Conformational State of Ovalbumin at Acidic pH as Evaluated by a Novel Approach Utilizing Intrachain Sulfhydryl-Mixed Disulfide Exchange Reactions†

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Received February 13, 1998; Revised Manuscript Received May 19, 1998

ABSTRACT: Ovalbumin contains four cysteine sulfhydryls (Cys11, Cys30, Cys367, and Cys382) and one cystine disulfide (Cys73-Cys120). A highly reactive aromatic disulfide, 2,2′-dipyridyl disulfide, reacts specifically with Cys367 of ovalbumin at pH 2.2 generating a mixed disulfide protein derivative [Tatsumi, E., and Hirose, M. (1997) *J. Biochem. 122*, 300−308]. The mode of conformational fluctuation in ovalbumin was investigated at pH 2.2 using the mixed disulfide derivatives of the cystine-intact and cystine-reduced protein forms. In the presence of a high concentration of urea, both the mixed disulfide derivatives underwent rapid cysteine sulfhydryl/mixed disulfide exchanges, thereby releasing the quantitative amount of 2-thiopyridone. A peptide mapping analysis for disulfide-forming cysteines revealed that this release was mostly accounted for by the nucleophile attack on the Cys367-mixed disulfide by the nearest cysteine residue in the primary structure, Cys382. At the acidic pH, the exchange reaction was practically restricted to the cysteine sulfhydryl/mixed disulfide exchanges; no other exchange reaction, such as the cysteine sulfhydryl/cystine disulfide exchange reaction, was detected. In the absence of urea, the cystine-reduced form, but not the cystine-intact form, underwent significant sulfhydryl/mixed disulfide exchange reactions at a physiological temperature, as determined by the release of 2-thiopyridone. A kinetic analysis for the generation of disulfide-forming cysteines with Cys367 at 37 °C revealed that the rate for the intrachain exchange reaction was quite different for the five cysteine sulfhydryls. The effective concentrations of the five cysteine sulfhydryls relative to the Cys367-mixed disulfide were determined by using three related model reactions: the obtained values were 11.4, 4.6, 15.2, 5.9, and 8.9 μM for Cys11, Cys30, Cys73, Cys120, and Cys382, respectively. Implications of the effective concentrations for the conformational state of acidic ovalbumin are discussed.

Globular proteins are transformed from their native state into different non-native states under various denaturing conditions, such as extreme pH, high denaturant concentrations, and elevated temperatures (*1*). Studies of the non-native state are important for the understanding of protein folding mechanism, since highly denatured protein is usually employed as the starting sample for refolding experiments. Furthermore, the equilibrium molten globule state having a partially denatured conformation has been shown to be similar to or identical with a kinetic intermediate at an early refolding stage (*2*−*7*). Methodological approach for investigating the non-native state is, however, very limited, since the state, unlike the native one, includes a vast number of conformational isomers.

As a useful approach, the intramolecular sulfhydryl/disulfide exchange reaction has been employed for analyzing the non-native conformational state. The rate for the exchange reaction depends on the accessibility, hence the effective concentration, of a disulfide relative to a sulfhydryl. In a protein with multiple sulfhydryls and disulfides, many combinations of the exchange reactions proceed, generating various disulfide isomers at equilibrium; the relative occurrence of the disulfide isomers should reflect the non-native conformational states. On the basis of this idea, the highly denatured state of OVA[1] (*8*) and the molten globule state of the partially disulfide-reduced (*9*, *10*) or disulfide-engineered (*11*−*13*) α-lactalbumin have been extensively investigated using the sulfhydryl/disulfide exchange approach. Such an approach, however, does not practically work at acidic pH where the rate for cysteine sulfhydryl/disulfide exchange reaction is usually very slow, although the partially denatured conformational state is most widely induced at acidic pH.

