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Increased Exposure of Hydrophobic Surface in Molten Globule State of α-Lactalbumin

FLUORESCENCE AND HYDROPHOBIC PHOTOLABELING STUDIES*

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The involvement of molten globule state as a distinct intermediate in the denaturation process in proteins is well documented. However, the structural characterization of such an intermediate is far from complete. We have, using fluorescence and fluorescence quenching, studied the molten globule state of bovine α-lactalbumin. Unlike the native state, where all the 4 tryptophans are buried in the protein, 2 tryptophans are exposed in the molten globule state. Using the hydrophobic photoactivable reagent [^{3}H]diazofluorene, we observe an increased hydrophobic exposure in the molten globule state. These structural characteristics conform to the current views on the molten globule state, *i.e.* it has similar secondary structure but a poorly defined tertiary structure. Our fluorescence studies indicate the involvement of a premolten globule state in the native to molten globule state transition. This premolten globule state exists at pH 5.0 and has a very compact structure involving increased hydrophobic interactions in the protein interior. These results are also supported by circular dichroism studies.

The denaturation process in proteins has received considerable attention recently. An intermediate state (A), often referred to as the "molten globule state," has been encountered while going from the native (N) state to the unfolded denatured (U) state (Ptitsyn, 1987; Kuwajima, 1989). The molten globule state has been described as a compact intermediate protein conformation that has a secondary structure content like that of the native state but poorly defined tertiary structure. The molten globule state being transient in nature is difficult to detect. However, it has been detected under favorable circumstances. Bovine α-lactalbumin (α-LA)[1] is probably one of the best known examples wherein a stable molten globule state can be detected and studied (Ptitsyn, 1987; Kuwajima, 1989; Baum *et al.*, 1989). It is a 123-amino acid globular protein with a single bound calcium ion (Kronman, 1989). The apo-α-LA exists in similar molten globule states under conditions of low ionic strength, low pH, high temperatures, and intermediate denaturant concentrations (Ptitsyn, 1987; Kuwajima, 1989; Baum *et al.*, 1989; Kronman, 1989). Recent NMR studies on baboon α-LA indicate that the secondary structural characteristics of the native protein structure are retained in the molten globule state (Baum *et al.*, 1989). In this paper we report further characterization of the molten globule state of bovine α-LA by fluorescence, fluorescence quenching, and hydrophobic photolabeling with a new reagent, [^{3}H]diazofluorene (DAF). The results obtained here indicate increased exposure of hydrophobic surface in the molten globule state. In addition, we observe the involvement of a highly compact intermediate state, referred to as premolten globule state, at pH 5 in the native protein to molten globule state transition. The premolten globule state is characterized by minimal exposure of hydrophobic surface, quite unlike the molten globule state, which indicates an increased exposure of hydrophobic surface.

MATERIALS AND METHODS

All reagents used were of the highest purity grade available. Fluorescence measurements were done on a Shimadzu RF-540 spectrofluorometer. CD measurements were done using a Jasco-600 spectropolarimeter. The path length of the cell used was 1 mm. The results are reported in terms of molar ellipticity [θ] in units of degree·cm^2/dmol. Photolysis was carried out on a Rayonet RMR-500 miniphotochemical reactor equipped with four 3000-Å lamps. Bovine α-LA was obtained from Sigma and purified by Sephadex G-100 column chromatography (Kim and Kim, 1986). The purified α-LA was found to be homogeneous on analysis by SDS-PAGE. Electrophoresis was done using 15% SDS gel slabs by the method of Laemmli (Laemmli, 1970). Buffers were decalcified using Chelex 100 and the purified α-LA was decalcified by repeatedly passing over a tris(carboxymethyl ethylene diamine)-agarose column (Koga and Berliner, 1985). [^{3}H]DAF, of specific activity varying from 184 to 593 μCi/μmol, was prepared from [2-^{3}H]fluorenone according to the procedure reported earlier (Pradhan and Lala, 1987).

Fluorescence Studies—The concentration of α-LA used in all experiments, unless otherwise specified, was 32 μg/ml. The excitation wavelength for all fluorescence measurements was 295 nm, the excitation and emission slits were set at 5 and 10 nm, respectively. Fluorescence measurements were corrected for any dilution factor. Correction factor was also applied for the attenuation of excitation light intensity by the added acrylamide (Parker, 1968). This correction was made by multiplying the measured fluorescence intensity by the factor given by the equation, factor = $2.3\ A/1\text{-}10^{-A}$, where A is the absorbance of a given concentration of acrylamide at the exciting wavelength and 10-mm light path. All the buffers used were of 50 mM concentration and contained 48 mM NaCl. Glycine buffer was used for pH range 2.0–3.6, sodium acetate for pH range 4–5, citrate phosphate for pH 6, and Tris for pH 7.4.

