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Highly Ordered Molten Globule-Like State of Ovalbumin at Acidic pH: Native-Like Fragmentation by Protease and Selective Modification of Cys367 with Dithiodipyridine[1]

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Structural characteristics of ovalbumin at acidic pH were investigated by a variety of analytical approaches. At pH 2.2, the protein appeared to assume a partially denatured, molten globule-like conformation as evaluated by the binding of a hydrophobic probe, anilino-1-naphthalene-8-sulfonate. The protein was, however, resistant to proteolysis with pepsin under conditions in which the urea-denatured form was extensively hydrolyzed. Furthermore, under more drastic proteolytic conditions, the acid ovalbumin was specifically proteolyzed at the N-terminal site of Ala351, which is located in close proximity to the canonical serpin cleavage site Ala352-Ser353 that is known to be the cleavage site at neutral pH with subtilisin and elastase in native ovalbumin. Among the four cysteine residues (Cys11, Cys30, Cys367, and Cys382), which are all known to be buried in the native ovalbumin molecule, only Cys367 was specifically modified with 2,2′-dithiodipyridine, generating a mixed-disulfide protein derivative. Upon incubation of the derivative with a high concentration of L-cysteine, the thiopyridine mixed disulfide did not undergo any bimolecular exchange reaction with the thiol in the absence of an added denaturant, indicating that the mixed disulfide group is inaccessible. The far-UV CD spectra indicated that the native secondary structure is retained in either the modified or non-modified protein; but as evaluated by the near-UV CD spectra, the asymmetric nature of aromatic side chains in the non-modified ovalbumin and of the mixed-disulfide group in the modified protein was almost lost at pH 2.2. These results are consistent with a highly ordered molten globule-like state for OVA at pH 2.2, in which side chains, but not the backbone chain, significantly fluctuate.

Key words: molten globule state, ovalbumin, protein conformation, protein fragmentation, serpin superfamily.

Globular proteins are transformed from their native states into different non-native states under various denaturing conditions, such as extreme pH, high denaturant concentrations, and elevated temperatures (*1*). Studies on the non-native states are important for the understanding of protein folding mechanisms, since highly denatured protein is usually employed as the starting sample for refolding experiments. Furthermore, the equilibrium molten globule state has been shown to be similar to or identical with a kinetic intermediate at an early refolding stage (*2, 3*). The molten globule state assumes a compact and considerably native-like secondary structure, but it is differentiated from the native state by the absence of close packing throughout the molecule, as reflected in the absence of a cooperative temperature transition and in a slightly increased hydrodynamic radius (*2, 4-7*). The state is characterized as a nonspecific assembly of secondary structure segments brought about by hydrophobic interactions (*2*); this results in the formation of a hydrophobic cluster, which can be detected as increased binding of a hydrophobic dye (*8*).

In addition to such a classical molten globule, the occurrence of more ordered as well as more disordered states has been demonstrated in several instances of partly denatured proteins. Partly denatured equine lysozyme retains a compact cluster within the core of the protein (*9*) and exerts a clear cooperative temperature transition (*10, 11*). Cooperative thermodynamic transition has also been confirmed with cytochrome *c* in the presence of perchloric anion at pH 1.8 (*12*). A "dry molten globule" has been theoretically hypothesized as a slightly denatured form (*13*); an equivalent state in which side chains are free to rotate has been detected as a denaturation intermediate of ribonuclease A (*14*). On the other hand, a more disordered state than the classical molten globule has been found and designated as pre-molten globule. Examples of the pre-molten globule include the guanidine hydrochloride-induced unfolding intermediates of β-lactamase (*15*) and carbonic anhydrase B (*16*), and acid-denatured cytochrome

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Abbreviations: ANS, anilino-1-naphthalene-8-sulfonate; IAEDANS, *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; IAM, iodoacetamide; Pyr-S-S-Pyr, 2,2′-dithiodipyridine; Pyr-SH, 2-thiopyridone. OVA represents ovalbumin.

c in a low salt condition (*17*).

Previous reports have shown that OVA assumes a unique partially denatured conformation at acidic pH (*18-20*). OVA consists of a single polypeptide chain of 385 amino acid residues that folds into a globular conformation with a high secondary structure content (30.6% α-helix and 31.4% β-strand), as shown in Fig. 1. It contains four free sulfhydryls (Cys11, Cys30, Cys367, and Cys382) and a disulfide bond between Cys73 and Cys120 (*21*). CD spectra have shown that the secondary structure content is almost exactly the same at pH 2 and 7, but that the native tertiary interactions are almost completely disrupted at the acidic pH (*18*). However, intrinsic viscosity and the sedimentation coefficient, $S^{\circ}_{20,w}$, which both depend on the protein hydrodynamic volume, are essentially the same at the two pH values (*19*). Furthermore, the protein exhibits a large endothermic transition during heat denaturation at acidic pH (*20*). These observations have suggested that the conformational state of OVA at pH 2 is more rigid than the classical molten globule.

In the present report, we analyzed the conformational state of the acid OVA utilizing alternative approaches, including ANS binding as well as other approaches unique to this protein. OVA, a member of the serpin superfamily, undergoes, in the native state, limited proteolysis with subtilisin (*22*) or elastase (*23*) at the canonical P_1-P_1' site located in helix-R (*23*). We investigated by pepsin fragmentation analysis whether the native cleavage behavior was retained at pH 2. As another unique feature, the four free cysteine sulfhydryls of OVA are all buried in the native protein molecule (Fig. 1) as reflected in its nonreactive nature to an alkylation reagent at near-neutral pH (*24*). A highly reactive aromatic disulfide, Pyr-S-S-Pyr (*25, 26*), however, reacts with one of the four sulfhydryl groups of OVA at pH 2 (*18*). This reaction should yield a modified protein. Here, we show that, at pH 2.2, OVA undergoes the native-like fragmentation by pepsin and selective modification of Cys367 by Pyr-S-S-Pyr, indicating a highly ordered conformation of acid OVA. The inaccessible nature of the thiopyridine mixed disulfide group to added L-cysteine is also consistent with highly ordered conformation. The overall conformation of acid OVA as evaluated by ANS binding and CD spectra appears to resemble a molten globule-like state. These results are consistent with a highly ordered molten globule-like state for OVA at pH 2.2, in which side chains, but not the backbone chain, significantly fluctuate.