Using OVA as a model protein, we have attempted to develop an alternative sulfhydryl/disulfide exchange approach

† This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan. E. Tatsumi was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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[1] Abbreviations: HPLC, high-performance liquid chromatography; IAEDANS, *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; IAM, iodoacetamide; Pyr-S-S-Pyr, 2,2′-dipyridyl disulfide; Pyr-SH, 2-thiopyridone; Pyr-S-S-Cys, *S*-(pyridin-2-ylthio)-L-cysteine. OVA and $_R$-OVA represent the ovalbumin form with the native cystine disulfide (Cys73-Cys120) and its cystine-reduced form, respectively. The subscripts attached to OVA and $_R$OVA represent non-native disulfides; -SS, -SS-Pyr, and -SS-Cys are intrachain non-native cystine disulfide, thiopyridine-mixed disulfide, and L-cysteine-mixed disulfide, respectively. Disulfide structures are shown using residue numbers in brackets: e.g., [73-120] for Cys73-Cys120; [367-Pyr] for thiopyridine-mixed disulfide form of Cys367; [367-Cys] for cysteine-mixed disulfide form of Cys367.

S0006-2960(98)00353-5 CCC: $15.00
Published on Web 08/15/1998

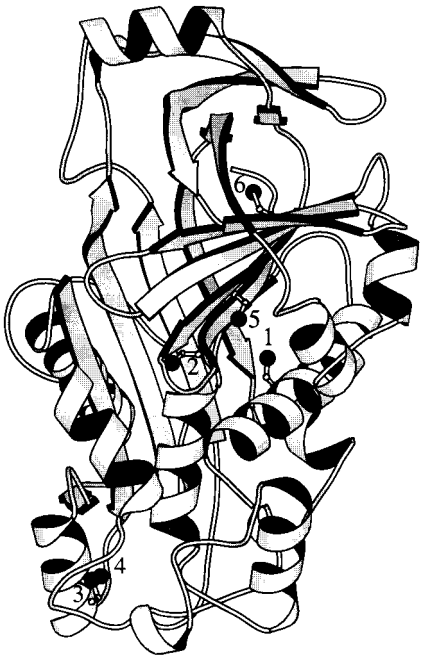

FIGURE 1: Schematic view of ovalbumin. The figure is based on the X-ray crystallographic data of ovalbumin (*14*) and was drawn using the MolScript program (*37*). The numbered shaded spheres represent the sulfur atoms: 1, Cys11; 2, Cys30; 5, Cys367; 6, Cys382. The sulfur atoms 3 (Cys73) and 4 (Cys120) form the native disulfide bond.

that allows us to analyze the non-native conformational states at acidic pH. OVA, a member of the serpin superfamily, consists of 385 amino acid residues that fold into a globular conformation with a high secondary structure content (30.6% α-helix and 31.4% β-strand), as shown in Figure 1 (*14*). The protein contains six cysteine residues of Cys11, Cys30, Cys73, Cys120, Cys367, and Cys382; in the native state, Cys11, Cys30, Cys367, and Cys382 exist as free cysteine sulfhydryls, while Cys73 and Cys120 form a cystine disulfide bond (*15, 16*). Several lines of evidence have shown that OVA assumes a highly ordered molten globule-like conformation at pH 2.2 (*17, 18*). At this acidic pH, an aromatic disulfide, Pyr-S-S-Pyr, which is known to be reactive with protein sulfhydryls even under acidic conditions (*19, 20*), reacts with only Cys367 of OVA (*18*). This reaction (reaction 1) should yield a mixed disulfide derivative of the protein:

$$\text{OVA} + \text{Pyr-S-S-Pyr} \rightarrow \text{OVA-SS-Pyr} + \text{Pyr-SH} \quad (1)$$

The high reactivity of Pyr-S-S-Pyr is due to the anomalously low pK value of −1.1 for the sulfhydryl of the leaving group of Pyr-SH and to the almost exclusive occurrence of the leaving group as the tautomeric thio form with the mobile hydrogen attached to the nitrogen (*19, 20*). Therefore, intrachain exchange reactions between the mixed disulfide and some of the other cysteine sulfhydryls, in which Pyr-SH is again the leaving group, should be induced even at acidic pH, if the sulfhydryls can contact the mixed disulfide in a non-native acidic state:

$$\text{OVA-SS-Pyr} \rightarrow \text{OVA-SS} + \text{Pyr-SH} \quad (2)$$

Any other exchange reaction such as the cysteine sulfhydryl/cystine disulfide exchange is unlikely to proceed at acidic pH (*21*−*24*). The exchange reaction of eq 2 (reaction 2) which should depend on the extent of conformational fluctuation can be monitored from the visible absorption of released Pyr-SH. As an alternative exchange reaction that includes the release of Pyr-SH, the highly reactive pyridine-mixed disulfide can be replaced by a much less reactive group of cysteine-mixed disulfide, according to the following bimolecular reaction (reaction 3):

$$\text{OVA-SS-Pyr} + \text{L-Cys} \rightarrow \text{OVA-SS-Cys} + \text{Pyr-SH} \quad (3)$$