Photolabeling Studies—All photolabeling experiments were carried out in the dark under safe red light. An ethanolic solution of [^{3}H]DAF was added to a solution of α-LA maintaining the ethanol concentration below 1% (v/v). The samples were photolysed for 3 min after incubating the probe with the protein for a period of 15 min. The protein to DAF molar ratio was maintained at 1:2.5, unless

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[1] The abbreviations used are: α-LA, α-lactalbumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; [^{3}H]DAF, [^{3}H]diazofluorene; CD, circular dichroism.

otherwise specified. The photolysed samples were extracted with equal volumes of ether twice, and then treated with trichloroacetic acid (final concentration of trichloroacetic acid is 10% (v/v)) and kept at 0 °C for 15 min, after which they were centrifuged at 12,000 rpm for 5 min. The precipitate was finally washed with cold acetone, kept at 0 °C for 15 min, and centrifuged at 12,000 rpm for 5 min. Analysis by trichloroacetic acid precipitation was found to be a quick method to determine the extent of labeling, although it did give values which were higher than those obtained on analysis by gels. However, the overall trend remained the same.

RESULTS

Intrinsic Fluorescence of α-LA—Bovine α-lactalbumin (α-LA) is a 123-amino acid protein which contains 4 tryptophans at positions 26, 60, 104, and 118. The fluorescence spectra of α-LA at pH 7.4 gives an emission maxima at 328 nm (Hanssens *et al.*, 1980; Kuwajima, 1989) indicating that at this pH all the tryptophans are buried in the hydrophobic interior of the protein. However, with lowering of pH, the emission maxima undergoes a red shift so that at pH 3.0 it appears at 343 nm, indicating exposure of tryptophans. A clear transition can be seen in Fig. 1*a*, wherein around pH 4.2 the protein undergoes a sharp increase in emission maxima which stabilizes around pH 3.0. The denatured protein (6 M guanidine HCl) at pH 7.4 gives rise to an emission maxima at 353 nm, the normal emission maxima for tryptophan in solution (Barstein *et al.*, 1973), as expected for a fully denatured protein. The α-LA at pH 3.0 is thus in a state which is different from the denatured state, and in accordance with earlier reports, the low pH state corresponds to the molten globule state (Kuwajima, 1989). The same state also seems to be prevalent at higher temperatures (Dolgikh *et al.*, 1985; Kuwajima and Sugai, 1978). The increase in temperature gradually eliminates the pH transition observed by fluorescence (Fig. 1*a*). Thus at 65 °C no change in the emission maxima (343 nm) is observed with change of pH. However, at pH 5, a blue shift is observed at higher temperatures. In order to see if this corresponds to any other intermediate state in the N ⇌ A transition, an alternative and more accurate method for monitoring small changes in emission maxima was used. This method involves measurement of the ratio of relative fluorescence intensity at 330 and 350 nm in proteins (Wharton *et al.*, 1988; Jiang and London, 1990). A decrease in I_{330}/I_{350} indicates a red shift in the emission maxima. The Fig. 1*b* indicates that there is no change in I_{330}/I_{350} at pH 3 as a function of temperature as α-LA is already in the molten globule state at this pH value. However, at neutral pH, increase in temperature leads to decrease in the I_{330}/I_{350} value, indicating a red shift or increased exposure of tryptophans in the molten globule state induced by temperature. The change in I_{330}/I_{350} values as a function of temperature at pH 5 are interesting, and up to approximately 35 °C, the high and stable value of I_{330}/I_{350} indicates that tryptophan residues at this pH are in a highly hydrophobic environment. These results confirm the observations made on the basis of Fig. 1*a*, *i.e.* a highly compact intermediate, wherein the hydrophobic interactions in the interior of the protein increase leading to a decreased exposure of hydrophobic surface relative to the native structure.

