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Pressure-assisted cold denaturation of hen egg white lysozyme: the influence of co-solvents probed by hydrogen exchange nuclear magnetic resonance

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Abstract

COSY proton nuclear magnetic resonance was used to measure the exchange rates of amide protons of hen egg white lysozyme (HEWL) in the pressure-assisted cold-denatured state and in the heat-denatured state. After dissolving lysozyme in deuterium oxide buffer, labile protons exchange for deuterons in such a way that exposed protons are substituted rapidly, whereas "protected" protons within structured parts of the protein are substituted slowly. The exchange rates k_{obs} were determined for HEWL under heat treatment (80ºC) and under high pressure conditions at low temperature (3.75 kbar, -13ºC). Moreover, the influence of co-solvents (sorbitol, urea) on the exchange rate was examined under pressure-assisted cold denaturation conditions, and the corresponding protection factors, *P*, were determined. The exchange kinetics upon heat treatment was found to be a two-step process with initial slow exchange followed by a fast one, showing residual protection in the slow-exchange state and *P*-factors in the random-coil-like range for the final temperature-denatured state. Addition of sorbitol (500 mM) led to an increase of *P*-factors for the pressure-assisted cold denatured state, but not for the heat-denatured state. The presence of 2 M urea resulted in a drastic decrease of the *P*-factors of the pressure-assisted cold denatured state. For both types of co-solvents, the effect they exert appears to be cooperative, i.e., no particular regions within the protein can be identified with significantly diverse changes of *P*-factors.

Key words
- Pressure-assisted cold denaturation
- Co-solvents
- Lysozyme
- Hydrogen exchange nuclear magnetic resonance

Introduction

The usually observed positive denaturation increment of heat capacity, ΔC_p, of proteins implies that the enthalpy change of protein denaturation is a temperature-dependent function (1,2). Hence, one can expect that the enthalpy of denaturation can, in principle, become zero and then even invert its sign at some low enough temperature, changing from the factor stabilizing the native protein structure to a factor destabilizing its structure. Therefore, one can imagine that protein denaturation can occur not only upon

heating but also upon cooling. In contrast to heat denaturation, cold denaturation should then proceed with a release of heat, i.e., a decrease of enthalpy. Unfortunately, for most proteins the low temperature part of the heat capacity function that is connected with cold denaturation takes place at too low temperatures to be traced to its completeness even in a supercooled solution. To be able to study the system at temperatures below 0ºC, one may apply moderate pressures which allow studies of aqueous solutions down to temperatures of about -15ºC. By this procedure, the pressure-assisted cold-denatured state of many proteins becomes accessible. The nature of the cold-unfolded state and its comparison to the heat- and pressure-induced unfolded state is still a matter of controversy, however, and only very little work has been carried out on pressure-assisted cold denaturation of proteins (3-8). According to Marques et al. (6), the reason for pressure-assisted cold denaturation lies in the inability of water molecules at the protein surface to arrange in the low density ice-like structures which are favored with decreasing temperature. With increasing pressure, the low density network is less stable, and a more dense state is favored. Hence, water molecules penetrate the protein core which then leads to an at least partial unfolding of the protein.

Nash and Jonas (8) examined the cold-denatured state of hen egg white lysozyme (HEWL) by hydrogen exchange nuclear magnetic resonance (NMR) and found that this kind of unfolding scenario is a "mild" one, leaving considerable residual structure of the protein intact. On the basis of this work, we wanted to probe the influence of different types of co-solvents (sorbitol, urea) on the pressure-assisted cold-denatured state. Many additives which preferentially bind to the surface of the protein, such as urea, tend to destabilize, whereas co-solvents, such as sugars, which are repelled from the surface, tend to stabilize the native state of the protein. Sugars like sorbitol stabilize the native conformation by the preferential hydration effect (9), raising the order of water molecules at the surface of the protein and thus shifting the transition temperature of heat-denaturation to higher values. Hence, probing the influence of co-solvents on the pressure-assisted cold-denatured state should also provide information about the role of water in this particular unfolding scenario.