This reaction can be utilized for the quenching of reaction 2, which is required for a subsequent chemical analysis of disulfide-forming cysteines in OVA-SS. By analyzing the rate for the formation of an individual non-native disulfide bond in OVA-SS, the effective concentrations of the corresponding protein sulfhydryl relative to the Cys367-mixed disulfide should be determined.

In the present report, the cystine-reduced form as well as the cystine-intact form were investigated as model proteins, since the native cystine disulfide is not essential for the native folding at near neutral pH (*25, 26*). For the sulfhydryl/mixed disulfide exchange approach to correctly work, it is prerequisite that the exchange reaction is practically restricted to reaction 2. This was confirmed by a peptide mapping analysis for disulfide-forming cysteines that had been generated by exchange reactions in the presence of a high concentration of urea at pH 2.2. In the absence of urea, $_{R}$-OVA-SS-Pyr, but not OVA-SS-Pyr, underwent the sulfhydryl/mixed disulfide exchange reaction at a physiological temperature, as determined by the release of Pyr-SH. A kinetic analysis for the generation of disulfide-forming cysteines with Cys367 revealed that the rate for the intrachain exchange reaction was quite different for Cys11, Cys30, Cys73, Cys120, and Cys382. The effective concentrations of the five cysteine sulfhydryls relative to the Cys367-mixed disulfide were determined by an indirect but feasible way. The conformational state of acidic $_{R}$OVA was discussed in relation to the effective concentrations and to previous structural data.

EXPERIMENTAL PROCEDURES

Materials. OVA was prepared as described before (*8*). IAEDANS was purchased from Aldrich Co., Inc. Other chemicals including dithiothreitol, urea, Pyr-S-S-Pyr, and IAM were guaranteed grade from Nacalai Tesque. Pyr-S-S-Cys was produced by incubating 3.5 mM L-cysteine and 4.5 mM Pyr-S-S-Pyr in 0.1% trifluoroacetic acid at 37 °C for 10 min. The sample was applied to a Cosmosil 5C_{18}-AR120A column (20 × 250 mm) equilibrated with 0.1% trifluoroacetic acid, and L-cysteine, cystine, and Pyr-SH were eluted in wash-through fractions. Pyr-S-S-Cys was eluted with 0−40% linear gradient of acetonitrile, being clearly separated from Pyr-S-S-Pyr. Major Pyr-S-S-Cys peak was collected, lyophilized, and further purified by rechromatography using the same column and solvent system. The identification of Pyr-S-S-Cys was performed by the amino acid analysis and by an optical quantification of released Pyr-SH upon incubation with 20 mM dithiothreitol in 50 mM Tris-HCl/1.0 mM Na-EDTA buffer, pH 8.2, at 37 °C for 10 min.

