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Different Subdomains are Most Protected From Hydrogen Exchange in the Molten Globule and Native States of Human α-Lactalbumin

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α-Lactalbumin (α-LA) is a two-domain, calcium-binding protein that forms one of the best studied molten globules. We present here amide hydrogen exchange studies of the molten globule formed by human α-LA at pH 2 and compare these results with a similar study of the native state at pH 6.3. The most persistent structure in the molten globule is localized in the helical domain, consistent with previous results. However, the helices most protected from hydrogen exchange in the molten globule are, in the native state, less protected from exchange than other regions of the protein. The molten globule appears to contain major elements of the native fold, but formation of the fully native state requires stabilization of structure around the calcium-binding site and domain interface.

Keywords: α-lactalbumin; hydrogen exchange; molten globule; protein folding; subdomain

Molten globules are partially folded forms of proteins postulated to be general intermediates in protein folding. Classic molten globules have few, if any, fixed tertiary interactions, and are observed at equilibrium for many proteins in mildly denaturing conditions (Kuwajima, 1989; Ptitsyn, 1992; Dobson, 1994). In addition, with rare exception (O'Shea *et al*., 1993), *de novo* designed proteins have properties more reminiscent of molten globules than native proteins (for a review, see Betz *et al*., 1993). Thus, understanding what distinguishes partially folded states such as molten globules from fully folded proteins is critical for understanding protein folding and protein design.

The best characterized molten globule is that formed by α-lactalbumin (α-LA), a 123-residue, two-domain calcium-binding protein (Figure 1). α-LA adopts a molten globule conformation readily under a wide variety of conditions including low pH, reduction of disulfide bonds, and the absence of calcium and other salts (Kuwajima, 1989; Ewbank & Creighton, 1991; Yutani *et al*., 1992). Numerous studies have led to the view that the α-LA molten globule has a bipartite structure, with (1) a disordered β-sheet domain and (2) an α-helical domain containing substantial secondary structure and a native tertiary fold, even though it lacks

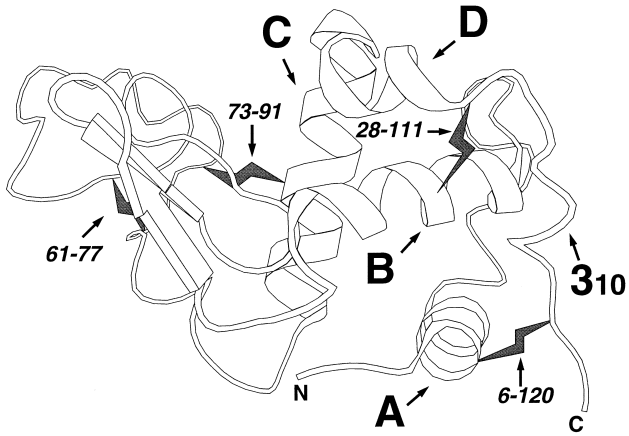

Figure 1. Schematic representation (Priestle, 1988) of human α-LA (Acharya *et al*., 1991). Helices are labeled A, B, C, D, and 3_{10}. Disulfide bonds are shown in black and are labeled by the cysteine residues that they connect.

Abbreviations used: α-LA, α-lactalbumin; HX, amide hydrogen exchange; H-^{2}H, hydrogen-deuterium; HSQC, heteronuclear single quantum coherence; NMR, nuclear magnetic resonance.

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extensive fixed tertiary interactions (Baum *et al*., 1989; Kuwajima, 1989; Alexandrescu *et al*., 1993; Peng & Kim, 1994; Xie & Freire, 1994; Wu *et al*., 1995).