Fig. 1. **Three-dimensional structure of OVA.** The figure is based on the X-ray crystallographic data of OVA (*41*) and was drawn using the MolScript program (*42*). Filled circles represent the sulfur atoms of the cysteine residues: 1, Cys11; 2, Cys30; 3, Cys73; 4, Cys120; 5, Cys367; 6, Cys382. Cys73 and Cys120 form the native disulfide bond. The pepsin cleavage site at pH 2.2 (N-terminal side of Ala351 in helix-R, see "RESULTS") is indicated by an arrow.

MATERIALS AND METHODS

Materials—OVA was prepared as described before (*27*). Pepsin from porcine stomach mucosa (1:60,000, Type P-7012) and IAEDANS were purchased from Sigma and Aldrich Company, respectively. Other chemicals including Pyr-S-S-Pyr and IAM were obtained from Nacalai Tesque.

ANS Binding Experiments—OVA was dissolved in 0.1 M K-phosphate or Na-citrate buffer having different pH values or in Buffer A (0.1 M potassium phosphate-HCl, pH 2.2) containing 8 M urea, then mixed with 0.025 volume of 0.8 mM ANS, and the fluorescence emission spectra were measured at 25°C with a fluorescence spectrophotometer (Hitachi, F-3000) at an excitation wavelength of 350 nm. The final concentrations of the protein and ANS were 2 and 20 μM, respectively.

Analysis of Resistance to Pepsin Digestion—The conformational state of OVA in acidic pH was evaluated by measuring the resistance to pepsin digestion. OVA was incubated at 1 mg/ml in Buffer A (0.1 M potassium phosphate-HCl, pH 2.2) at 25°C for 30 min in the presence or absence of 6 M urea. An aliquot was mixed with 2 volumes of pepsin (1 μg/ml) in Buffer A, giving 0.33 mg/ml OVA and 0.67 μg/ml pepsin, and incubated at 25°C. At various incubation times, 9 μl of 1.0 M Tris-base and 33 μl of SDS buffer (0.25 M Tris-HCl, pH 7.0, 4% SDS, 40% glycerol, 80 mM 2-mercaptoethanol) were added to 90 μl of the reaction mixture. These samples were pretreated in a boiling water bath for 2 min, electrophoresed on a 10% polyacrylamide gel according to the standard method of Laemmli (*28*), and then stained with Coomassie Brilliant Blue R-250. The amount of intact OVA was determined from the band intensity measured with a densitometer (Shimadzu, CS-9000).

Characterization of Proteolytic Fragments—The conformational state of OVA was also examined using pepsin at a prolonged incubation time and at a higher protease concentration in the absence of urea. OVA was incubated at 0.33 mg/ml in Buffer A with 16.7 μg/ml of pepsin at 25°C for 20 h, and then the digestion was terminated by the addition of 0.1 volume of 1.0 M Tris-base, giving a final pH value of 7.0.

To determine the N-terminal sequence of fragments, the sample was mixed with the SDS buffer, pretreated in a boiling water bath in the same way, and then electropho-

resed on a 16.5% polyacrylamide gel according to the method by Schägger and von Jagow (*29*). The protein was electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Lab., Trans-Blot Transfer Medium) with a semi-dry transfer unit (Sartoblot II S) as described (*30*). The peptides blotted onto the membrane were stained with 0.1% Ponceau solution. The membrane was washed with a 1% acetic acid solution and distilled water, then dried. The peptides bands were cut out and subjected to sequence analysis with a protein sequenator (Applied Biosystems, model 492).

To isolate fragments, the digested sample (9 ml) was applied to a reverse-phase HPLC column (Cosmosil $5C_4$-AR-300) and peptides were eluted with an acetonitrile linear gradient (25-80%) in 0.1% trifluoroacetic acid. The eluted peptides were detected by measuring the absorbance at 220 nm, dried *in vacuo*, and dissolved in 20 μl of 50% acetonitrile. An aliquot of the sample was analyzed for N-terminal sequence with the sequenator. Another aliquot was subjected to molecular mass determination. The sample (1 μl) was mixed with 1 μl of 0.2 mM angiotensin (1,297.5 Da) and 1 μl of 0.2 mM insulin (5,734.5 Da) as internal standards and with 7 μl of 1% α-cyano-4-hydroxy-cinnamic acid, dissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid. The molecular masses of the peptides were analyzed by matrix-assisted laser desorption and ionization mass spectrometry using a time-of-flight spectrometer (PerSeptive Biosystems, Voyager RP).

Preparation of Thiopyridine Mixed-Disulfide OVA—To modify selectively a reactive cysteine residue, we employed modification conditions such that the number of modified cysteine residues was less than 1.0. OVA (10 mg/ml) was incubated at 25°C with 0.5 mM Pyr-S-S-Pyr in Buffer A for 2 h. The number of cysteines modified with Pyr-S-S-Pyr was determined from the released Pyr-SH using the molar extinction coefficient of 7,345 $M^{-1} \cdot cm^{-1}$ at 343 nm. Excess Pyr-S-S-Pyr and the released Pyr-SH were removed by gel filtration using a Sephadex G-25 column (Pharmacia Biotech, NAP-25) equilibrated with Buffer A.