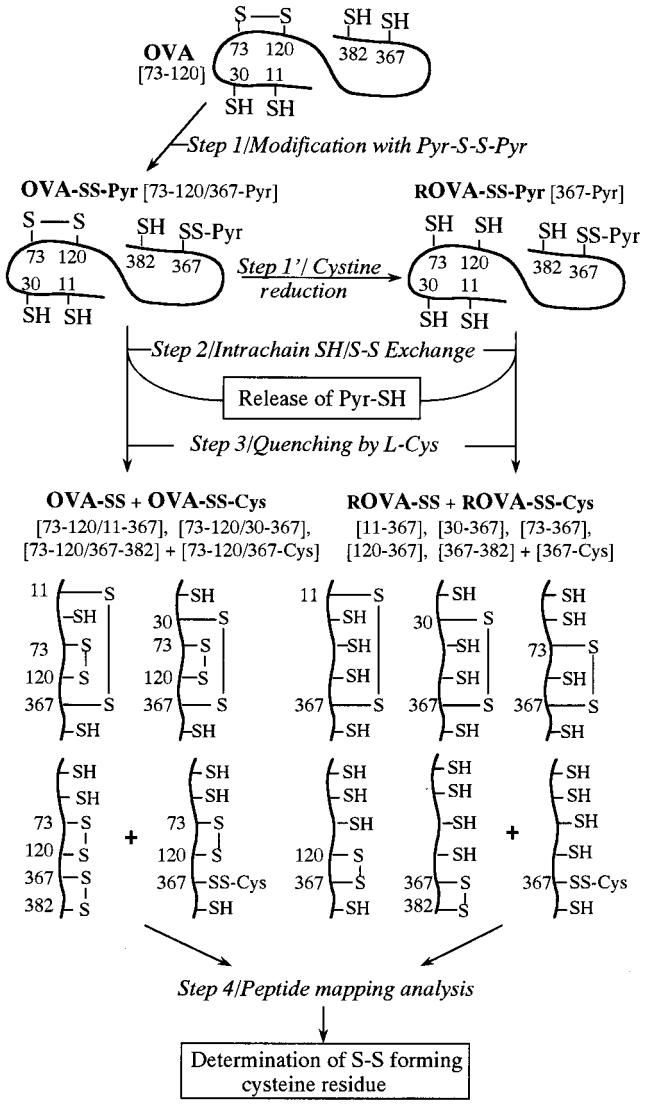

FIGURE 2: Strategy for analyzing the non-native conformational state of ovalbumin at acidic pH using the intrachain sulfhydryl/mixed disulfide exchange reactions. As step 1, Cys367 of OVA with the native cystine ([73−120]) is modified at pH 2.2 with Pyr-S-S-Pyr giving a mixed disulfide form of OVA-SS-Pyr ([73-120/367-Pyr]), according to reaction 1. In step 1′, the native cystine is reduced at near-neutral pH with dithiothreitol, producing $_R$OVA-SS-Pyr ([367-Pyr]). OVA-SS-Pyr and $_R$OVA-SS-Pyr are subjected to some denaturing conditions at acidic pH as step 2. In this step, the intrachain sulfhydryl/mixed disulfide exchange occurs by reaction 2 between the cysteine sulfhydryls and the mixed disulfide, thereby releasing Pyr-SH. Other exchange reactions, such as the reaction between the cysteine sulfhydryls and cystine disulfide, are not induced. The exchange reactions are quenched in step 3 by addition of excess L-cysteine according to reaction 3. Consequently, OVA-SS consisting of three different disulfide isomers ([73-120/11-367], [73-120/30-367], [73-120/367-382]) and OVA-SS-Cys ([73-120/367-Cys]) should be produced from OVA-SS-Pyr. From $_R$OVA-SS-Pyr, $_R$OVA-SS consisting of five disulfide isomers ([11-367], [30-367], [73-367], [120-367], and [367-382]) and $_R$OVA-SS-Cys ([367-Cys]) are produced. The disulfide-forming cysteines can be determined by the peptide mapping analysis as step 4. Cys367 should form a disulfide in all the disulfide isomers, while the extent of disulfide formation of the other cysteines should depend on the their effective concentrations relative to the Cys367-mixed disulfide.