It is important to understand the similarities and differences between the molten globule and a native protein. Amide-proton hydrogen exchange probes the structure and dynamics of proteins (Linderstrøm-Lang, 1955; Woodward *et al*., 1982; Englander & Kallenbach, 1983; Wagner & Wüthrich, 1986). We report here hydrogen exchange studies of both the molten globule and native states of human α-LA. For a number of proteins, a correlation has been found between the location of the amide protons most protected from solvent exchange in the native protein and the protein folding core (Woodward, 1993). For α-LA, however, we show here that the region of the native structure that is most protected from exchange in the molten globule is not the region most resistant to solvent exchange in the native protein. Our results can be attributed to the additional interactions necessary to stabilize the native state in this two-domain protein.

Exchange rates in the molten globule were measured indirectly because broad lines and poor dispersion complicate interpretation of NMR spectra of the α-LA molten globule (Baum *et al*., 1989; Alexandrescu *et al*., 1993). Hydrogen exchange was carried out for a variable time period at pH* 2, 5°C, conditions at which α-LA is a molten globule. (pH* refers to meter readings in ^2H_2O solutions using a glass pH electrode, without correction for isotope effects.) Solvent exchange was quenched by freezing and lyophilization, and the sample was analyzed subsequently under conditions where human α-LA is native (Nozaka *et al*., 1978) and gives rise to high quality ^{15}N-^1H HSQC spectra for which assignments are complete (our unpublished results). In these experiments, only amide protons that are highly protected from hydrogen exchange in the native state can be probed. Nonetheless, 34 amide protons can be assayed in the molten globule: 24 from α-helices and 10 from the β-sheet domain (Figure 2). None of the β-sheet amide protons that are assayed are significantly protected from solvent exchange in the molten globule. Protected protons