A very valuable method of gaining information at the molecular level is by ^{1}H-NMR via determination of the amide proton exchange rates. The amide protons in proteins are labile, and therefore, if the protein is dissolved in heavy water, they are replaced by deuterons. The rate of exchange depends on pressure, temperature, pD, and the structure of the protein. Exposed protons are substituted fast, while protons within structured regions of the protein are protected against exchange. The substitution of amide protons for deuterons is visible in the ^{1}H-COSY spectrum as a decrease of the C_α-amide crosspeak intensity. Plotting the intensities of these crosspeaks against time and fitting the data to a single exponential function yields the observed rate constant k_{obs}. The rate constant k_{rc} to be expected for the protein in random coil formation can be calculated using literature data (10). The ratio of k_{rc} and k_{obs} is called protection factor P. This value is a measure of structure around an amide residue: high P-factors indicate high structural order, whereas P-factors close to one indicate that the space around the residue resembles a random coil kind of conformation. In the native state of proteins, protection factors are often found to be greater than 10^6. In general, persistency of structure is a stringent requirement for the observation of significant protection. For example, a protection factor of 5 corresponds, within the simplest of models, to 80% occupancy of a structured state affording full protection, rapidly interconverting with one or more states providing no protection (11).

Thus *P*-factors allow us to draw conclusions about the structure of the unfolded state and offer the opportunity to examine the influence of co-solvents on the conformation of the molecule. In the present study, *P*-factors were determined for the heat-denaturation (80ºC, pD 3.4) of HEWL in the presence and absence of 500 mM sorbitol, and for the pressure-assisted cold-denatured state (-13ºC, 3.75 kbar) without addition of co-solvents as well as in the presence of sorbitol (500 mM) and urea (2 M).

Material and Metods

HEWL, sorbitol, urea, and citric acid were purchased from Fluka, Buchs, Switzerland. HEWL was used without further purification. Maleic acid and disodium citrate were purchased from Merck, Darmstadt, Germany, and sodium maleate from Aldrich Chemical Company Inc., Milwaukee, WI, USA. For the heat-induced unfolding, 5 and 10% (w/v) HEWL solutions in 20 mM citrate buffer (pD 3.4) were prepared. The heat denaturation experiments in the presence of 500 mM sorbitol were carried out using 5% (w/v) HEWL in 20 mM citrate buffer (pD 3.4). The samples for studying pressure-assisted denaturation consisted of 5% (w/v) HEWL in 20 mM maleic acid buffer solution (pD 2.4).

For heat treatment, the HEWL solutions were added to NMR-tubes and heated in a water bath (80ºC) for a well-defined time and subsequently cooled in an ice bath. Samples for pressure-assisted cold denaturation, placed in a tube with an O-ring sealed plug, were put in a homemade, precooled pressure vessel (at -13ºC), which was previously filled with a suitable pressure medium (octane). The pressure was increased with a piston pump (HIP High Pressure Equipment Co., Frankfurt, Germany) and the samples were kept at 3.75 kbar at -13ºC. Under these conditions, HEWL appears to be in the pressure-assisted cold-denatured state (8). After the incubation time, the pressure of the vessel was slowly released, causing the sample fluid to freeze. This procedure guaranteed that the samples were under pressure-assisted, cold-denaturing conditions within minutes and that the exchange was stopped at a well-defined time.

The samples were then stored in a freezer and their 2D-^{1}H-COSY NMR-spectrum was measured at a convenient time with a Bruker 400 DRX spectrometer (Rheinstetten, Germany). The spectra consisted of 1025 x 256 data points with a spectral width of 4800 Hz and were measured with a total of 16 scans. The spectra were analyzed with the XWINNMR software from Bruker and scaled to two signals of non-exchangeable aromatic protons. The intensities of the C_α-amide correlation peaks were plotted as a function of time and fitted to a single exponential decay function, thus yielding the observed exchange rate k_{obs}. Each fit consisted of at least 5 data points. The protection factors, *P*, were calculated as the average of two sets of measurements. The difference of the averaged value and the single values was used as a measure of the error bar. k_{rc} was calculated by the method of Bai et al. (10) and the influence of the pressure was taken into account as indicated by Nash and Jonas (8). The k_{rc}-values were not corrected for the influence of sorbitol and urea because the effects of co-solvents on the exchange rates can be neglected at low pH (12,13).