Fluorescence Quenching of α-LA—The low pH induced protein folding in α-LA was further investigated by fluorescence quenching. Since quenching occurs by collision between the fluorophore and the quencher, changes in protein struc-

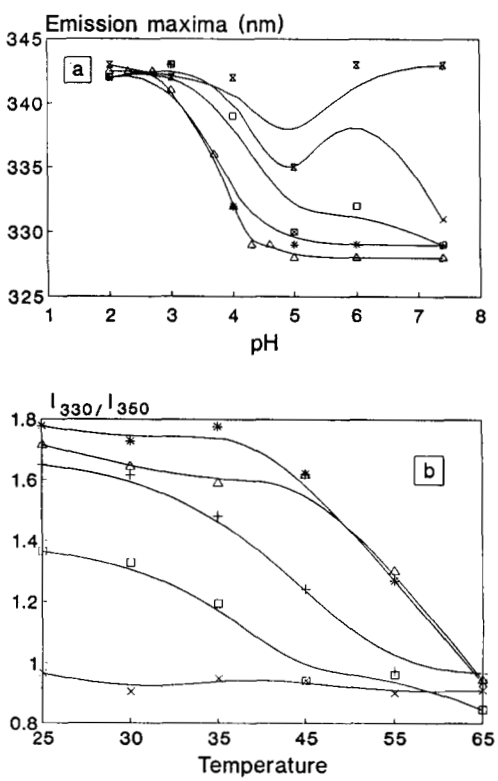

FIG. 1. *a*, effect of pH on the fluorescence emission maxima of α-LA at different temperatures; 25 (△), 35 (*), 45 (□), 55 (×), and 65 (⋈) °C. *b*, effect of increasing temperature on the fluorescence of α-LA at various pH; pH 7.4 (△), 6.0 (+), 5.0 (*), 4.0 (□), and 3.0 (×). Emission maximum shifts were monitored by the ratio of fluorescence intensity at 330 nm to that at 350 nm (I_{330}/I_{350}).

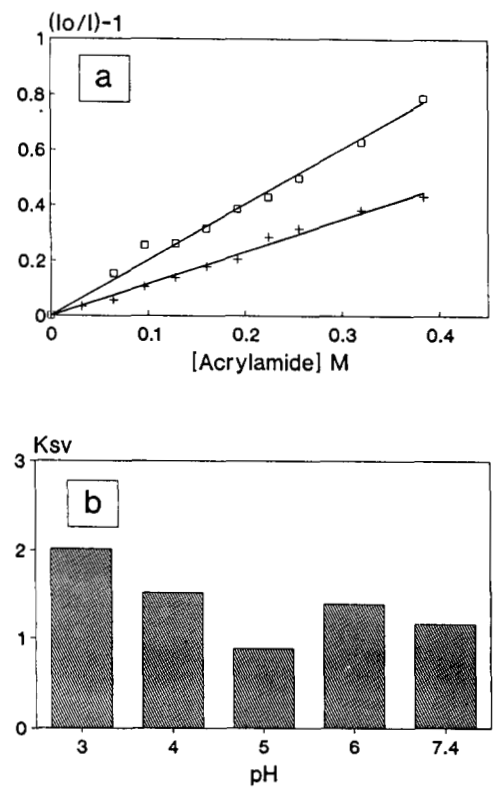

FIG. 2. *a*, Stern-Volmer plot for fluorescence quenching of α-LA by acrylamide at pH 3.0 and 7.4. Increasing aliquots of a 8 M solution of acrylamide were added to the α-LA solution (32 μg/ml protein concentration). Fluorescence measurements were further corrected for inner-filter effect due to acrylamide and analyzed by the Stern-Volmer plot; pH 7.4 (+) and 3.0 (□). *b*, a *bar* plot of Stern-Volmer constants (K_{sv}) obtained from quenching of α-LA by acrylamide in solution at different pH values.