Strategy and Experimental Conditions for Sulfhydryl/Mixed Disulfide Exchange Analysis. Figure 2 schematically shows the strategy for analyzing the mode of intrachain sulfhydryl/mixed disulfide exchanges in OVA and $_R$OVA at acidic pH. In step 1, Cys367 of OVA is specifically modified with Pyr-S-S-Pyr yielding a mixed disulfide derivative OVA-SS-Pyr ([73-120/367-Pyr]) according to reaction 1. This was performed by incubating OVA at 10 mg/mL in buffer A (0.1 M potassium phosphate-HCl, pH 2.2) at 25 °C with 0.5 mM Pyr-S-S-Pyr for 2 h or with 1.5 mM Pyr-S-S-Pyr for 1.5 h. The number of cysteines modified with Pyr-S-S-Pyr was determined from the released Pyr-SH using the molar extinction coefficient of 7345 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 Inc., NAP-25) equilibrated with buffer A. For the preparation of cystine-reduced, mixed disulfide form ($_R$OVA-SS-Pyr) as step 1′, the gel filtration buffer was substituted by buffer B (50 mM Tris-HCl buffer, pH 8.2/1 mM Na-EDTA). Then, the sample was incubated with 15 mM dithiothreitol at 37 °C for 1 h. Obtained $_R$OVA-SS-Pyr was passed through a Sephadex G-25 column equilibrated with buffer A. OVA-SS-Pyr and $_R$OVA-SS-Pyr were stored at 4 °C and used within 2 h for a subsequent analysis. In step 2, these protein derivatives were subjected to the sulfhydryl/mixed disulfide exchanges according to reaction 2. For the exchange experiments in an acid/urea solution, OVA-SS-Pyr and $_R$OVA-SS-Pyr were denatured by incubation at 1.0 mg/mL in buffer A containing 6 M urea for various times at 25 °C. Bulk exchange reactions were monitored by measuring the absorption of the released Pyr-SH using the same molar extinction coefficient of 7345 at 343 nm. As step 3, the sulfhydryl/mixed disulfide exchanges were quenched by addition of 1.5 volume of buffer A containing 6 M urea and 83.3 mM L-cysteine, according to reaction 3. After 10 min of incubation at 25 °C, urea and excess L-cysteine were removed by gel filtration using a Sephadex G-25 column (Pharmacia Biotech Inc., NAP-5) equilibrated with buffer A. The protein sample was mixed with 9 volumes of buffer B containing 10 M urea and 222 mM IAM, alkylated by incubation in this neutral/urea buffer at 37 °C for 10 min, and then subjected to the analysis for the disulfide-forming cysteines, as step 4. The disulfide-forming cysteines were determined by selective IAEDANS alkylation and subsequent peptide mapping analysis as described previously (*8*). Briefly, in the sample run, the alkylated protein was fully reduced with dithiothreitol and the newly generated sulfhydryls were alkylated with a fluorescent reagent of IAEDANS. The sample was subjected to extensive proteolysis by trypsin, chymotrypsin, and *Achromobacter* protease I and analyzed by reverse-phase HPLC as monitored by fluorescence (excitation, 340 nm; emission, 520 nm) and by absorption at 220 nm. In the standard run, the protein was treated in the same way except that the first alkylation step with IAM was skipped. The disulfide-forming number for each of the six cysteine residues (N_{Cys-i}, i = 11, 30, 73, 120, 367, or 382) was determined using the equation: $N_{Cys-i} = (A_{Cys-i}/B_{Cys-i})/(A_{pep}/B_{pep})$, where A_{Cys-i} and B_{Cys-i} represent fluorescent peak areas for cysteine peptides in the sample and standard runs, respectively, with respect to a cysteine residue, Cys-*i*. A_{pep} and B_{pep} are the areas for peptide absorption peaks at 220 nm in the sample and standard runs, respectively. For measuring A_{pep} and B_{pep}, we employed the sum of the areas of two non-cysteine peptide peaks that were clearly separated from other bulk peaks in the HPLC analysis.

When the exchange reactions at the acidic pH were examined in the absence of urea (step 2), OVA-SS-Pyr and $_R$OVA-SS-Pyr were incubated in buffer A at various temperatures for 2 h or for various times at 37 °C. Bulk exchange reactions were monitored optically in the same way. For the determinations of disulfide-forming cysteines, the mixtures were vigorously mixed with 1.5 volumes of buffer A containing 83.3 mM L-cysteine and 10 M urea, incubated at 25 °C for 10 min (step 3), and analyzed by the peptide mapping procedure (step 4) in the same way.