Results

Heat denaturation of HEWL

Figure 1 shows a plot of the relative intensities of four representative C_α-amide crosspeaks as a function of time. The data points can be separated into three kinetic phases: first, a roughly linear, slow decrease that we interpret as the phase until the final thermal equilibrium is established, which is followed by two approximately exponential

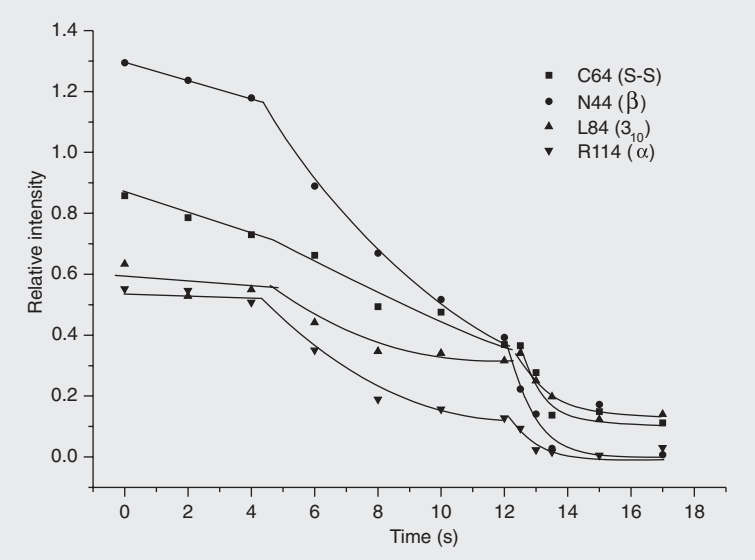

Figure 1. Cross-peak intensities of the amino acid residues C64, N44, L84, and R114 of hen egg white lysozyme as a function of time under heat-denaturing conditions (10% lysozyme, 20 mM citrate buffer, pD 3.4, at 80ºC). Residue C64 contains a disulfide bridge (S-S). N44 is part of a ß-sheet (ß), L84 and R114 are in helical regions of the protein (a 3_{10} and an α-helix, respectively). The exchange-behavior can be separated into three phases: an equilibration phase where the system reaches thermal equilibrium, a slow, transient exchange phase and a final, fast exchange phase.

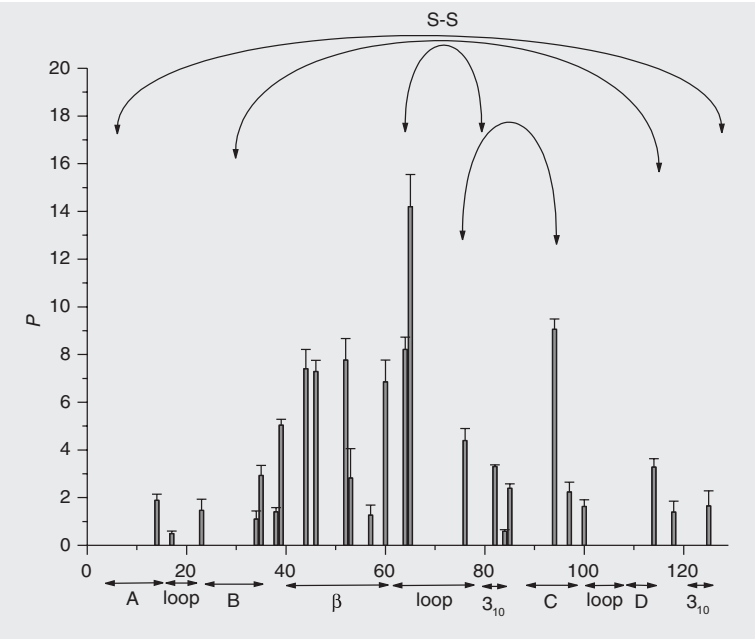

Figure 2. Protection factors, *P*, for the heat-induced transient state of hen egg white lysozyme as a function of residue number. Secondary structure elements are indicated at the bottom: "A-D" denote the four α-helices, "ß" the ß-sheet and "loop" the loop-regions. "3_{10}" denotes the 3_{10}-helices and "S-S" cross-linked cysteine residues. The transient state exhibits residual secondary structure with highest *P*-factors found at the cross-linked cysteines C64 and C94 as well as in the three-stranded ß-sheet.