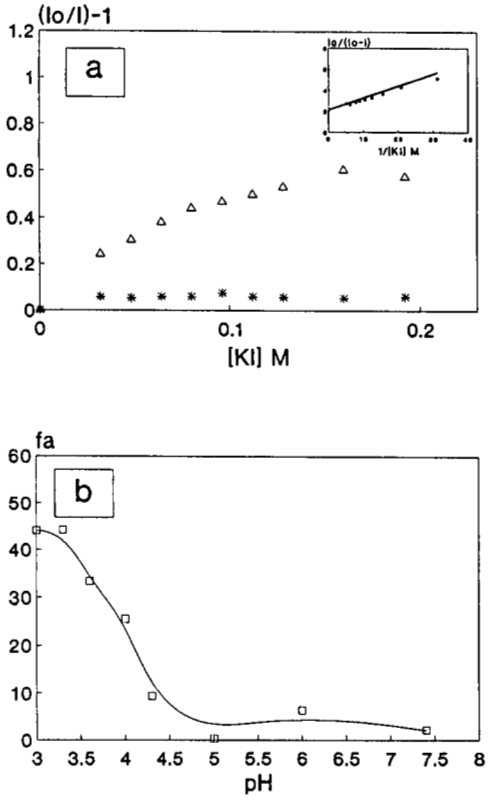

FIG. 3. *a*, Stern-Volmer plots for quenching of α-LA at pH 3.0 (△) and 7.4 (*) by iodide. Increasing aliquots of a 4 M solution of KI, containing a small amount of 10 mM $Na_2S_2O_3$ in order to prevent formation of I_2 and I_3^-, were added to the α-LA solution and fluorescence intensity measured and data analyzed by Lehrer's equation. *Inset* shows the Lehrer analysis of quenching of α-LA using I^- at pH 3.0. *b*, plot of the number of Trp of α-LA accessible (f_a) to the quencher, I^- as a function of pH.

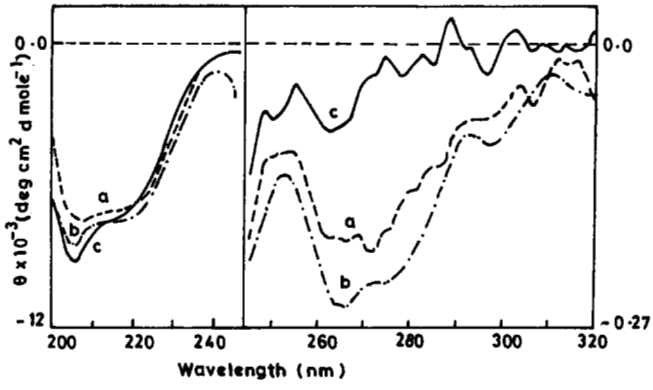

FIG. 4. *a*, far UV circular dichroism spectra of α-LA in solution; pH 7.4 (*a*), 5.0 (*b*), and 3.0 (*c*). Protein concentrations used were 0.428 mg/ml. *b*, near UV circular dichroism spectra of α-LA; pH 7.4 (*a*), 5.0 (*b*), 3.0 (*c*). Protein concentrations used were as described in *a*.

ture can be followed by judicious choice of quenchers. In the event of all tryptophans being accessible to a quencher, the fluorescence process can be described by the Stern-Volmer equation (Stern and Volmer, 1919).

$$(I_0/I) - 1 = K_{SV}[Q] \quad (1)$$

where I_0 is the initial fluorescence, I is the fluorescence at the quencher concentration $[Q]$, and K_{SV} is the Stern-Volmer constant. A plot of "$(I_0/I) - 1$" *versus* $[Q]$ gives rise to a linear plot which passes through the origin. However, if all the

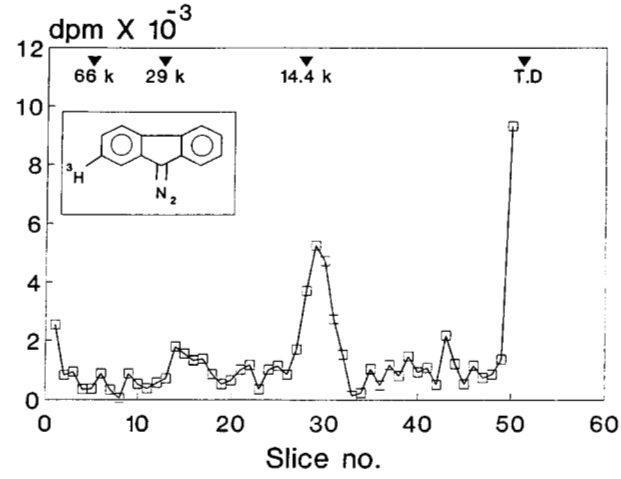

FIG. 5. **Electrophoretic profile of α-LA photolysed with [^{3}H] DAF at pH 7.4.** 1 mg/ml solution of α-LA (69.4 nmol) in 10 mM Tris, pH 7.4, was incubated with an ethanolic solution of [^{3}H]DAF (174 nmol, 102 μCi) of specific activity 593 μCi/μmol for 15 min and then photolysed for 3 min using a RMR-500 Rayonet minireactor fitted with four 3000-Å lamp. The photolysed material was then extracted with ether and precipitated with trichloroacetic acid. Finally, the pellet obtained was analyzed on 15% SDS-urea gels. The gel was stained with Coomassie Blue and then destained. The radioactive lanes were then cut into 2-mm slices, solubilized using 250 μl of 30% H_2O_2, and then counted using a toluene-based scintillant. The position of standard molecular weight markers is indicated by *arrowheads*. α-LA band appeared at 14.4 kDa. *Inset* shows the structure of [^{3}H]DAF.