Localization of the Mixed Disulfide Cysteine—To localize the cystine residue forming a mixed disulfide with Pyr-S-S-Pyr, the modified OVA (7.1 mg/ml) was mixed with 6.1 volumes of Buffer A containing 7 M urea 58.1 mM L-cysteine (giving a final L-cysteine concentration of 50 mM) and then allowed to stand for 10 min at 25°C. Urea and excess L-cysteine were removed by gel filtration using a Sephadex G-25 column (Pharmacia Biotech, NAP-5) equilibrated with Buffer A. The protein sample was mixed with nine volumes of 50 mM Tris-HCl buffer, pH 8.2, containing 1 mM Na-EDTA, 10 M urea, and 0.22 M IAM (giving a final IAM concentration of 0.2 M) and alkylated by incubation in this neutral/urea buffer at 37°C for 10 min.

The disulfide-forming half-cystines were determined by selective IAEDANS alkylation and subsequent peptide mapping analysis as described previously (*27*). Briefly, in the sample run, the alkylated protein was fully reduced with dithiothreitol and the newly generated sulfhydryls were alkylated with a fluorescent reagent, IAEDANS. The sample was proteolyzed and analyzed by reverse-phase HPLC monitored by fluorescence measurement (excitation, 340 nm; emission, 520 nm). In the standard run, the protein was treated in the same way except that the first alkylation step with IAM was skipped. The numbers of disulfide-forming half-cystines were determined as the relative ratio of labeled half-cystines ($R_{SH\text{-}i}$, $i=1$, 2, 5, or 6 for Cys11, Cys30, Cys367, or Cys382) to the average value of $R_{SH\text{-}3}$ (for Cys73) and $R_{SH\text{-}4}$ (for Cys382) was determined using the following equation:

$$R_{SH\text{-}i} = 2 \times (A_{SH\text{-}i}/B_{SH\text{-}i})/(A_{SH\text{-}3}/B_{SH\text{-}3} + A_{SH\text{-}4}/B_{SH\text{-}4}),$$

where $A_{SH\text{-}i}$ and $B_{SH\text{-}i}$ represent the HPLC peak areas for the half-cystines labeled with IAEDANS in the sample and standard runs, respectively, with respect to one of the half-cystines, SH-*i*.

Accessibility of Thiopyridine Mixed-Disulfide Group—The modified OVA (7.1 mg/ml) was mixed with 6.1 volumes of Buffer A containing varying concentrations of urea and 50 mM L-cysteine, and incubated at 25°C. The time course of the release of Pyr-SH, which corresponds to the mixed disulfide group replaced with L-cysteine, was monitored by measuring the absorbance at 343 nm.

CD Spectra—The CD spectra of OVA were recorded at 0.1 mg/ml protein for the far-UV and at 1.0 mg/ml for the near-UV region in various buffer systems with a Jasco J-720 spectropolarimeter. A cuvette of 0.2-cm light path for far-UV or of 1-cm light path for near-UV CD spectra was used. The temperature was kept at 25°C with a circulating water bath. The data are given as the averages of triplicate measurements. The far-UV data are expressed as mean residue ellipticity (degree·cm^2/decimol) using a value of 111 as the mean residue weight of OVA, and those for the near-UV CD spectra are expressed as molar ellipticity.

RESULTS

ANS Binding—The conformational state of OVA was analyzed by ANS binding experiments at various pH values. Figure 2 shows the emission spectra of ANS in the presence of OVA. In the native state at pH 7.0, OVA showed very low levels of ANS binding. At pH 2.2, how-

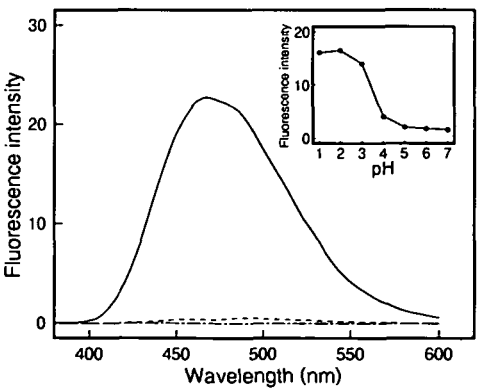

Fig. 2. **ANS binding.** OVA in Buffer A, pH 2.2 (solid line), in 0.1 M K-phosphate buffer, pH 7.0 (dashed line) or in Buffer A containing 8 M urea (dash-dotted line) was mixed with ANS solution, giving final concentrations of 2 μM OVA and 20 μM ANS, and the fluorescence emission spectra were measured with an excitation wavelength of 350 nm at 25°C. In the inset, OVA was diluted with 0.1 M K-phosphate or Na-citrate buffer having different pH values in the same way and the fluorescence emission was measured at 470 nm with an excitation wavelength of 350 nm. The fluorescence intensity is shown in arbitrary units.

ever, greatly increased fluorescence emission with a peak at 472 nm was observed. In the presence of 6 M urea at pH 2.2, ANS fluorescence was almost undetectable. These observations are consistent with a molten globule-like state for OVA at pH 2.2. Figure 2 (inset) shows that the transition between the native and molten globule-like state occurred in the pH range from 3 to 4.

Resistance to Pepsin Digestion—It has been demonstrated that subtilisin (*22*) and elastase (*23*) specifically hydrolyze native OVA at the canonical serpin cleavage site in helix-R. These proteases hydrolyze the intact 45 kDa form into two fragments: the N-terminal 41 kDa fragment (Gly1-Glu346) and a small C-terminal peptide consisting of 33 amino acid residues (Ser353-Pro385). Trypsin, however, does not digest it because of the lack of a Lys or Arg residue in the helix-R region. We utilized pepsin as a probe for evaluation of the conformational state of OVA at acidic pH. OVA was incubated in Buffer A for 30 min in the presence or absence of 6 M urea, and diluted 3-fold with Buffer A containing pepsin to give final concentrations of 0.33 mg/ml OVA and 0.67 μg/ml pepsin. The time course of the digestion was followed by SDS-PAGE. As shown in Fig. 3A, OVA was almost entirely resistant to pepsin during incubation for up to 120 min, while the urea-denatured protein was extensively digested during the same incubation. These findings suggested that OVA at pH 2.2 assumed a more ordered conformation than the urea-denatured protein.