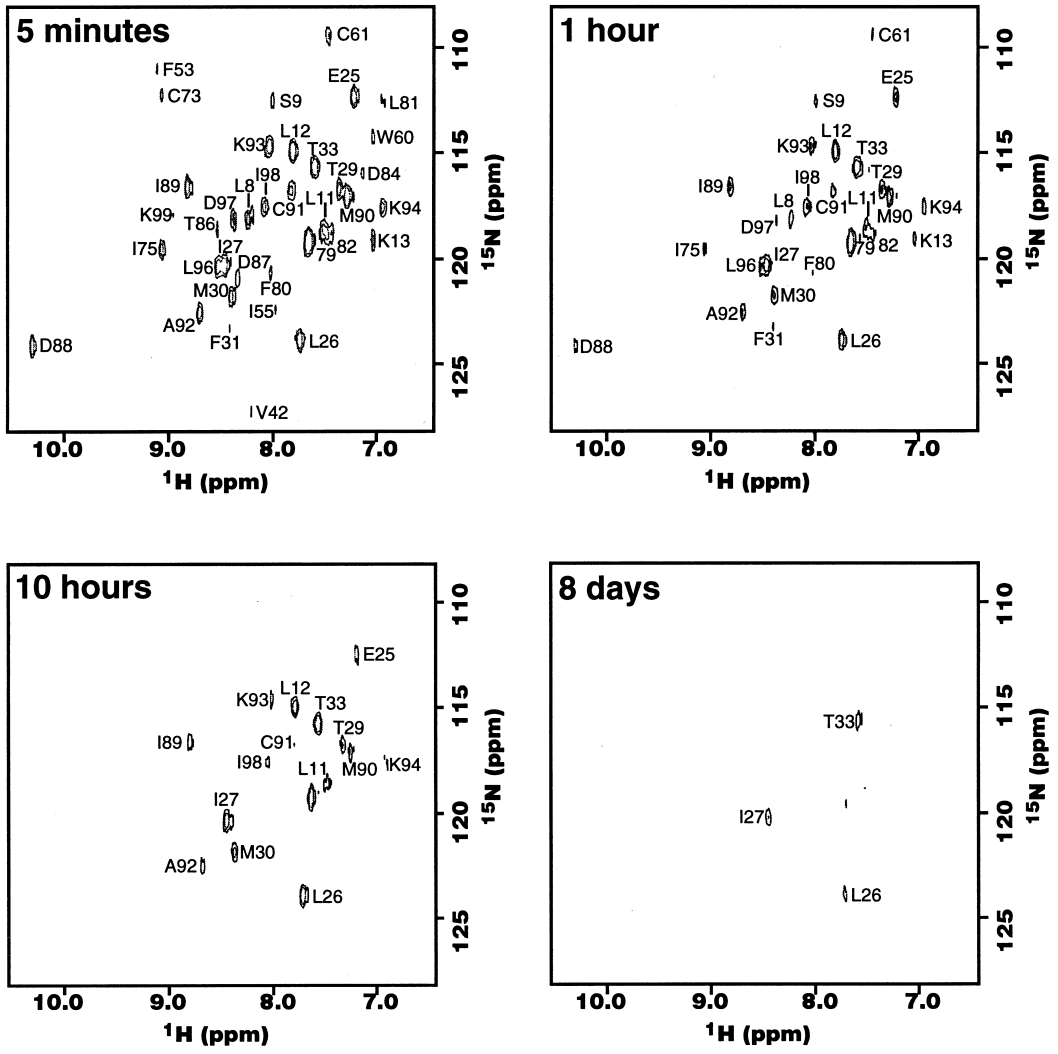
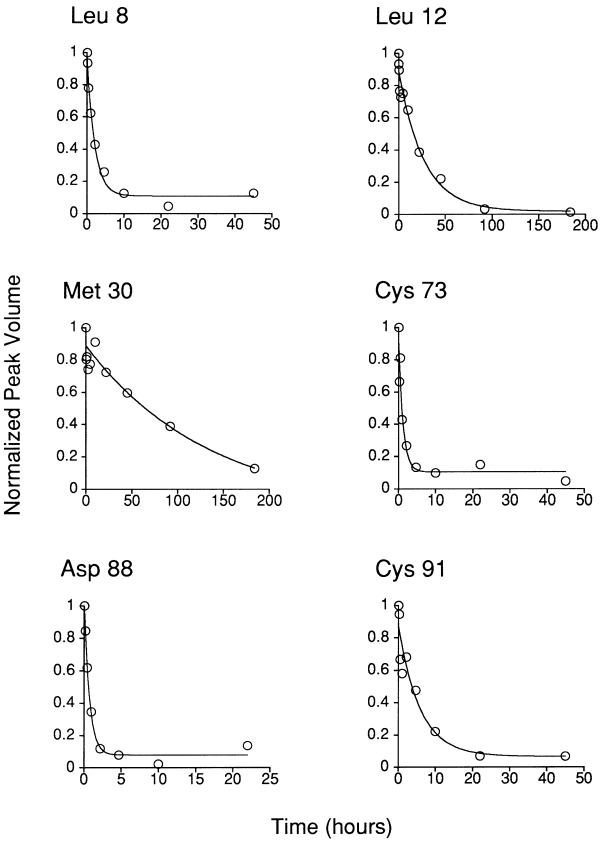


Figure 2(A) (*legend opposite*)



(B)

Figure 2. A, ^{15}N-^{1}H HSQC spectra of native α-LA after exchange in the molten globule for the periods of time indicated. (Resonance assignments, by Redfield *et al*., are forthcoming.) B, Time-course of signal decay resulting from hydrogen exchange in the molten globule for the following representative amide protons: Leu8, Leu12, Met30, Cys73, Asp88, and Cys91.

are detected within the α-helical domain, with local differences in protection factors (Table 1, Figure 3A). The B-helix contains the amide hydrogens most protected from exchange, with protection factors for four residues exceeding 100. The A-helix also persists in the molten globule, with three of its five probes protected more than tenfold, and one by a factor of 50. Only four of the 12 probes from the C-helix are protected more than tenfold, and none is protected by more than a factor of 20.