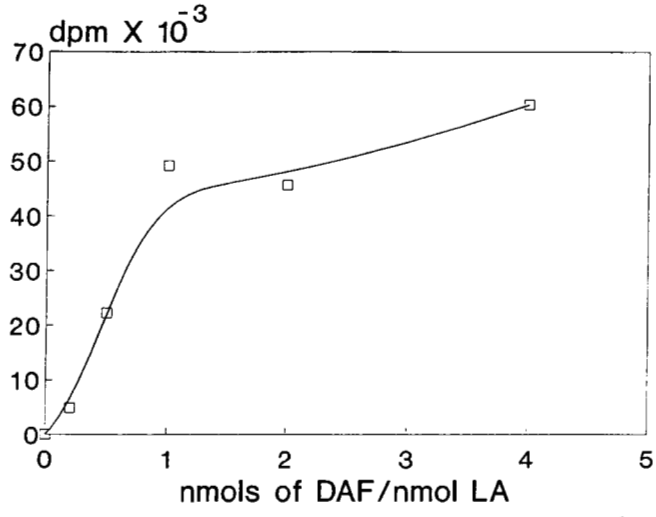

FIG. 6. **Concentration dependent labeling of α-LA with [^{3}H] DAF at pH 7.4.** 25 μl of α-LA (18 μg, 1.25 nmol) was incubated with increasing concentrations of an ethanolic solution of [^{3}H]DAF of specific activity 184 μCi/μmol in ratios of 1:0.2, 1:0.5, 1:1, 1:2, and 1:4 for 15 min and photolysed for 3 min, extracted with ether, and finally precipitated with trichloroacetic acid and counted for radioactivity.

tryptophan in a protein are not accessible to a quencher, *i.e.* buried in the hydrophobic interior of a protein, the Stern-Volmer plot deviates from linearity and the quenching process can be described by a modified equation (Lehrer, 1971).

$$I_0/(I_0 - I) = 1/K_{SV}\cdot[Q]\cdot f_a + 1/f_a \quad (2)$$

where I refers to change in fluorescence intensity on addition of the quencher and f_a refers to the fraction of tryptophan accessible to the quencher. A water-soluble quencher like iodide cannot partition into the hydrophobic interior of a protein and quenches fluorescence associated with tryptophan residues located on the surface of a protein. The use of

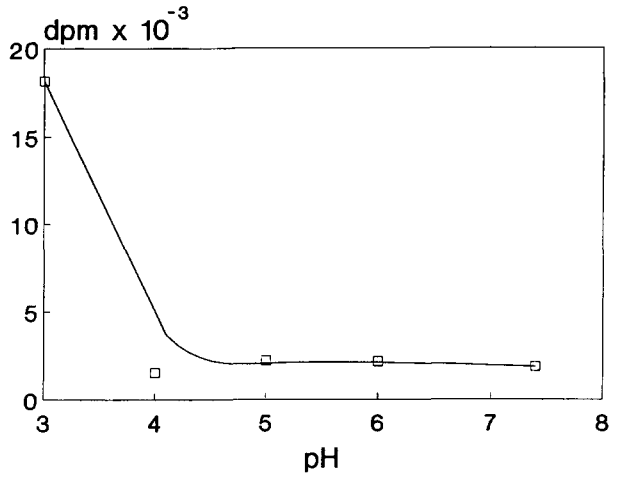

FIG. 7. **Plot of labeling of α-LA with [^{3}H]DAF as a function of pH.** 0.5 ml of α-LA (100 μg, 6.94 nmol) was taken in buffer of pH 3, 4, 5, 6, and 7.4 and then incubated with an ethanolic solution of [^{3}H]DAF (7.78 μCi, 17.7 nmol) of specific activity 279 μCi/μmol for 15 min and photolysed for 3 min. It was extracted as described under "Materials and Methods" and analyzed on 15% SDS-PAGE.

quenchers like iodide can thus provide information about the number of tryptophans which are buried in the protein. However, quenchers like acrylamide can penetrate into the protein and give rise to normal Stern-Volmer kinetics (Eftink and Ghiron, 1976). The increase in slope of the Stern-Volmer plot indicates easy accessibility of tryptophan residues to the quencher. The change in fluorescence quenching by these water-soluble quenchers can thus be used to follow the variations in the protein structure.