Upon digestion with a 20 times higher concentration of pepsin for a 10 times longer incubation time, however, the original intact 45.0 kDa band decreased and two new fragments were generated, as shown in Fig. 3B; the molecular mass of the larger one was 41.0 kDa, but that of the smaller one could not be calculated, since its mobility was slightly greater than that of the smallest marker peptides used. We analyzed the N-terminal sequence of these fragments, after the peptides had been transferred from SDS-PAGE gel onto a polyvinylidene difluoride membrane. The N-terminal sequence was detected only for the smaller fragment; the determined sequence was Ala-Ala-Ser-Val-Ser-Glu-, which corresponds to Ala351 to Glu356. The N-terminal Gly of OVA is known to be acetylated (*31*). Therefore, the fact that the N-terminal sequence of the 41.0 kDa fragment could not be detected was consistent with the view that the N-terminal sequence of the fragment comprised that of the intact protein.

To perform further analyses, the pepsin-digested sample was fractionated by reverse-phase HPLC. Only one peak was detected at an acetonitrile concentration of 46% as a fragment newly generated by the pepsin digestion. The N-terminal sequence of the peak was Ala-Ala-Ser-, which corresponded to the N-terminal sequence of the smaller fragment. To determine the exact molecular mass of the smaller fragment, we employed matrix-assisted laser desorption and ionization mass spectrometry. Figure 4 shows that the determined molecular mass was 3,882.2 Da. This value was almost exactly the same as that of the amino acid sequence of Ala351 to Pro385 (3,881.6 Da).

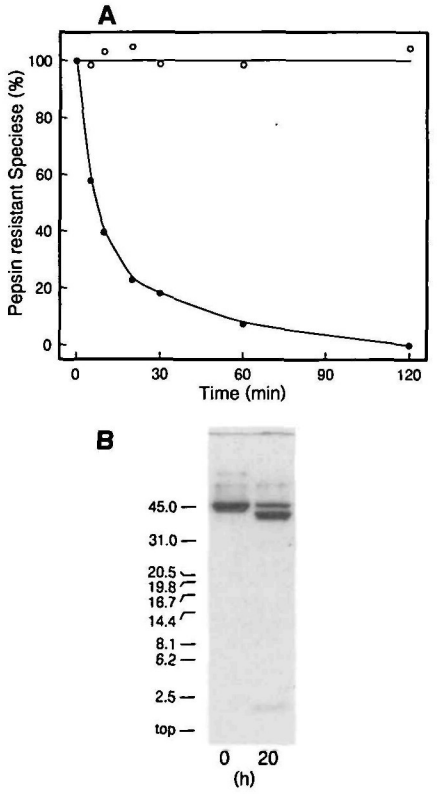

Fig. 3. **Susceptibility of OVA to pepsin.** In panel A, OVA was incubated in the presence (closed circles) or absence (open circles) of 6 M urea, diluted with Buffer A, digested with pepsin (0.67 μg/ml) at 25°C for various times, and then analyzed for SDS-PAGE as described in the text. The band intensity was measured with a densitometer. The averages of duplicate experiments are expressed as the percent of remaining intact OVA compared to the non proteolyzed control. In panel B, OVA in Buffer A was digested with a higher concentration of pepsin (16.7 μg/ml) at 25°C for 20 h, and then electrophoresed as described in the text. On the left side, the migrations of carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (20.5/19.8 kDa), horse heart myoglobin (16.7 kDa), lysozyme (14.4 kDa), and myoglobin fragment (8.1/6.2/2.5 kDa) are shown.

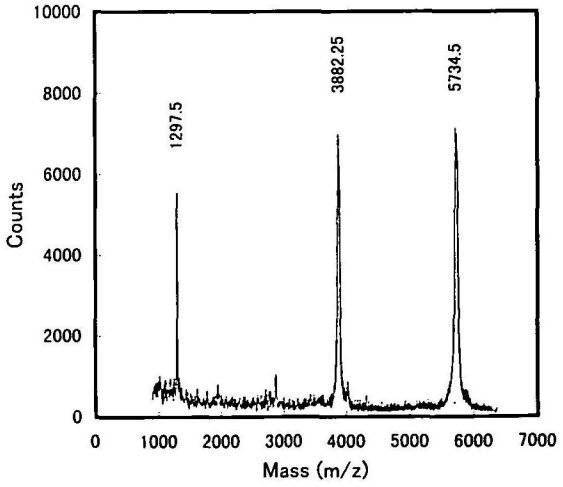

Fig. 4. **Molecular mass of the small pepsin fragment.** The small pepsin fragment of OVA (see Fig. 3B) was purified by reverse-phase HPLC and the molecular mass was determined by matrix-assisted laser desorption ionization mass spectrometry as described in the text. Angiotensin (1,297.5 Da) and insulin (5,734.5 Da) were included as internal standards. The calculated molecular mass of the smaller fragment in Fig. 3B was 3,882.25 Da.

Localization of the Cysteine Residue Forming a Thiopyridine Mixed Disulfide—A highly reactive aromatic disulfide, Pyr-S-S-Pyr (*25, 26*) has been suggested to react with one of the four sulfhydryl groups of OVA at pH 2 (*18*). This reaction should yield a modified protein:

$$OVA^{SH}_{SH} + Pyr\text{-}S\text{-}S\text{-}Pyr \longrightarrow OVA^{SH}_{S\text{-}S\text{-}Pyr} + Pyr\text{-}SH \quad (1)$$

where $OVA^{SH}_{S\text{-}S\text{-}Pyr}$ represents a mixed-disulfide form of OVA with Pyr-S-S-Pyr. The localization of the cysteine residue forming a thiopyridine mixed disulfide was determined.

