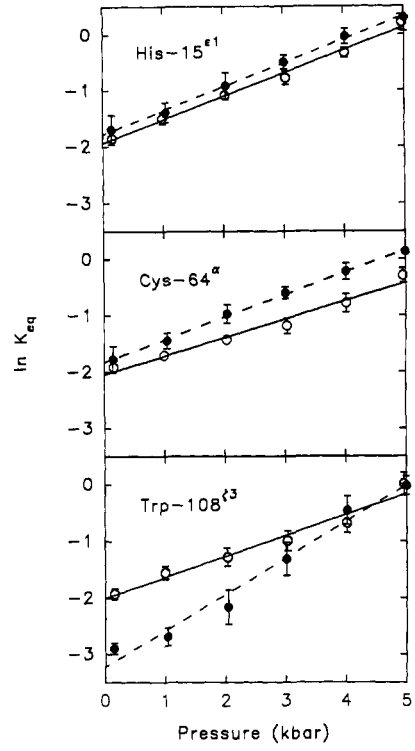

FIGURE 3: Plots of ln K_{eq} against pressure at 68.5 °C and pH 3.9 for the His-15$^{\epsilon 1}$, Cys-64$^{\alpha}$, and Trp-108$^{\epsilon 3}$ residues of lysozyme. Full symbols denote lysozyme in the presence of tri-NAG, and open symbols denote lysozyme in the absence of bound tri-NAG.

Table I: ΔV Values Calculated from the Pressure Dependence of the ln K_{eq} for Five Amino Acid Residues of Lysozyme, in the Presence and Absence of Tri-*N*-acetylglucosamine (tri-NAG)

residue	ΔV (cm^3/mol) without tri-NAG	ΔV (cm^3/mol) with tri-NAG
His-15$^{\epsilon 1}$	−11.8 ± 0.5[a]	−12.0 ± 0.3
Leu-17$^{\delta 2}$	−10.2 ± 0.5	−10.6 ± 0.9
Trp-28$^{\epsilon 3}$	−10.8 ■ 0.7	−10.4 ± 0.5
Cys-64$^{\alpha}$	−9.3 ± 0.7	−10.9 ± 0.4
Trp-108$^{\epsilon 3}$	−10.3 ■ 0.6	−18.1 ± 1.7

[a] The ΔV values are given as the mean ± standard deviation from seven independent experiments.

N-acetylglucosamine, the average K_{eq} for the three residues is 0.139, in good agreement with the value of 0.108 at 69 °C determined by Wedin et al. (1982).

In the presence of the substrate analog the K_{eq} at atmospheric pressure for the Trp-108$^{\epsilon 3}$ residue is 0.037, a substantially lower value that indicates stabilization of this residue in the native form.

Table I summarizes the means and standard deviations for the ΔV values calculated from the slopes of the ln K_{eq} versus pressure plots, for all five residues. In the absence of tri-*N*-acetylglucosamine, the ΔV values range from −9.3 ± 0.7 to −11.8 ± 0.5 cm^3/mol. Differences between the mean values that exceed the standard deviations can be considered statistically significant; therefore, the ΔV values for the three nonpolar residues, Leu-17$^{\delta 2}$, Trp-28$^{\epsilon 3}$, and Trp-108$^{\epsilon 3}$, are essentially equal, while those for His-15$^{\epsilon 1}$ and Cys-64$^{\alpha}$ are smaller and larger, respectively. In the presence of tri-*N*-acetylglucosamine, Leu-17$^{\delta 2}$, His-15$^{\epsilon 1}$, and Trp-28$^{\epsilon 3}$ have the same ΔV values as in the absence of the substrate analog, whereas Cys-64$^{\alpha}$ and Trp-108$^{\epsilon 3}$ have more negative ΔV values. In fact, the ΔV for Trp-108$^{\epsilon 3}$ decreases dramatically from −10.3 ± 0.6 to −18.1 ± 1.7 cm^3/mol.

DISCUSSION

From the experimental results obtained, it is clear that the pressure-induced denaturation begins simultaneously for the residues located in the different regions of lysozyme. In order to discuss the ΔV values for the individual residues, it is important to show the 3-D structure of lysozyme in Figure 4 and indicate the specific locations of the residues studied. At this point, a brief summary of the main results of the X-ray study of lysozyme by Kundrot and Richards (1987) will help in understanding the subtle differences in the ΔV values obtained for the various residues of lysozyme in the absence of tri-NAG. By analyzing the X-ray data obtained for lysozyme at 1 bar and 1 kbar, Kundrot and Richards found differential compressibilities for the different regions of