The fluorescence quenching of α-LA was studied as a function of pH using acrylamide and iodide. The quenching data using acrylamide could be easily described by the Stern-Volmer equation (Fig. 2*a*). The results obtained indicate that degree of quenching of α-LA increases as the pH is lowered (Fig. 2*b*) which indicates greater exposure of tryptophans at the lower pH value. At pH 7.4, α-LA was barely quenched by iodide. This is expected in view of the fact that emission maxima of α-LA at pH 7.4 appears at 328 nm, which indicates buried tryptophans. However, at pH 3.0 α-LA was substantially quenched (Fig. 3*a*). The fluorescence data could not be interpreted using the Stern-Volmer equation (Stern and Volmer, 1919) and consequently the data was analyzed by Lehrer's method (Lehrer, 1971). Such an analysis indicated that at pH 3.0 the accessible fraction of quenched tryptophans (f_a) is 47%, suggesting that 2 out of 4 tryptophans in α-LA are more exposed to the solvent at the lower pH value (Fig. 3*a*). The Fig. 3*b* gives a variation in f_a as a function of pH. This data indicates that the accessible tryptophan fraction increases as the pH is lowered. A closer examination of Figs. 2*b* and 3*b* indicates that at pH 5, α-LA is minimally quenched, once again endorsing the involvement of an extremely compact intermediate state, wherein the hydrophobic core of the protein interacts more strongly than in the native state. This observation is also supported by circular dichroism (CD) studies described below.

The CD spectrum of α-LA in the molten globule state is similar to that of the native protein in the far UV (peptide) region, but is nearly absent in the near UV (aromatic) region (Robbins and Holmes, 1970; Ewbank and Creighton, 1991). This is consistent with an almost similar secondary but a disrupted tertiary structure (Kuwajima, 1989; Dolgikh *et al.*, 1985). Fig. 4 gives the CD spectrum of α-LA at pH 3, 5, and 7.4 in the peptide and the aromatic region. While the CD in the peptide region (200–245 nm) looks quite similar at all these pH values (Fig. 4*a*), the CD in the aromatic region (245–300 nm) at pH 5 is more stronger than that at pH 7 (Fig. 4*b*), indicating that asymmetry of tryptophans at pH 5 has been altered.

Hydrophobic Photolabeling of α-LA with [^{3}H]DAF—We have used DAF as a hydrophobic photoactivable reagent to probe exposure of the hydrophobic surface in the molten globule state. While this reagent was primarily designed to label the membrane hydrophobic core (Pradhan and Lala, 1987) it can also be used to label hydrophobic pockets in proteins. DAF is a hydrophobic reagent and on photolysis gives rise to a carbene, which inserts into neighboring molecules by inserting into any bond including the C-H bond (Anjaneyulu and Lala, 1984; Pradhan and Lala, 1987). The high reactivity of this carbene precursor and its hydrophobicity makes it an ideal reagent for probing hydrophobic surface in proteins.

DAF on incubation with apo-α-LA followed by photolysis at pH 7.4 led to cross-linking of this reagent to α-LA as revealed by precipitation of the labeled protein with trichloroacetic acid. The DAF-labeled protein on passage through the Sephadex G-25 column indicated that the bulk of radioactivity eluted with the α-LA using 10 mM Tris, pH 7.4, containing 0.1% sodium dodecyl sulfate as the eluting buffer (data not shown). Electrophoretic analysis of the DAF-labeled α-LA confirmed the covalent attachment of DAF to α-LA (Fig. 5). The presence of a radioactive band around 29 kDa suggests that part of α-LA exists as a dimer. The photolabeling of α-LA with increasing concentration of DAF (Fig. 6) indicated saturation around DAF to α-LA at a molar ratio of 1:1, although at higher ratios, *e.g.* above 3:1, DAF tends to precipitate making photolabeling difficult at these ratios. The presence of Ca^{2+} or 100 mM Na^+ led to decrease in labeling of apo-α-LA by 10–50% (data not shown). This is in accordance with decreased binding of the fluorescent polarity probe, 4,4′-bis-[1-(phenylamino)naphthalene-8-sulfonate to Ca(II)-α-LA (Musci and Berliner, 1984) and other fluorescence measurements which indicate that 100 mM NaCl has the same effect as Ca^{2+} on apo-α-LA (Hanssens *et al.*, 1984; Mitani *et al.*, 1986). The decrease of labeling in the presence of Ca^{2+} also supports the suggestion that binding of Ca^{2+} to apo-α-LA leads to a decrease in exposure of hydrophobic surface based on chromatography of α-LA on phenyl-Sepharose (Lindahl and Vogel, 1984).