To prepare the modified protein, OVA was incubated at 10 mg/ml with 0.5 mM Pyr-S-S-Pyr in Buffer A. After 2 h incubation, the number of half-cystines modified with Pyr-S-S-Pyr, as determined from the absorbance of the released Pyr-SH, was 0.759 per OVA molecule. The localization of the cysteine residue forming the mixed disulfide with Pyr-S-S-Pyr was analyzed by a peptide mapping procedure (*27*). Figure 5A shows the elution profile of $OVA^{SH}_{S\text{-}S\text{-}Pyr}$ in a standard run designed to detect all the half-cystine-containing peptides. In a control experiment, we confirmed that the disulfide-forming half-cystines of non-modified OVA were Cys73 and Cys120, which form the native disulfide bond (Fig. 5B). For the modified protein, both Cys367 and Cys382 along with Cys73 and Cys120 were apparently detected as disulfide-forming half-cystines (Fig. 5C). However, the total level of disulfide-forming half-cystine was not consistent with the optically estimated number of modified half-cystines (0.759); the total level of the disulfide-forming half-cystines was 1.39 (0.05 for Cys11, 0.07 for Cys30, 0.68 for Cys367, and 0.59 for Cys382). This apparent discrepancy can be explained by the highly reactive nature of the pyridine mixed disulfide group in $OVA^{SH}_{S\text{-}S\text{-}Pyr}$.

The rate of the sulfhydryl/disulfide exchange reaction depends on the pH and on the pK values of both the sulfhydryls of the nucleophile and leaving groups. According to the reported semi-empirical equation (*32-34*), the rate constant, k, for the sulfhydryl-disulfide exchange reactions can be expressed as follows:

$$-S_{nuc} + S_c\text{-}S_{lg} \longrightarrow S_{nuc}\text{-}S_c + -S_{lg}$$

$$\log k = 5.2 + pH - 0.5\ pK_{nuc} - 0.27\ pK_c - 0.73\ pK_{lg} - \log\left(1 + 10^{pH - pK_{nuc}}\right) \quad (A)$$

where the subscripts of nuc, c, and lg represent nucleophile, central, and leaving groups, respectively, and k is in units of $s^{-1} \cdot M^{-1}$. The pK value for the leaving Pyr-SH is -1.1

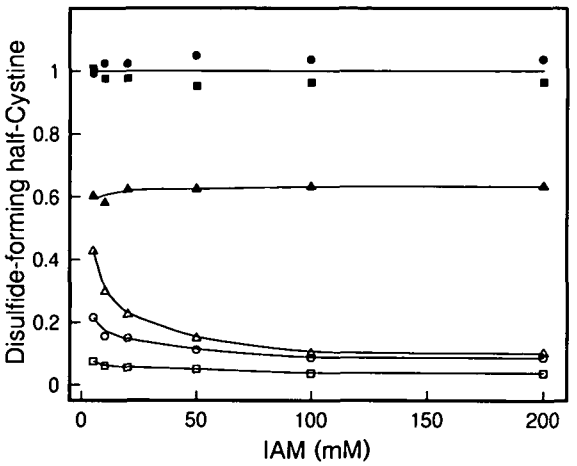

Fig. 6. **Quenching of free cysteine sulfhydryl by IAM.** The free protein sulfhydryls after replacement of the mixed disulfide group with 50 mM L-cysteine were quenched with the indicated IAM concentrations (37°C, 10 min), and the half-cystines involved in disulfide formation were determined by selective IAEDANS alkylation followed by peptide mapping analysis. The disulfide-forming half-cystines for Cys11 (open squares), Cys30 (open circles), Cys73 (closed circles), Cys120 (closed squares), Cys367 (closed triangles), and Cys382 (open triangles) were determined by selective alkylation with IAEDANS and peptide mapping analysis as described in the text. The number of thiopyridine groups in a sample determined from the absorbance of released Pyr-SH was 0.746 per OVA molecule.