Our data agree with previous work showing that specific helices are folded in the molten globule of α-LAs from guinea pigs, cows and humans (Baum *et al*., 1989; Alexandrescu *et al*., 1993; Chyan *et al*., 1993). An additional finding in the present study is that amides in the A-helix, which were not probed in previous ^{1}H NMR studies of α-LA, are significantly protected from exchange. The protection that we observe for a number of helices strongly supports a view of the molten globule with extensive native-like secondary structure. In previous studies, analysis of the propensities of cysteine residues to form disulfide bonds provided clear evidence that the helical domain of human α-LA retains an overall native-like fold even as a molten globule (Peng & Kim, 1994; Wu *et al*., 1995). The protection from amide proton exchange that we observe for the B-helix, taken together with the favorable equilibrium constant for forming the 28-111 disulfide bond (Peng *et al*., 1995), suggest that there is a correlation between secondary and tertiary structure in α-LA, even in the absence of extensive side-chain packing interactions.

For native α-LA, amide proton exchange rates were measured directly (Table 1). Relatively similar numbers of amide protons are protected from exchange in the α-helical and the β-sheet regions. The most highly protected amide protons are located primarily around two disulfide bonds: 61-77, within the β-sheet domain; and 73-91, connecting the β-sheet domain to the long C-helix (Table 1, Figure 3B). In the helical domain, the C-helix is most protected, followed by the B and then the A-helix. The D and 3_{10}-helices exchange too quickly to be measured. It is striking that the C-helix, which is only marginally protected from exchange in the molten globule, is the most highly protected from exchange in native α-LA (Figure 4). Examination of the α-LA structure (Figure 1), however, provides an explanation for this observation. The calcium binding site is located in a loop between the N terminus of the C-helix and the β-sheet and interdomain disulfide bonds. In the molten globule state formed at low pH, calcium does not bind strongly. Loss of calcium, therefore, disrupts the domain interface and results both in loss of structure within the β-domain and in relative destabilization of the C-helix to which it is linked by many side-chain contacts and the 73-91 disulfide bond (cf. Loh *et al*., 1993).

Based on the large number of proteins that are molten globules under mildly denaturing conditions (Dobson, 1994), and the relative ease with which *de novo* design efforts yield partially folded proteins (Betz *et al*., 1993; Davidson & Sauer, 1994) it seems that the protein folding problem can be sub-divided into understanding: (1) how a protein can fold into a molten globule with a native-like tertiary fold in the absence of extensive packing interactions (Peng & Kim, 1994; Morozova *et al*., 1995; Wu *et al*., 1995) and (2) how fixed tertiary structure is formed (Handel *et al*., 1993; O'Shea *et al*., 1993; Lumb & Kim, 1995). The present results shed light on both of these issues. For α-LA, and perhaps for other proteins, the helices that can fold and still maintain dynamic flexibility form early in protein folding, without fixed tertiary packing. Importantly, studies of both α-LAs and the homologous lysozymes indicate that early kinetic folding intermediates resemble equilibrium molten globules, with structure concentrated in the helical domain (Kuwajima *et al*., 1985; Ikeguchi *et al*., 1986; Radford *et al*., 1992; Hooke *et al*., 1994; Balbach, *et al*. 1995). In order to form the fully folded

Table 1. Amide proton exchange rates and protection factors for human α-lactalbumin in the molten globule and native states