Once it was established that DAF labels α-LA and can thus provide an indication of exposure of hydrophobic surface, the labeling of α-LA was studied as a function of pH. Fig. 7 indicates a very high degree of labeling at pH 3. This data indicates increased exposure of hydrophobic surface in the molten globule state prevalent at low pH. A preliminary analysis of the DAF-labeling site at pH 3 and 7.4 was also carried out. α-LA contains only 1 methionine at position 90, so that cyanogen bromide fragmentation leads to a 10-kDa (CN-1) fragment corresponding to the N-terminal of α-LA and a 4-kDa (CN-2) fragment corresponding to the C-terminal of α-LA (Brew and Hill, 1970). α-LA labeled by DAF at both pH 3 and 7.4 was subjected to cyanogen bromide fragmentation. Electrophoretic analysis of α-LA labeled at pH 7.4 indicated that all the radioactivity was associated with the CN-1 fragment. Similar analysis of α-LA labeled at pH 3 indicated that both CN-1 and CN-2 fragments are labeled although the bulk of the labeling was still associated with CN-1 (data not shown). These results clearly indicate labeling of α-LA in the molten globule state at multiple sites and not increased labeling at a single site.

DISCUSSION

We have shown here that the native to molten globule state transition in α-LA can be easily detected by following changes in intrinsic fluorescence of α-LA as a function of pH and temperature. The molten globule state has been further characterized by fluorescence quenching using acrylamide and iodide. Both the quenchers indicate exposure of buried tryptophans in the molten globule state. The exposure of tryptophan residues in the molten globule state of α-LA have been studied in the past using solvent perturbation studies (Kronman *et al.*, 1972; Kronman and Holmes, 1965) and charge transfer complex formation (Robbins and Holmes, 1972). While these techniques did indicate the exposure of tryptophan residues in the native to molten globule state transition, the number of tryptophan residues exposed has varied from 1 to 2 (Kronman *et al.*, 1972; Kronman and Holmes, 1965; Robbins and Holmes, 1972). Iodide quenching of the intrinsic fluorescence resolves this controversy and clearly indicates that 2 out of 4 tryptophans are exposed in the molten globule state.

The fluorescence as well as fluorescence quenching data point towards involvement of another intermediate state, prevalent at pH 5, in the formation of molten globule state. In this intermediate state, the normally buried tryptophans residues in the native state of α-LA move into a still further hydrophobic environment before being exposed in the molten globule state. The strong CD in the near UV region at pH 5 strongly supports the involvement of such an intermediate compact state in the native to molten globule state transition. The formation of such an intermediate compact state have also been observed recently in the case of *Pseudomonas* exotoxin A (Jiang and London, 1990). It is possible that in the native to molten globule state transition, which primarily involves loss of tertiary structure of the native protein, the hydrophobic interactions in the protein interior resist this disruption, leading to such an intermediate compact state, before being transformed into slowly fluctuating tertiary structure characteristic of a molten globule state (Dolgikh *et al.*, 1981).

The photolabeling of α-LA with DAF indicates that this reagent can be effectively used to study exposure of hydrophobic surface in proteins. The increased labeling of α-LA at pH 3.0 points to exposure of hydrophobic surfaces in the molten globule state. It would have been interesting to see if DAF labeling at pH 5.0 is minimal in view of the proposed formation of the intermediate compact state of α-LA. However, the low level of labeling between pH 4 and 7 makes it difficult to make a definite conclusion. A more conclusive result will have to await preparation of [^{3}H]DAF of higher specific activity. Finally, labeling of multiple sites in α-LA by DAF indicates that the sequencing of the labeled protein should provide useful information for characterization of increased hydrophobic surface in the molten globule state. Our attempts in this direction are in progress.

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