decays, a slow one followed by a fast one. The resulting *P*-factors for the slow kinetic component, with maximum values in the range between 8 and 14, are shown in Figure 2. The *P*-factors for the fast kinetic component are very low, all of them being even lower than one. This is probably due to the fact that, after heating, it took some seconds to cool the sample down again. This causes rate constants that tend to be slightly too fast, and the faster the respective exchange, the bigger this source of error. Hence, for the fast exchange component we can just note that the *P*-factors are very low and in the range of random coil values, which would be in agreement with literature data (11). We attribute the slow exchange component to a transient state and the fast, final exchange component to the final unfolding of the protein. The *P*-factors of the transient state show highest *P*-values at and in the vicinity of the cross-linked cysteines C64 and C94, and notable protection is found also in the ß-sheet region between residues 41 and 60.

The exchange kinetics under heat-denaturing conditions was also determined in the presence of 500 mM sorbitol (data not shown). The addition of sorbitol decreased the quality of the 2-D spectra in the C_α-amide region, so that not as many crosspeaks could be resolved as in the latter case, and the resulting data points were just fitted to one kinetic component after thermal equilibrium was reached. Figure 3 shows a plot of the difference ΔP of the *P*-factors in the presence and absence of 500 mM sorbitol versus the sequence of the protein. Within the resolution of the experiment one can say that addition of sorbitol has little, if any, effect on the resulting *P*-factors, as can be seen from the minor changes in *P*-values.

Cold denaturation of HEWL

Figure 4 shows the *P*-factors of the pressure-assisted cold-denatured state of HEWL at -13ºC and 3.75 kbar. The values obtained

under these conditions were several times higher than those obtained under heat-induced unfolding conditions, demonstrating that this kind of denaturation is less harsh than the one induced by heat. Residues at or in the vicinity of disulfide bridges (C64, C94, C115) showed the highest protection factors, while the highest values of other residues were around 20 for both helical and ß-sheet regions. Thus, one can conclude that cross-linked cysteines are a very important factor of structural rigidity under these conditions; helices and ß-sheets do not show great differences with respect to highest *P*-factors.

Upon addition of 500 mM sorbitol, the *P*-factors increased (Figure 5). The presence of sorbitol decreased the quality of the spectra as it was the case for heat-induced denaturation, but due to the much slower exchange rate under the cold-denaturing conditions, the loss of kinetic data was not that severe. Sorbitol led to a general increase of *P*-factors, with high Δ*P*-values for residues with high *P*-factors and low Δ*P*-values for residues with low ones. The same tendency, but with the opposite sign, was observed in the presence of 2 M urea (Figure 6). Sorbitol obviously raised the structural order and rigidity of the pressure-assisted cold-denatured state, while urea had the opposite effect. Figure 7 exhibits the corresponding relative changes of *P*-values. Interestingly, both co-solvents seem to exert their influence on the structure of the protein as a whole, i.e., no distinct regions within the protein exhibiting significantly diverse changes of *P*-factors could be observed.

Discussion

Heat-induced denaturation

The comparison of the *P*-factors determined here for the heat-induced denaturation process with those reported by Buck et al. (11) shows that they are of the same magnitude, but they differ on average by about 40%. Especially the *P*-values for the ß-sheet (residue 41 to 60) differ and were of much higher value in the present study for