	Molten globule (pH* 2, 5°C)		Native (pH* 6.3, 3 mM Ca^{2+}, 15°C)	
Residue	k_{ex} (h^{-1})	k_{int}/k_{ex}	k_{ex} (h^{-1})	k_{int}/k_{ex} (10^{-3})
Leu8	0.43	3.5	0.080	6.8
Ser9	0.25	12	0.56	7.6
Gln10			0.29	23
Leu11	0.078	18	0.31	4.0
Leu12	0.037	50	0.026	18
Lys13	0.35	6.0	0.66	2.5
Glu25	0.14	13	0.059	8.8
Leu26	0.0054	280	0.018	31
Ile27	0.0039	220	0.0039	86
Thr29	0.076	15	0.18	41
Met30	0.010	120	0.058	78
Thr33	0.0045	520	0.071	230
Val42	0.24	1.9	0.20	1.8
Tyr50			0.13	8.7
Phe53			0.047	22
Gln54			0.086	45
Ile55	0.34	2.0	0.052	17
Ser56			0.52	7.8
Trp60	0.35	7.8	0.040	17
Cys61	3.0	0.50	0.035	230
Lys62			0.079	97
Ser63			0.097	93
Cys73	1.0	1.1	0.038	160
Ile75	1.1	0.97	0.0099	37
Cys77	2.6	1.1	0.20	100
Lys79	0.33	9.2	0.039	46
Phe80	0.59	2.8	0.023	100
Leu81			0.028	32
Asp82	1.1	1.7	0.040	23
Asp84			0.084	12
Thr86	1.7	0.32	0.59	3.9
Asp87	1.0	2.9	0.59	39
Asp88	1.4	5.2	0.025	39
Ile89	0.063	17	0.011	33
Met90	0.099	7.2	0.028	61
Cys91	0.17	14	0.035	390
Ala92	0.44	7.6	0.035	240
Lys93	0.20	14	0.014	200
Lys94	0.089	19	0.023	160
Leu96	0.94	0.72	0.0093	49
Asp97	1.2	1.5	0.037	25
Ile98	0.22	4.8	0.023	16
Lys99			0.067	23
Asp102	0.20	6.3	0.42	2.0
Trp104 (indole)			0.06	0.25

Amide proton exchange was performed on uniformly (>95%) ^{15}N-labeled recombinant α-LA. α-LA was expressed from BL21 (DE3) pLys S (Novagen) harboring the plasmid encoding human α-LA under T7 control (Studier *et al*., 1990; Peng *et al*., 1995; Wu *et al*., 1995), grown in minimal M9 media containing one gram/liter [^{15}N]ammonium sulfate (99.5% ^{15}N; Isotec; Miamisburg, Ohio), and supplemented with 0.5 mg/liter thiamine (McIntosh & Dahlquist, 1990). Inclusion bodies containing α-LA were solubilized, partially purified, oxidatively refolded and purified as described previously for a related protein (Peng & Kim, 1994). The identity of α-LA was confirmed by laser desorption mass spectrometry (Finnigan LASERMAT). All protein concentrations were determined by tryptophan, tyrosine and cystine absorbance (Edelhoch, 1967).

α-LA is monomeric under the conditions of hydrogen-deuterium exchange and NMR, as shown by equilibrium sedimentation, using Beckman 6-sector, 12 mm pathlength epoxy cells, and an An-60 Ti rotor in a Beckman XL-A 90 analytical ultracentrifuge. Samples were dialyzed exhaustively against the reference buffer (either 100 mM imidazole, pH 6.3, 3 mM $CaCl_2$ (native state) or 10 mM HCl (molten globule)). For the native state, data were collected at 15°C and 25,500 rpm for three initial concentrations (20, 75 and 150 μM). The measured molecular weight is 15.0 kDa ± 5% (95% confidence; calculated, 14.1 kDa) with random residuals. The density of the buffer was measured manually and the partial specific volume was calculated to be 0.736 using constants from Laue *et al*. (1992). For the molten globule, non-ideality was observed as a decrease in the apparent molecular weight as a function of increasing protein concentration, likely resulting from the large ratio of net charge to molecular weight at the low ionic strength of the buffer, thus requiring the use of the second virial coefficient as a fitting parameter (Williams *et al*., 1958). Therefore, data at 5°C from three wavelengths (251, 260 and 296 nm), rotor speeds (22,000, 26,000 and 30,000 rpm), and initial concentrations (20, 40 and 100 μM) were fit simultaneously using HID4000 (Johnson *et al*., 1981). 15 data sets were fit to a single molecular weight, 15 intercepts, 14 offsets and a single second virial coefficient, with no systematic variation in the residuals. The measured molecular weight is 13.9 kDa (±5%, 95% confidence) as compared to the calculated molecular weight of 14.1 kDa.