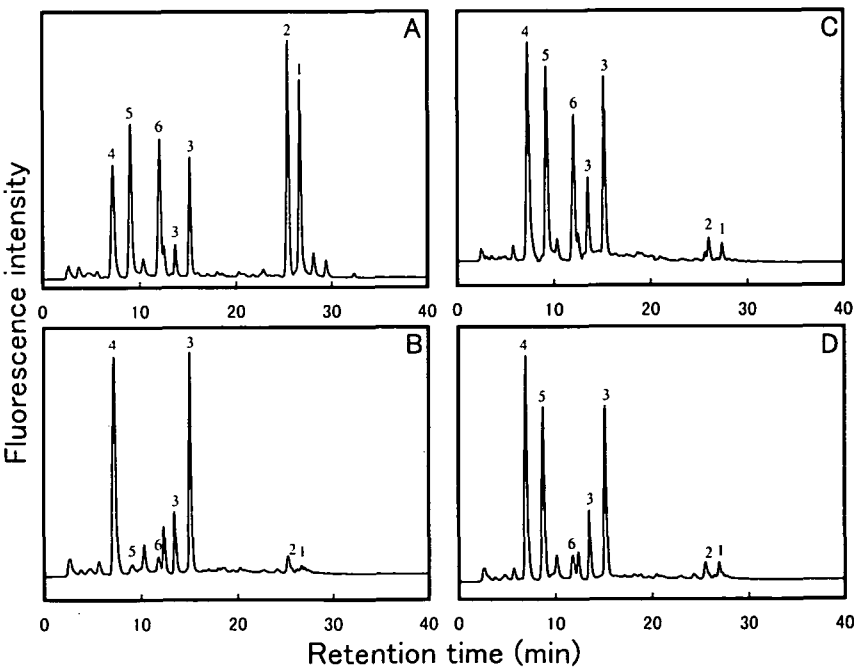

Fig. 5. **Identification of the half-cystine modified with Pyr-S-S-Pyr.** The number of thiopyridine groups in the sample was 0.759 per OVA molecule. To localize the mixed disulfide-forming half-cystine, the pyridine mixed-disulfide group of the modified OVA was replaced by incubation with 50 mM L-cysteine in Buffer A containing 6 M urea according to reaction (*3*) (see text). The free sulfhydryls of the modified OVA with (panel D) or without (panel C) replacement by L-cysteine were alkylated by IAM and the half-cystines involved in disulfide formation were identified by selective IAEDANS alkylation and subsequent peptide mapping analysis. In the standard run (panel A), the protein was treated in the same way except that the first alkylation step with IAM was skipped as described in the text. For comparison, the elution profile of the labeled disulfide-forming half-cystines of non-modified OVA is given in panel B. The numbers above the peaks of 1, 2, 3, 4, 5, and 6 represent the elution of half-cystine peptides for Cys11, Cys30, Cys73, Cys120, Cys367, and Cys382, respectively.

(*26*), while the pK values of protein sulfhydryls vary from 8.5 to 9.1 (*32*). The rate constant for the exchange at pH 8.8 between the cysteine sulfhydryl and the thiopyridine mixed-disulfide is about 10^7 times faster than that between the cysteine sulfhydryl and the cystine disulfide. For the correct localization of the cysteine residue forming a thiopyridine mixed-disulfide, the free cysteine sulfhydryls in $OVA^{SH}_{S\text{-}S\text{-}Pyr}$ must be quenched by alkylation with IAM at pH 8.8. The anomalously low pK value for the leaving group (Pyr-SH), however, causes a very rapid intramolecular cysteine sulfhydryl/mixed-disulfide exchange reaction:

$$OVA^{SH}_{S\text{-}S\text{-}Pyr} \longrightarrow OVA^{S}_{S} + \text{Pyr-SH} \quad (2)$$

where OVA^{S}_{S} represents an ovalbumin species having an additional intrachain disulfide. In Fig. 5C, the intramolecular exchanges (reaction 2) may occur more rapidly than the sulfhydryl quenching reaction by IAM, which results in an overestimation of disulfide-forming half-cystines. We, therefore, first replaced the mixed disulfide group of thiopyridine in 6 M urea at pH 2.2 by L-cysteine, which has a much higher pK value of 8.7 than Pyr-SH, generating another mixed-disulfide form of OVA ($OVA^{SH}_{S\text{-}S\text{-}Cys}$):

$$OVA^{SH}_{S\text{-}S\text{-}Pyr} + \text{Cys} \longrightarrow OVA^{SH}_{S\text{-}S\text{-}Cys} + \text{Pyr-SH} \quad (3)$$

and then the free protein sulfhydryls were quenched by IAM:

$$OVA^{SH}_{S\text{-}S\text{-}Cys} + \text{IAM} \longrightarrow OVA^{S\text{-}AM}_{S\text{-}S\text{-}Cys} \quad (4)$$

where $OVA^{SH}_{S\text{-}S\text{-}Cys}$ and $OVA^{S\text{-}AM}_{S\text{-}S\text{-}Cys}$ represent the cysteine mixed-disulfide form of ovalbumin and alkylated protein, respectively. We have confirmed that the pyridine mixed disulfide group can be completely replaced by L-cysteine at a concentration higher than 50 mM (Tatsumi, E. and Hirose, M., unpublished data). Figure 5D clearly demonstrates that only Cys367 participated in disulfide formation in the $OVA^{S\text{-}AM}_{S\text{-}S\text{-}Cys}$, other than Cys73 and Cys120. More quantitatively, as shown in Fig. 6, the intrachain sulfhydryl/mixed disulfide exchanges at near-neutral pH could be completely quenched by IAM in $OVA^{SH}_{S\text{-}S\text{-}Cys}$. The disulfide-forming Cys11, Cys30, and Cys382 significantly decreased with increasing IAM concentration to minimum levels at IAM concentrations higher than 0.1 M. For Cys367, the level slightly increased with IAM concentration, but was almost constant at a value of about 0.63 at IAM concentrations higher than 0.1 M. The ratios of the disulfide-forming half-cystines relative to the average of Cys73 and Cys120 were 0.04 for Cys11, 0.09 for Cys30, 0.63 for Cys367, and 0.10 for Cys382 against the total value of 0.746 that was calculated from the absorbance of released Pyr-SH. We therefore concluded that Cys367 was specifically modified with Pyr-S-S-Pyr, forming a mixed disulfide.

Bimolecular Exchange Reactions—The preceding data demonstrate that Cys367 among the four cysteine residues was selectively reactive with Pyr-S-S-Pyr, which formed a thiopyridine mixed disulfide derivative. We examined whether or not the mixed disulfide group was accessible to

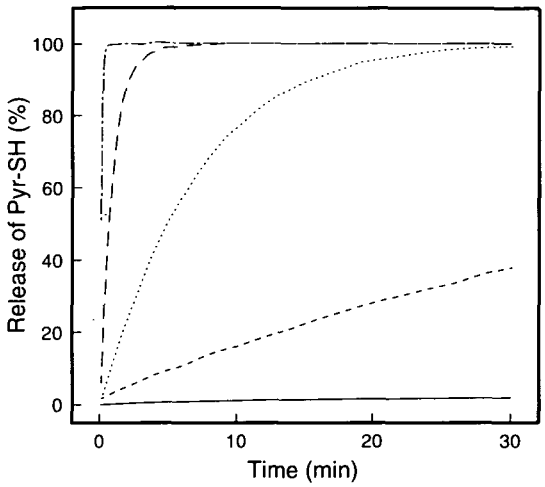

Fig. 7. **Accessibility of the mixed-disulfide-bonded Cys367.** The modified OVA was mixed with Buffer A containing varying concentrations of urea and 50 mM L-cysteine, giving a final urea concentration of 0 M (solid line), 2 M (short-dashed line), 3 M (dotted line), 4 M (long-dashed line), or 6 M (dash-dotted line), and incubated at 25°C. The time course of the release of Pyr-SH, which corresponds to the mixed disulfide group replaced with L-cysteine, was monitored by measuring the absorbance at 343 nm. The number of thiopyridine groups in a sample was 0.786 per OVA molecule, which was taken as 100%. The dead time for the manual mixing was 5 s.