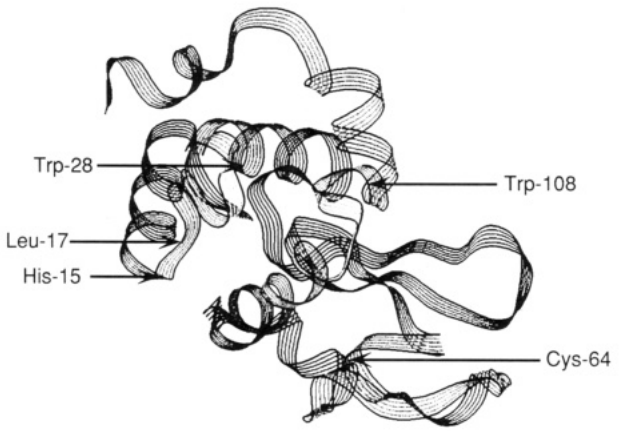

FIGURE 4: Schematic ribbon 3-D structure of lysozyme indicating the specific locations of residues studied (see the text for details).

lysozyme. Domain 1, which contains residues 1–39 and residues 89–129, exhibited a compressibility of 5.7×10^{-3} kbar^{-1} while domain 2, including residues 40–88, was essentially incompressible. The interdomain region was also compressible. We note that only one residue, Cys-64$^{\alpha}$, studied in the present work is located in the incompressible domain 2.

In the absence of tri-NAG, the ΔV values are essentially identical at $-10.2 \sim 10.8$ cm^3/mol for the three nonpolar residues Leu-17$^{\delta 2}$, Trp-28$^{\epsilon 3}$, and Trp-108$^{\epsilon 3}$. Interestingly, ΔV for Cys-64$^{\alpha}$ is somewhat smaller at -9.3 ± 0.7 cm^3/mol than for the other residues, probably because Cys-64$^{\alpha}$ is located in the incompressible domain 2. In addition, our results indicate (see Figure 2) that this residue is more resistant to pressure denaturation than the other residues as at 5 kbar the concentration of its denatured form is ~42% whereas for all the other residues of the denatured form is ~53%. One possible origin of this resistance to denaturation may be related to the fact that Cys-64$^{\alpha}$ is a disulfide linked residue in the loop region of lysozyme. We shall comment further on the significance of this difference in the pressure response of this residue when considering the results obtained for lysozyme with bound tri-NAG and its direct relevance to the conclusions by Weber and co-workers (Li et al., 1976) in their high-pressure fluorescence study of lysozyme in the absence and presence of tri-NAG. His-15$^{\epsilon 1}$ is the only charged residue that is analyzed in the present work, and it may well be that in the native state it is partially protected from D_2O while in the denatured state electrostriction could lead to a larger decrease in volume when compared to the uncharged residues.

In their experiments on lysozyme using fluorescence techniques Weber and co-workers (Li et al., 1976) obtained a $\Delta V = -19.7$ cm^3/mol which is more negative than our average value of -10.5 cm^3/mol. However, this difference is not surprising as our experiments were carried out at pH 3.9 and 68.5 °C, whereas the fluorescence study (Li et al., 1976) was performed at pH 7.6 and a temperature of 23 °C. According to Zipp and Kauzmann (1973) the ΔV must become less negative with increasing temperature.

In the context of the general question of possible similarities or differences between thermal denaturation and pressure denaturation, it is appropriate to mention the NMR results obtained by Dobson and co-workers (Wedin et al., 1982) at atmospheric pressure who determined the equilibrium constant for the denaturation of lysozyme over the temperature range 70–78 °C at pH 3.8 using the residues His-15$^{\epsilon 1}$, Leu-17$^{\delta 2}$, Trp-28$^{\epsilon 3}$, and Trp-108$^{\epsilon 3}$. The fact that a linear temperature dependence was obtained for all the resonances indicates that thermal denaturation can be adequately described by a two-state model. Therefore, our finding that statistically significant though small differences in ΔV values do exist for the same residues investigated suggests that pressure denaturation resulting from changes in intermolecular distances may provide novel information about the details of the folding/unfolding process.

In the presence of tri-NAG, the ΔV values are essentially unchanged for the His-15$^{\epsilon 1}$, Leu-17$^{\delta 2}$, and Trp-28$^{\epsilon 3}$ residues, but we find a more negative value for Cys-64 and much more negative value for Trp-108$^{\epsilon 3}$. Both these residues are near the active site and can be affected by the binding of the substrate analog. It is well known that tri-NAG binds to lysozyme in a cleft at the surface of the enzyme and occupies about half of the cleft. The hydrogen bonds between the tri-NAG and lysozyme are as follows: sugar residue A is hydrogen bonded to Asp-101; residue B also to Asp-101; the sugar residue C is hydrogen bonded to Trp-62 and Trp-63 and to the main chain at residues 59 and 107.