Hydrogen-deuterium exchange was initiated by dissolving lyophilized samples either in ^2H_2O, pH* 2 (molten globule) or native exchange buffer (100 mM d_4-imidazole (Cambridge Isotope Laboratories, >98% D), 3 mM $CaCl_2$ with a final pH* of 6.3). The protein concentration was 80 μM for exchange in the molten globule and 800 μM for exchange in the native state. For the molten globule, exchange was quenched by freezing in liquid nitrogen and subsequent lyophilization at regular intervals from ten seconds to nine days. Dry samples were stored at −80°C and dissolved in native exchange buffer for analysis. Rates of H-^{2}H exchange were measured from ^{15}N-^1H HSQC spectra collected at 15°C, consisting of 48 increments of 16 transients defined by 1024 complex data points, and an ω2 spectral width of 6024 Hz, an ω1 spectral width of 1130 Hz, and a recycle delay of one second (Bax *et al*., 1990; Norwood *et al*., 1990). ^{15}N decoupling during acquisition was achieved with WALTZ-16 (Shaka *et al*., 1983). The residual HOD peak was suppressed with a low power purge pulse (Messerle *et al*., 1989). For the native state, rates of H-^{2}H exchange were measured similarly to those in the molten globule, except that one spectrum was collected for each time point with 64 transients per increment and 128 increments of $t1$.

Amide proton decays were followed by measuring peak volumes in ^{15}N-^1H HSQC spectra. The volumes were normalized to peak areas of non-exchangeable resonances (corresponding to the γ2 and δ protons of Ile95, and to the α proton of Cys61) in one-dimensional ^1H spectra collected immediately prior to each set of HSQC spectra for the molten globule, or before each spectrum of the native state. The amide proton exchange rates, k_{ex}, were determined (Kaleidograph, Abelbeck software) by fitting the data to the three parameter curve, $I(t) = I(\infty) + I(0)\exp(-k_{ex} * t)$, where $I(t)$ is the intensity at time (t) after addition of deuterated solvent to the protein. Values for $I(\infty)$ were, on average, tenfold lower than $I(0)$ for the molten globule, and 10- to 30-fold lower for the native state. Predicted intrinsic rates were calculated as described by Bai *et al*. (1993) for main-chain amide protons and as described by Wüthrich (1986) for the Trp 104 indole.

conformations, however, additional interactions are necessary to lock in the unique tertiary contacts of the native structure, which involve specific interdomain contacts for both lysozymes and α-LAs (Van Dael *et al*., 1993; Ewbank & Creighton, 1993; Dobson *et al*., 1994; Pardon *et al*., 1995; L. C. Wu, Z.-y. Peng, B. A. Schulman and P. S. Kim, unpublished results). In the case of α-LAs, these contacts are stabilized by the binding of calcium, which promotes the formation of the native state (Figure 4). Interestingly, such additional specific interactions are crucial for forming native-like features in *de novo* designed proteins (Handel *et al*., 1993; O'Shea *et al*., 1993; Lumb & Kim, 1995).