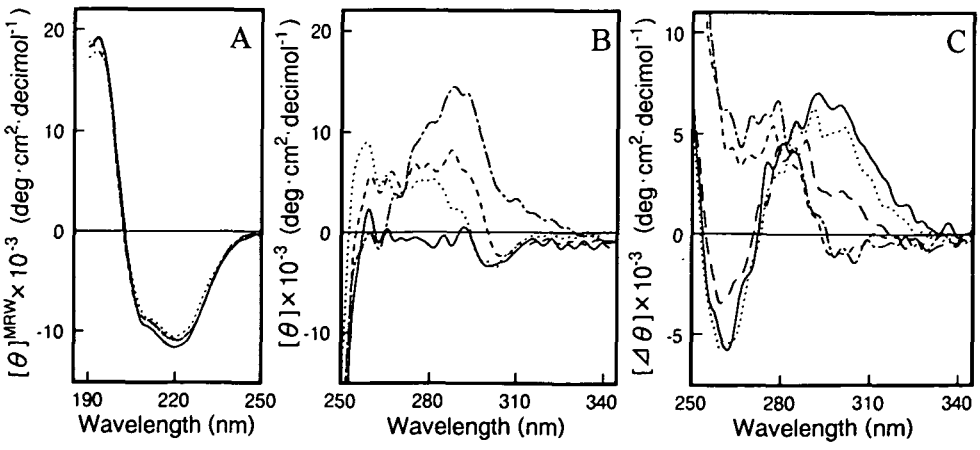

Fig. 8. **Effects of pH on CD spectra.** The number of thiopyridine groups in modified OVA was 0.80 per molecule. The modified OVA (7.1 mg/ml in Buffer A) was diluted 7.1-fold with 0.1 M K-phosphate buffer, giving a final pH of 7.0 (dash-dotted line) or 2.2 (dotted line). The samples were immediately analyzed at 25°C for far-UV (panel A) and near-UV (panel B) CD spectra as described in the text. The CD spectra for non-modified OVA were also recorded at pH 7.0 (dashed line) and pH 2.2 (solid line) in the same way. In panel C, the differential near-UV CD spectra (the modified protein minus non-modified protein) are compared at different pH values. The near-UV CD spectra were recorded in 0.1 M K-phosphate buffer at pH 2.0 (dash-dotted line), 3.0 (short-dashed line), or 7.0 (solid line) or in 0.1 M Na-citrate buffer at pH 4.0 (long-dashed line) or 5.0 (dotted line) in the same way.

bimolecular exchange with L-cysteine. The modified OVA (1 mg/ml) was incubated with 50 mM L-cysteine in Buffer A at 25°C. The possible replacement of the mixed disulfide group with L-cysteine was monitored by following the time course of the release of Pyr-SH. As shown in Fig. 7, the mixed-disulfide group of Cys367 did not undergo any exchange reaction with L-cysteine under the conditions employed, indicating that the mixed disulfide is inaccessible at pH 2.2. In order for the thiopyridine group to exchange with L-cysteine, destabilization of the protein molecule by addition of a denaturant was required. Figure 7 shows that release of Pyr-SH occurred in the presence of a urea concentration of 2 M or above, and that the rate of the release increased with increasing concentration of the denaturant.

CD-Spectral Analyses—The conformational state of OVA was analyzed by CD spectroscopy at various pH values. As shown in Fig. 8A, almost exactly the same far-UV CD spectra were observed for the modified and non-modified OVA at pH 2.2 and 7.0. The protein secondary structures may, therefore, be essentially the same for the modified and non-modified OVA at the two different pH values. In contrast, near-UV CD spectra were significantly variable at different pH values; at pH 7.0, both the protein forms showed a strong positive ellipticity with a peak at 290 nm, but the strong positive ellipticity was significantly decreased at pH 2.2 (Fig. 8B). When the differential near-UV CD spectra (the modified protein minus non-modified protein) were compared at different pH values, they showed a strong positive peak at around 290 nm and a negative peak at around 260 nm at pH 5.0 to 7.0. At acidic pH (pH 2.0 and 3.0), however, these two clear peaks were almost lost. These data strongly suggested that the asymmetric nature of the mixed disulfide group was almost lost at acidic pH.