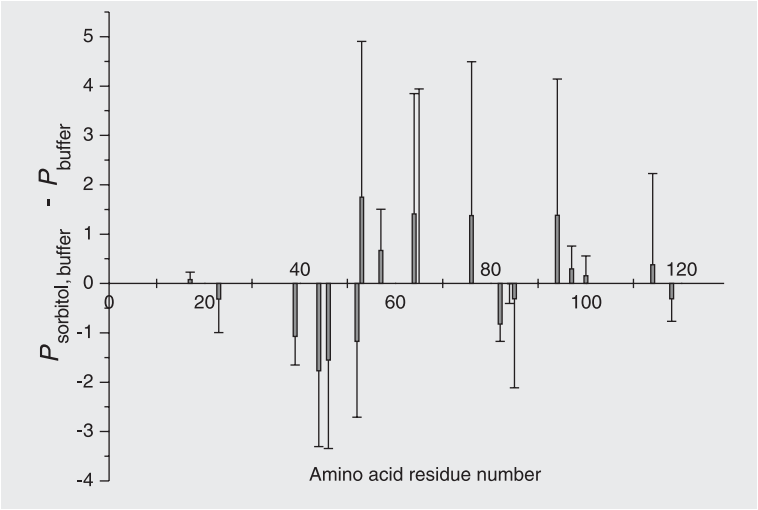

Figure 3. Difference in *P*-factors, $\Delta P = P_{sorbitol,\ buffer} - P_{buffer}$, between heat-induced denatured state in the presence and absence of 500 mM sorbitol as a function of amino acid residue number. Within the accuracy of the experiment, the addition of 500 mM sorbitol led to no significant change of *P*-factors.

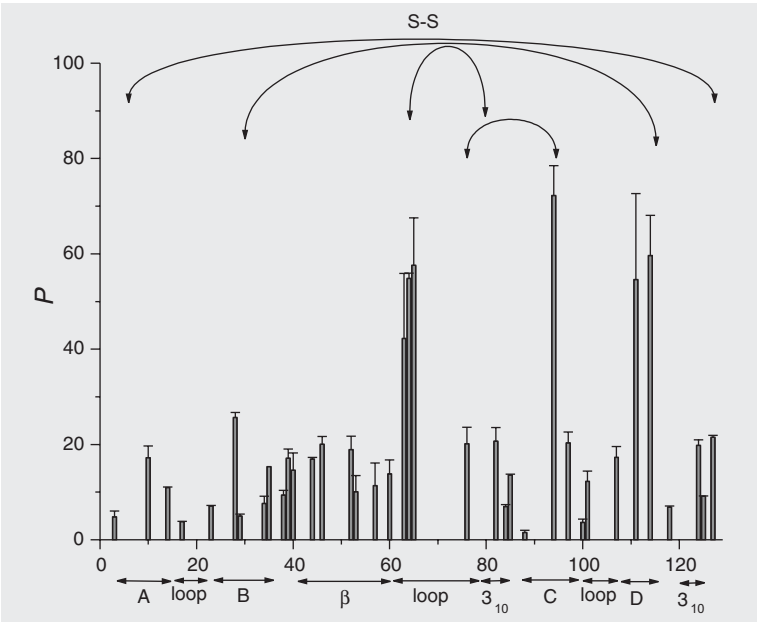

Figure 4. *P*-factors of the pressure-induced cold-denatured state of hen egg white lysozyme as a function of residue number (HEWL; 5% HEWL, 20 mM maleate buffer, pD 2.4, at -13ºC, and 3.75 kbar). Highest protection is found at or in the vicinity of the cross-linked cysteines C64, C94 and C115. On the average, the *P*-factors are about five times higher than those obtained under heat-denaturing conditions. For other explanations, see legend to Figure 2.

