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Structure of the Pressure-Assisted Cold Denatured State of Ubiquitin

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The pressure-assisted cold denatured state of ubiquitin in aqueous solution was investigated by high resolution NMR. Hydrogen exchange kinetics were measured for backbone amide protons in the cold denatured protein to determine its structure. In contrast to cold denatured ribonuclease A and lysozyme, cold denatured ubiquitin shows little persistent secondary structure. The behavior of ubiquitin supports the idea of a relationship between the residual structure of pressure-assisted cold-denatured states and the structure of early folding intermediates provided they exist.

The cold denatured state of proteins is of considerable interest, because of the complicated molecular interactions leading to it (1). Because of the difficulty in obtaining cold denaturation at temperatures above the freezing point of water, it is less well characterized than many other methods of denaturation. Previous work in this group (2,3) has used high pressure to lower the freezing point of water by as much as 20 °C, thus making cold denaturation feasible. By combining pressure-assisted cold denaturation with modern hydrogen exchange methods, the structure of a cold denatured protein can be characterized (3). In ribonuclease A (3) and lysozyme (4), extensive residual structure persists (protection factors $P \sim 10-100$), suggesting the formation of a compact unfolded state.

Protection against hydrogen exchange in cold-denatured lysozyme and RNase A is similar to that observed in early (refolding time < 10 ms) folding intermediates for these proteins (5,6), leading to the idea that the cold denatured state is structurally similar to such intermediates. To help test this idea, ubiquitin, which has markedly different folding kinetics from either RNase A or lysozyme (5), was investigated. In particular, ubiquitin shows much less evidence of early structure formation than lysozyme (5); it resembles a random coil more closely.

MATERIALS AND METHODS

Bovine red blood cell ubiquitin was obtained from Sigma Chemical and used without further purification. Ubiquitin was prepared to 2.0 mM in a buffer consisting of 20 mM citric acid and 1.5 mM TSP in D_2O, with a pH* (uncorrected meter reading) of 3.0 at ambient conditions. Conditions for pressure-assisted cold denaturation were obtained from 1D proton NMR using a previously described pressure vessel and NMR probe (7). Exchange of backbone amide protons was accomplished by simultaneously raising the pressure to 2.25 kbar and lowering the temperature to −16 °C, conditions under which ubiquitin is fully denatured. Since ubiquitin is highly stable under ambient conditions at a pH of 3.0, exchange of amide protons that are non-labile in the native state is negligible except under the extreme temperature and pressure conditions. Consequently, the time during which exchange occurs can be precisely controlled.

Quantitative hydrogen exchange data were obtained by preparing 10 samples with varying degrees of exposure to cold denaturing conditions (10 minutes to 36 hours). The degree of exchange was determined from the 2D homonuclear correlation (COSY) NMR spectrum. NMR spectra were taken on a Varian Unity 500 MHz spectrometer in the magnitude mode, consisted of 512 FIDs of 2048 points and 8 transients each, and were processed by unshifted sine bell apodization and software solvent subtraction to reduce the effects of residual HDO. Kinetic data were obtained by least squares linear fitting of $\ln (I/I_{ref})$ vs. t where I is the volume integral of the relevant amide proton crosspeak and I_{ref} is the average value of the volume integrals of crosspeaks of 3 nonexchanging protons.

To characterize the structure of ubiquitin under the extreme conditions of temperature and pressure used, established rates for chemical exchange from random coil proteins (8) had to be determined for the pressure and temperature in question. There are three contributions to the total chemical exchange rate: two represent specific acid and base catalyzed processes, and one the slight reactivity of solvent water:

$$k_{ch} = k_a[D^+] + k_b[OD^-] + k_w$$

To obtain accurate values for the reaction rate at elevated pressure and low temperature, the rate constants were corrected for temperature using established activation energies (8) and for pressure using established activation volumes (9,10). The water catalyzed rate, though often negligible (8,11), could not be ignored because of the

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significant negative activation volume for water catalysis (−9 cm^3/mol) (10). The rates were further adjusted for the change in $[D^+]$ and $[OD^-]$ with pressure (12) and temperature (9), and for the change in ionization of citrate with pressure (12). Finally, once these general temperature and pressure corrected rates were established, side chain correction factors (8) were applied to account for nearest-neighbor side chain effects on the reaction rates. The resulting random coil chemical exchange rate (k_{ch}) was then compared to the observed rate (k_{obs}) to characterize the structure of ubiquitin.

RESULTS AND DISCUSSION

Hydrogen exchange data are typically characterized by the protection factor P, given by $P = k_{ch}/k_{obs}$. Low P values ($P \sim 1$) indicate near-random-coil behavior, whereas P values of 10^6 or more can occur in native proteins. Intermediate values of P are characteristic of partially structured forms of a protein, such as molten globules and other compact unfolded states. Figure 1 shows the protection factors for cold denatured ubiquitin, as a function of residue number. Ubiquitin shows little deviation from a random coil in its hydrogen exchange kinetics, with no P values above 5 and most below 2. Such values are typical of highly denatured proteins such as the urea denatured state of lysozyme (13). Under other circumstances, however, such as the A state produced by 60% methanol solution at a pH of 2.0 (14), ubiquitin shows significantly more protection from exchange.