DISCUSSION

In a previous report (*18*), Koseki *et al.* have shown that OVA assumes a partially denatured conformation at acidic pH. As evaluated from the CD spectra, the secondary structure contents are almost exactly the same at pH 2.2 and 7.0, but the native tertiary interactions are almost completely disrupted at the acidic pH (*18*). Conformational changes around tryptophan residues, as detected by measuring the absorbance differences at 292 nm and the intrinsic fluorescence intensities, have been shown to occur at around pH 4 (*18*). In the present report, the conformational state of OVA at the acidic pH was investigated in more detail by alternative approaches. ANS binds to hydrophobic clusters of non-polar groups and its binding in the molten globule state is usually much stronger than that in the native and denatured states; the binding of ANS is accompanied by a large increase in its fluorescence emission (*8*). OVA indeed showed greatly increased ANS binding at acidic pH as compared with the native and the urea-denatured forms (Fig. 2). The conformational transition evaluated in terms of ANS binding occurred in a pH range (pH 3 to 4, Fig. 2, inset) similar to that of the conformational changes revealed by the absorbance difference and intrinsic fluorescence intensity (*18*). The far-UV CD spectra of modified and non-modified OVA are indicative of almost exactly the same secondary structure at pH 7.0 and 2.2 (Fig. 8A), but the loss of the near-UV CD in the non-modified protein (Fig. 8B) is consistent with a disordered conformation of aromatic side chains. We confirmed by ANS binding and pepsin fragmentation analysis that the modified OVA takes essentially the same global conformation as the non-modified protein (Tatsumi, E. and Hirose, M., unpublished data). The observed differential near-UV CD spectra (Fig. 8C), therefore, can be accounted for by the introduced mixed-disulfide group. We observed that the thiopyridine group in the mixed disulfide derivative shows absorbance at around 290 nm (Tatsumi, E. and Hirose, M., unpublished data). The positive peak observed at near neutral pH in the differential near-UV CD spectra may be accounted for by the absorption by the thiopyridine group. Protein disulfides, which show weak absorption, sometimes exhibit ellipticity in the near-UV region (*35*). The negative peak around 260 nm observed in the differential spectra at near-neutral pHs might be due to the disulfide bond of the mixed-disulfide group. The two clear CD peaks were both greatly diminished at lower pH values of 3.0 and 2.2. Therefore, the mixed disulfide group in the modified protein may significantly fluctuate at the acidic pH. Results from other experimental approaches, however, demonstrate that OVA at pH 2.2 assumes a more ordered structure than the classical molten globule.

First, OVA is highly resistant to pepsin digestion. Proteases with broad substrate specificity have been shown to be useful probes for partly disordered protein conformation (*36, 37*). α-Lactalbumin, which is protease-resistant in the native state, undergoes significant proteolysis with pepsin and thermolysin in the molten globule states that are respectively induced at acidic pH and in the presence of trifluoroethanol (*37*). The molten globule-like state of OVA induced at pH 2.2 is resistant to pepsin digestion (Fig. 3A) under similar proteolysis conditions to those used for α-lactalbumin. Furthermore, under more drastic proteolytic conditions, OVA undergoes a limited proteolysis (Fig. 3B) at the N-terminal side of Ala351, which is located in close proximity to the canonical serpin cleavage site, Ala352-Ser353 (see "RESULTS"). OVA, a member of the serpin superfamily, is digested in the native state with serine proteases at highly specific cleavage sites of the helix-R region (Fig. 1, Ser344-Ser353). For example, it is well-known that native OVA undergoes limited proteolysis by subtilisin at the N-terminal sides of Ala347, Ala351, and Ser353, to produce a nicked form of OVA, called plakalbumin (*22*). Elastase cleaves the N-terminal side of Ser353, which is the reactive site of serine protease inhibitor (*23*). These specific cleavages are consistent with the fact that helix-R is exposed at the surface of the OVA molecule (Fig. 1). Pepsin has broad substrate specificity, cleaving at the C-terminus of bulky and hydrophobic amino acid residues (*38*). The native-like fragmentation with pepsin strongly suggests that disorder in the tertiary structure is very limited at pH 2.2.

Second, Pyr-S-S-Pyr reacts with only Cys367 among the four cysteine sulfhydryls of OVA (Cys11, Cys30, Cys367, and Cys382) at pH 2.2 (Figs. 5 and 6). All four free sulfhydryls of OVA are buried in the native state (Fig. 1) and are not reactive to iodoacetic acid at pH 8.2 (*24*). Cys367, which is involved in strand 4 in the β-sheet, is surrounded by helix-A and helix-H in the native state (Fig. 1). The selective modification of Cys367 with Pyr-S-S-Pyr

suggests reduced stereochemical rigidity of the native tertiary structure in these secondary structure segments. According to equation (A), the theoretical second-order rate constant at pH 2.2 for the modification reaction should be 12,600 $s^{-1} \cdot M^{-1}$. We, however, found that the observed rate constant for the modification reaction is only 0.780 $s^{-1} \cdot M^{-1}$ (Tatsumi, E. and Hirose, M., unpublished data). This suggests that the accessibility of the sulfur atom of Cys367 in the acid OVA is about 1/16,000 of that of a hypothetical fully-exposed cysteine residue. The other non-modified cysteine residues (Figs. 5 and 6) may be even less accessible than Cys367. The introduced mixed-disulfide group does not undergo any bimolecular exchange reactions with added L-cysteine (Fig. 7). These observations support a highly ordered conformation for the acid OVA.

Third, OVA exhibits a large endothermic transition during heat denaturation at acidic pH (*20*). Specifically, we found that plots of the calculated ΔH values *versus* corresponding melting temperatures have a linear relationship from pH 2.0 to 6.0 (Tatsumi, E. and Hirose, M., unpublished data). Therefore, the heat capacity change of OVA between the molten globule-like and denatured states may be exactly the same as that between the native and denatured states. The heat capacity change of the ordered molten globule state (in the presence of perchloric anion at pH 1.8) of cytochrome *c* is much smaller than that of the native form (*12*). OVA at the acidic pH, therefore, appears to be in a more ordered state than the molten globule state of cytochrome *c*.

A "dry" molten globule state has been theoretically hypothesized as a slightly denatured state in which solvent molecules have not yet penetrated the hydrophobic cores of the proteins (*13*); an equivalent state, in which side chains are free to rotate (*14*), but individual peptide NH is resistant to exchange with solvent (*39*), has been detected as a denaturation intermediate of ribonuclease A. Similar observations have been reported for partly denatured equine lysozyme; amides in three of the four major helices of the native protein are significantly protected (*9*) and tertiary interactions are highly retained (*10*). A compact denaturation intermediate of barstar observed in an initial burst phase of the refolding does not show any increase in ANS binding suggesting that the intermediate does not possess solvent-exposed hydrophobic clusters (*40*). In contrast, OVA displayed at pH 2.2 the loss of tertiary interactions and an increase in ANS binding (Figs. 2 and 8). The highly ordered molten globule-like state of OVA at pH 2.2 may be less ordered than the "dry" molten globule state.

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