the residues N46 and D52, while the values found in the C-helix and in the 3^{10}-helix (residues 79 to 84) were in good agreement. Qualitatively both datasets agree; however, in both studies highest protection was observed for the cysteine residues C64 and C94 (the C115-crosspeak could not be resolved in our experiments) and all other values - except for the values in the ß-sheet mentioned above - were lower. The differences between these two data sets may be due to several reasons: first, the heat denaturation experiments cannot be carried out under ideal conditions. It takes some seconds until the sample reaches the temperature of the water bath (apparent in Figure 1 as the first kinetic component), and it takes several seconds to cool the sample down again. The resulting error is difficult to assess because it depends on the exchange rate of the individual amide proton and the exchange rate is a function of temperature. Moreover, the *P*-factors reported by Buck et al. (11) were determined at 69ºC and at pH 2.0, while the exchange rates in the present study were measured at 80ºC and pD 3.4, respectively. Although the influence of pH and temperature is taken into account by calculating the *P*-factors, it is known that HEWL is destabilized in a low pH, which could explain the higher *P*-values in the ß-sheet found by us. On the other hand, the temperature of 69ºC in the study by Buck et al. (11) was lower than the transition temperature T_m of HEWL (~70.3ºC at pD 3.4), which means that less than 50% of the molecules are unfolded under these conditions, whereas at our chosen temperature of 80ºC all HEWL molecules are unfolded in equilibrium. This might also be the reason why we found two transitions, whereas Buck et al. (11) did not observe a second component. We note that this intermediate is not visible in the differential scanning calorimeter (DSC) scan (data not shown).

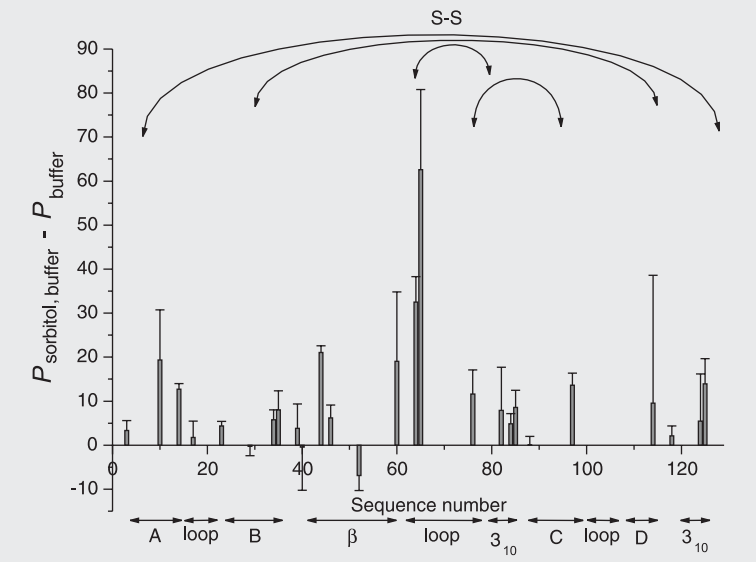

Figure 5. Differences in *P*-factors, $\Delta P = P_{sorbitol, buffer} - P_{buffer}$, upon pressure-assisted cold denaturation of hen egg white lysozyme in the presence and absence of 500 mM sorbitol. Addition of sorbitol led to a drastic increase of *P*-factors. For other explanations, see legend to Figure 2.

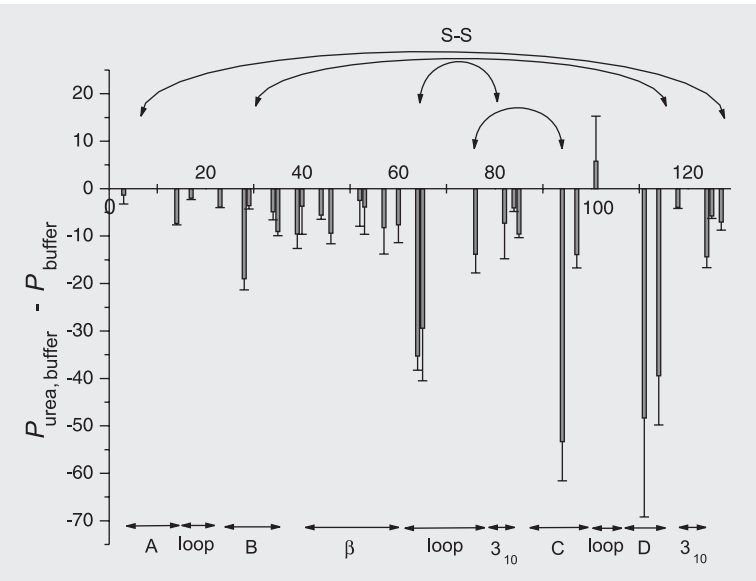

Figure 6. Differences in *P*-factors, $\Delta P = P_{urea, buffer} - P_{buffer}$, upon pressure-assisted cold denaturation of hen egg white lysozyme in the presence and absence of 2 M urea as a function of residue number. Upon addition of 2 M urea, the *P*-factors obtained decreased drastically. For other explanations, see legend to Figure 2.