Previous work on cold denatured proteins (3,4) has shown that persistent residual structure, corresponding to $P \sim 100$, can occur in the cold denatured state. In the case of ribonuclease A and particularly that of lysozyme, the degree of protection and the locations in the protein that are markedly protected are similar in both the cold denatured protein and the partially folded species formed in the early stages of protein folding. For example, cold denatured lysozyme (4) shows appreciable protection ($P > 10$) in the four alpha helices comprising the α domain, and a region of the β-domain from residue 60 to 78. The remainder of the β domain, including the two β sheets, is essentially unprotected (4). This mirrors the extent of protection established for lysozyme during the dead time ($t < 3.5$ ms) of a stopped flow hydrogen exchange experiment (5). "Dead time inhibition factors", analogous to protection factors (though differing in some details) larger than 10 were confined to the helices and the region of the β domain from residues 60 to 78.

In ubiquitin, the same dead time inhibition study (5) showed no evidence for protection from exchange, with the largest dead time inhibition factors being approximately 2 (Figure 1). Moreover, there was no correlation of even these modestly elevated factors with significant elements of secondary structure, as there were in lysozyme (5). The similarity of cold denatured state protection factors in some proteins to those observed in refolding studies suggests the cold denatured state is populated by species comprising elements of secondary structure that are comparatively stable, and which occur during protein folding. In the case of ubiquitin, the folding reaction appears to proceed in one highly cooperative step (5), with little of the multiphasic behavior observed in proteins like lysozyme (5,15) or ribonuclease A (6). Although a possible intermediate for ubiquitin refolding has been characterized by other means (16), the intermediate thus identified shows no protection from hydrogen exchange. The results for the cold denatured state of ubiquitin parallel these results:

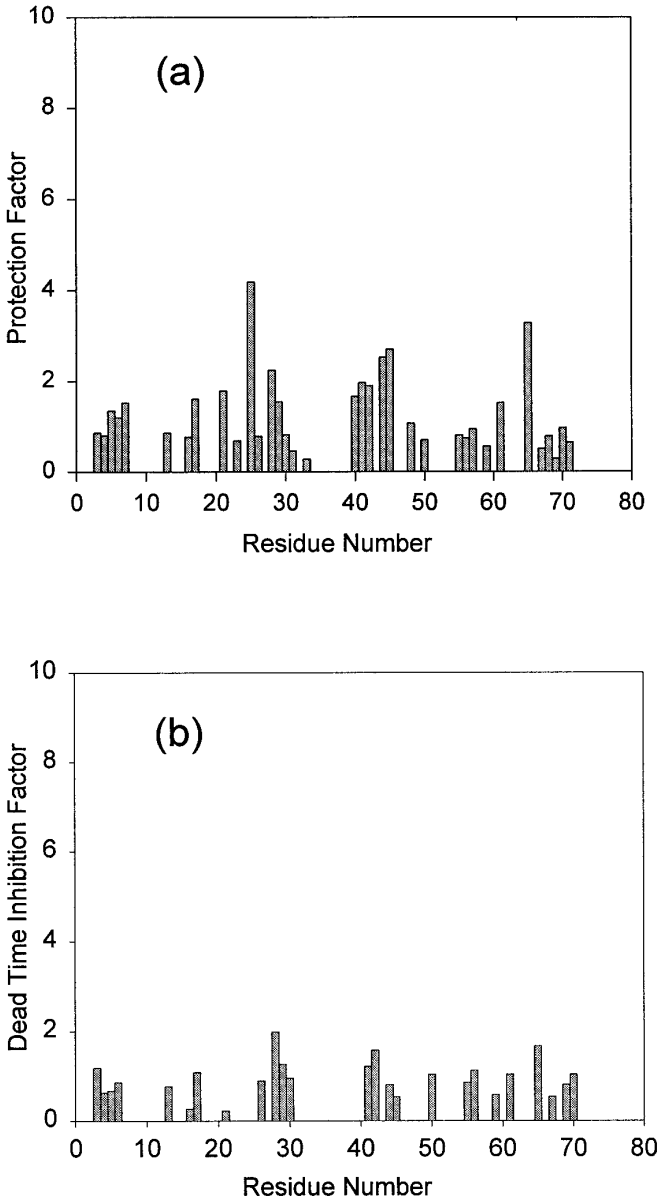

FIG. 1. (a) Protection factors for cold-denatured ubiquitin (T = −16 °C, P = 2250 bar) as a function of residue number. (b): "Dead time inhibition factors" (5) for ubiquitin during initial stages ($t < 3.5$ ms) of refolding from the urea denatured state.

there is no partially folded state that is stable enough to be characterized by hydrogen exchange methods when ubiquitin is cold denatured.

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