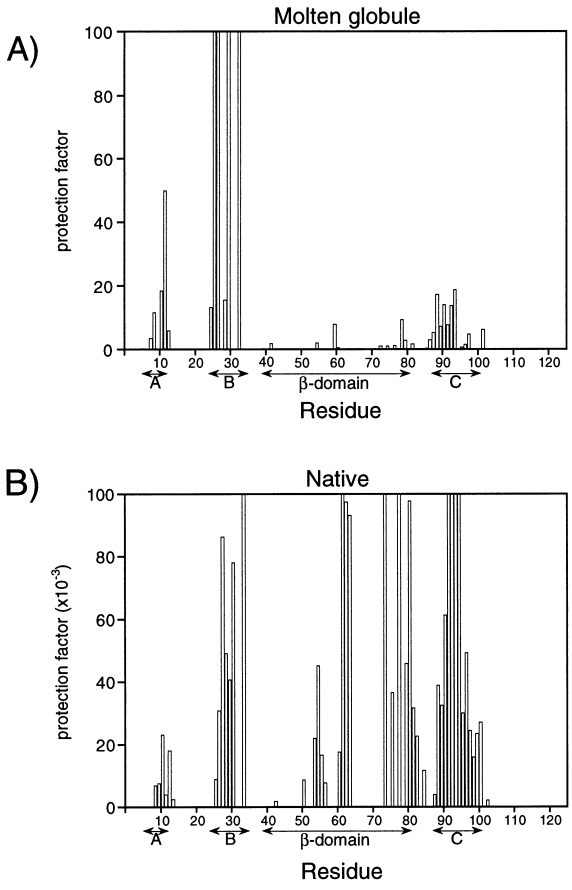


Figure 3. Histograms showing the distribution of protection factors from amide hydrogen exchange for (A) the molten globule and (B) the native state of human α-LA. The maximum protection factors shown are 100 for the molten globule and 10^5 for the native state to highlight differences.

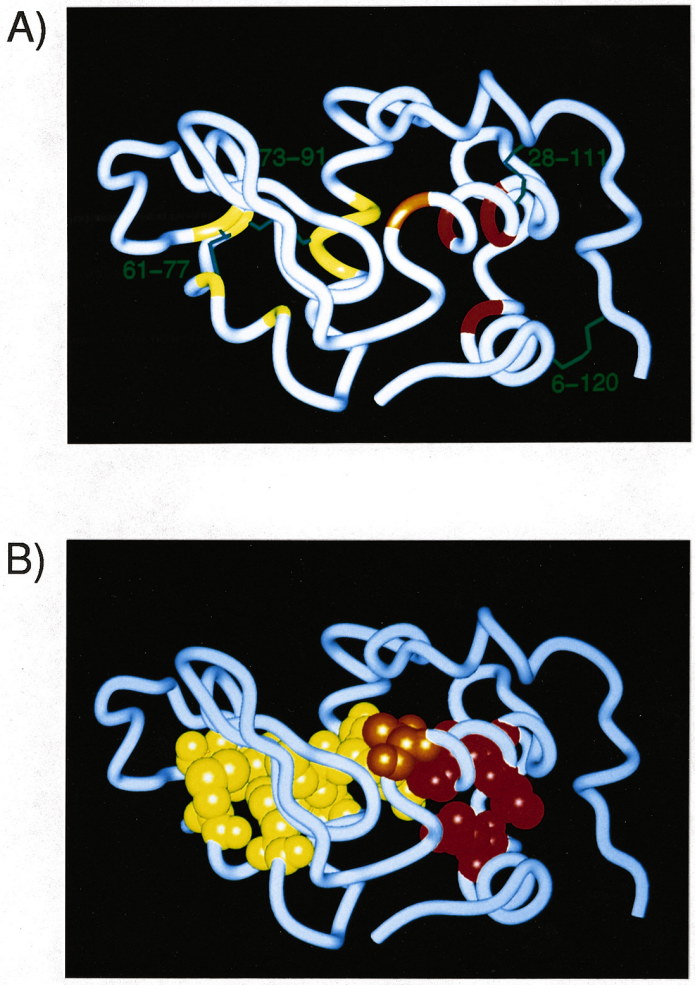


Figure 4. The most protected amide protons in the molten globule and native states are highlighted on the structure of native human α-LA (Acharya *et al*., 1991). Residues with amide protons protected by factors greater than 50 in the molten globule are shown in red, and those protected by factors greater than 10^5 in the native state are shown in yellow. Thr33, which is protected by these criteria under both conditions, is colored orange. Residues with protected amide protons are shown on a backbone drawing in A and in CPK models in B.

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