We are attributing the *P*-factors of the slow kinetic component to a transient state. The following fast kinetic component can be attributed to the final, total unfolding of HEWL. In fact, it is well known that HEWL forms a kinetic intermediate upon heat dena-

turation, and an equilibrium intermediate has also been found in recent years. Van Stokkum et al. (14) found an intermediate state in their Fourier transform infrared spectroscopy experiments and attributed this to a partially unfolded state with an intact secondary structure, but collapsed or highly flexible tertiary structure. Hirai et al. (15) combined DSC and small angle X-ray scattering measurements and found that the spatial-conformational changes of the tertiary structure follow a two-state transition as visible in DSC, while close-range intramolecular changes occur earlier and follow a more complex transition. Our findings also indicate a transient state with residual structure (although we cannot judge from these experiments if it is an equilibrium one), which is followed by the more or less complete unfolding of HEWL with *P*-factors around or even lower than 1.

Our results show that sorbitol hardly affects the compactness and rigidity of the intermediate state between the native and denatured conformation. This finding confirms that heat denaturation is mainly driven by the large positive configuration entropy term ΔS_{conf}, and that hydration effects play a minor role in this unfolding scenario.

Pressure-assisted cold denaturation

The *P*-factors determined in the present study for the cold-denatured state at -13ºC and 3.75 kbar qualitatively agree with those reported by Nash and Jonas (8), with high protection found at the cross-linked cysteine C64 and in the D-helix around C115, but the absolute values found in the current study were higher for most residues and in particular the *P*-factor determined for the cross-linked cysteine C94 was of much higher value. This may partially be due to the fact that Nash and Jonas (8) used 1.5 mM 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid as chemical shift reference. We did not use it as calibrating agent, because we observed that chemicals like 3-(trimethylsilyl)-1-propanesulfonic acid have a detergent-like behavior and thus might influence cold unfolding. The overall results, however, are in good agreement, showing that pressure-assisted cold denaturation is a mild form of unfolding, leaving residual structure to a large extent intact.