Our results show that for Trp-108$^{\epsilon 3}$ the equilibrium is shifted toward the native state (see Figure 2) when tri-NAG is bound, as expected from the stabilization of lysozyme by bound tri-NAG toward unfolding (Segawa & Sugihawa, 1984). Interestingly, the other more remote regions of lysozyme are not affected as revealed by the His-15$^{\epsilon 1}$, Leu-17$^{\delta 2}$, and Trp-28$^{\epsilon 3}$ behavior. With increasing pressure, the equilibrium between the native and denaturated forms of lysozyme, as reflected by the Trp–108 residue, approaches that found for the other residues. It is important to point out that binding of tri-NAG changes the ΔV for Trp-108$^{\epsilon 3}$ from -10.3 ± 0.6 to -18.1 ± 1.7 cm^3/mol. This large change in ΔV is most likely related to the fact that tri-NAG occupies about half of the active site and produces a significant free volume (Weber, 1990) in the immediate vicinity of the Trp-108$^{\epsilon 3}$ residue. It is important to note that Cys-64$^{\alpha}$ also senses the binding of the substrate analog, but in this case denaturation is favored at high pressure. This finding has a direct relevance to the results obtained by Weber and his co-workers (Li et al., 1976), who observed two distinct regions of fluorescence change in lysozyme which suggested the existence of more than one pressure-denatured forms of lysozyme. However, upon addition of tri-NAG to lysozyme, the two-step pressure change in fluorescence was replaced by a smooth single step. In spite of the fact that the pressure range in their study was 11 kbar, and different experimental conditions of pH and temperature were used, nevertheless, we see a connection between our results to their findings. As we already mentioned, the Cys-64$^{\alpha}$ residue shows only ~42% denaturation at 5 kbar in contrast to ~53% found for the other residues. After tri-NAG was added, the denaturation reflected by the Cys-64$^{\alpha}$ residue is favored at high pressure (see Figure 2), and the Cys-64$^{\alpha}$ residue behaves the same way as the other residues. This may be related to the finding of Weber and co-workers (Li et al., 1976) that bound tri-NAG essentially removes the two-step fluorescence change with pressure for lysozyme.

Since the results for Cys-64$^{\alpha}$ were obtained in the limited pressure range up to 5 kbar, we can only conclude that the behavior of Cys-64$^{\alpha}$ in the absence and presence of tri-NAG suggests the possible existence of multistep denaturation in lysozyme and a single-step process in lysozyme with bound tri-NAG. To unequivocally prove or disprove the existence of multiple pressure-denatured forms of lysozyme can only be accomplished by performing the high-pressure NMR experiments under experimental conditions comparable to those used in the fluorescence study of Weber and co-workers (Li

et al., 1976). This will require maximum pressures of 10 kbar. The building of such a high-resolution NMR probe which will allow variable temperature measurements in the pressure range from 1 bar to 10 kbar is in progress. The planned experiments should prove highly informative about the unfolding process judging from the small but statistically significant differences in the behavior of the various residues observed in the limited pressure range of 5 kbar in the present study. In addition, the information content of pressure effects on chemical shifts of the residues studied will also be explored.

REFERENCES

Campbell, I. D., Dobson, C. M., & Williams, R. J. P. (1975) *Proc. R. Soc. Lond., A 345*, 41–59.

Delepierre, M., Dobson, C. M., & Poulsen, F. M. (1982) *Biochemistry 21*, 4756–4761.

Dobson, C. M., & Evans, P. A. (1984) *Biochemistry 23*, 4267–4270.

Dobson, C. M., Evans, P. A., & Williamson, K. L. (1984) *FEBS Lett.* 168, 331–334.

Jolles, P. (1969) *Angew. Chem., Int. Ed. Engl. 8*, 227–239.

Jonas, J. (1978) *NATO ASI Ser., Ser. C, 41*, 65–110.

Jonas, J. (1987) *NATO ASI Ser., Ser. C, 41*, 193–236.

Kundrot, C. E., & Richards, F. M. (1987) *J. Mol. Biol. 193*, 157–170.

Li, T. M., Hook, J. W., III, Drickamer, H. G., & Weber, G. (1976) *Biochemistry 15*, 5572–5580.

McDonald, C. C., Phillips, W. D., & Glickson, J. D. (1971) *J. Am. Chem. Soc. 93*, 235–246.

Poulsen, F. M., Hoch, J. C., & Dobson, C. M. (1980) *Biochemistry 19*, 2597–2607.

Powning, R. F., & Irzykiewicz, H. (1966) *Biochim. Biophys. Acta 124*, 218–220.

Redfield, C., & Dobson, C. M. (1988) *Biochemistry 27*, 122–136.

Redfield, C., Poulsen, F. M., & Dobson, C. M. (1982) *Eur. J. Biochem. 128*, 527–531.

Segawa, S., & Sugihara, M. (1984) *Biopolymers 23*, 2489–98.

Weber, G. (1990) in *Festschrift in honor of Professor Cao Tan-Qin, Retrospect and Prospect of Protein Research*, (Ping, L.-Z., Zian-Xian, L., & You-Shang, Z., Eds.) pp 221–225, Well Scientific, Teaneck, NJ.

Weber, G., & Drickamer, H. G. (1983) *Q. Rev. Biophys. 16*, 89–112.

Wedin, R. E., Delepierre, M., Dobson, C. M., & Poulsen, F. M. (1982) *Biochemistry 21*, 1098–1103.

Zipp, A., & Kauzmann, W. (1973) *Biochemistry 12*, 4217–4228.