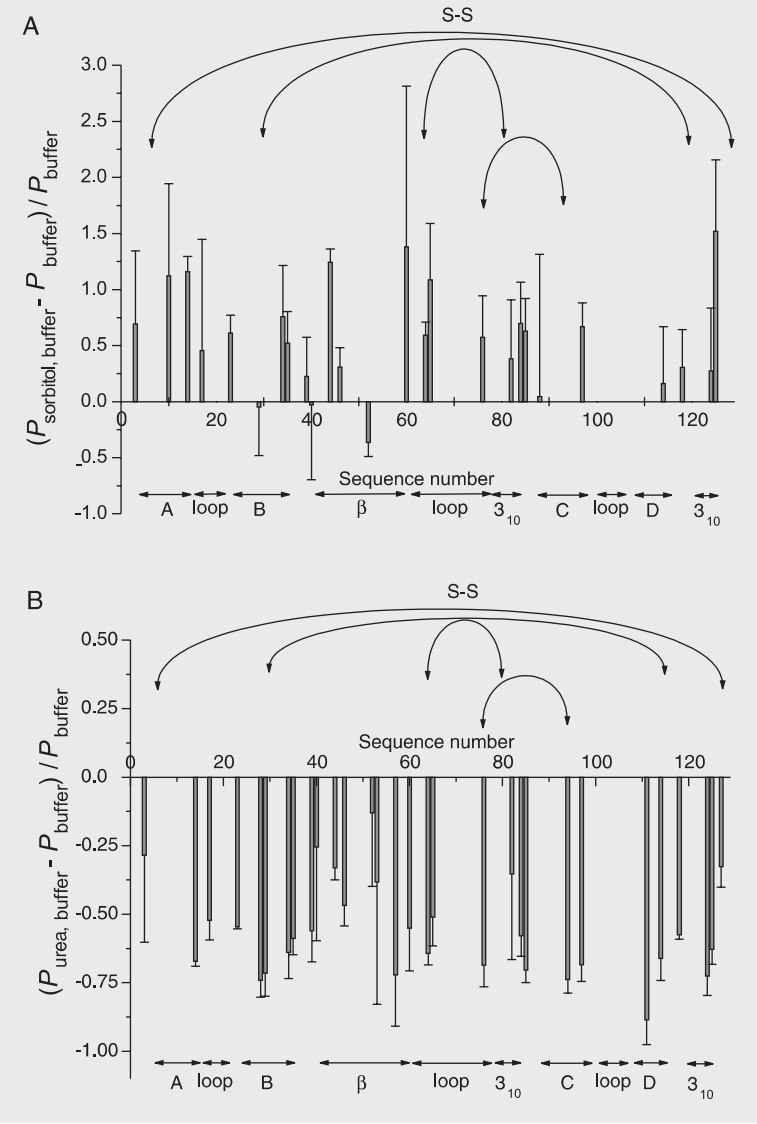

Figure 7. Relative changes in *P*-factors upon the addition of 500 mM sorbitol (A) and 2 M urea (B) for the pressure-assisted cold-denatured state. In both cases, no particular region within the protein can be identified which shows a significant diverse change of *P*-factors. Obviously, both co-solvents influence the structure of hen egg white lysozyme in the pressure-assisted cold-denatured state uniformly and cooperatively. For other explanations, see legend to Figure 2.

Our main aim was to study the influence of kosmotropic and chaotropic substances on the cold-denatured state. The models explaining the phenomenon of cold denaturation point out the particular role of water in this unfolding scenario, but data at the molecular level, which relate cold destabilization directly to hydration effects, are lacking. Co-solvents like sorbitol and urea exert their influence on protein stability essentially by the preferential hydration and binding effect, respectively, and thus might allow to draw conclusions on the role of hydration in the pressure-assisted cold-denatured state. In contrast to heat-induced denaturation, which is mainly driven by the large, positive ΔS_{conf} change, leading to a disruption of internal, non-covalent bonds, cold denaturation is driven by an increase in hydration of polar as well as hydrophobic residues (3), which leads to a swelling of the protein and a concomitant (partial) disruption of the native structure.

Our results show that the structure of the pressure-assisted cold-denatured state of HEWL, concerning regions of highest protection, is comparable to the structure of the transient state of heat-induced unfolding. Both conformations exhibit highest protection at or in the vicinity of the cross-linked cysteines C64 and C94. The pressure-assisted cold-denatured state also shows high protection in the vicinity of the cross-linked cysteine C115 in the D-helix. On an absolute scale, the *P*-factors of the pressure-assisted cold-denatured state are much higher than those of the partially heat-unfolded state, showing that the structure of the latter is much more compact and might be described by a molten globule kind of structure.

The strong influence of the co-solvents on the *P*-factors underlines the role of hydration effects in the cold unfolding scenario. Sorbitol exerts a stabilizing effect on the structure of the partially unfolded protein, leading to a higher compactness of the protein under cold-denaturing conditions. As the internal non-covalent bonds are not disrupted to the same extent as in the case of heat denaturation, the stabilizing effect by preferential hydration by sorbitol leads to a less flexible structure. Urea has, as expected, the opposite effect: preferential binding of urea to the protein causes a more open, water accessible conformation. We find that, within the accuracy of the experiments, the co-solvents exert their influence on the protein structure cooperatively, meaning that stabilizing or destabilizing the hydration of amino acid residues seemingly does not lead to changes of distinct secondary or tertiary structure elements, but to a gradual stabilization or destabilization of the whole, global structure of HEWL. This argues for a lower overall flexibility in the presence of sorbitol and a higher flexibility in the presence of urea. Hence, the conclusion that the corresponding pressure-assisted cold-denatured states are partially unfolded states does not imply that distinct regions of the protein are more or less unfolded, but rather that both co-solvents influence the structure of HEWL in the pressure-assisted cold-denatured state essentially uniformly and cooperatively.

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