Determination of Effective Concentrations. The effective concentrations of cysteine sulfhydryls relative to the mixed disulfide were determined using the rate constants for three bimolecular model reactions (see eqs 7−9 in Results and Discussion). For the first model reaction (eq 7), $_R$OVA-SS-Pyr was incubated at 1.0 mg/mL (22 μM) with various higher concentrations (1−4 mM) of L-cysteine at 37 °C in buffer A containing 6 M urea and the release of Pyr-SH was measured by absorption at 343 nm. The pseudo-first-order rate constants obtained from the time course of Pyr-SH release were plotted as a function of L-cysteine concentrations, and the second-order rate constant for the reaction was obtained from the slope of the plot. For the second model reaction (eq 8), L-cysteine was incubated at 22 μM with 0.22−1.1 mM Pyr-S-S-Cys under the same conditions, and the second-order rate constant was determined in the same way. For the third model reaction (eq 9), $_R$OVA (cystine-reduced, nonmodified protein) was incubated at 22 μM with 2.2 mM Pyr-S-S-Cys in buffer A containing 6 M urea incubated at 37 °C for varying times. The reaction was quenched by 50 mM L-cysteine, and disulfide-forming cysteine was quantified by the peptide mapping procedure in the same way. The pseudo-first-order rate constants for the six cysteine residues were obtained from the time courses of their disulfide formation; for the second-order rate constants, the pseudo-first-order rate constants were divided by the Pyr-S-S-Cys concentration.

RESULTS AND DISCUSSION

Reliability of Sulfhydryl-Mixed Disulfide Exchange Approach. Previous reports have shown that OVA assumes a highly ordered molten globule-like conformation (*17*, *18*) at pH 2.2 as evaluated by conventional spectroscopic techniques and that the cystine disulfide Cys73-Cys120 is not essential for the native folding at near-neutral pH (*25*, *26*). We have also shown that Pyr-S-S-Pyr reacts specifically with Cys367 of OVA at pH 2.2 yielding a mixed disulfide derivative OVA-SS-Pyr (*18*). No conformational alteration is detected in OVA by the introduction of thiopyridine-mixed disulfide group (*18*). These prompted us to investigate the mode of conformational fluctuation by intrachain sulfhydryl/mixed disulfide exchange analyses in both the cystine-intact and cystine-reduced forms of the protein at acidic pH, according to the strategy shown in Figure 2. The strategy, however, includes several experimental steps. It was investigated whether the experimental steps correctly work for the intrachain sulfhydryl/mixed disulfide exchange analysis.

The specific modification of Cys367 with Pyr-S-S-Pyr in step 1 according to reaction 1 that had been established in a previous report (*18*) was reconfirmed in the present study (see the data for Cys367 in Figures 4, 5, and 7). To prepare the mixed disulfide form of $_R$OVA as step 1′, the native cystine in OVA-SS-Pyr was reduced by incubation with dithiothreitol at pH 8.2. Figure 3 clearly demonstrates the production of $_R$OVA-SS-Pyr ([367-Pyr]), in which almost all of the cystine disulfides were reduced (more quantitatively, see the data at incubation time 0 in Figures 5 and 7).

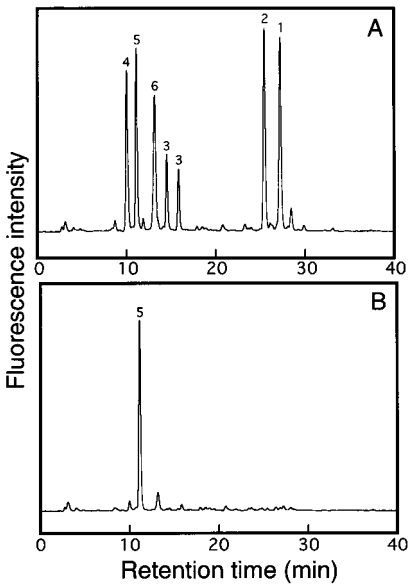

FIGURE 3: Identification of the disulfide-forming cysteine residue in $_R$OVA-SS-Pyr. The number of introduced thiopyridine groups was 0.88 per protein molecule as determined by absorption of the released Pyr-SH in step 1 in Figure 2. To localize the mixed disulfide-forming cysteine, the thiopyridine-mixed disulfide group of $_R$OVA-SS-Pyr was replaced by L-cysteine according to reaction 3 (see text). In panel A, all six cysteine residues were alkylated with IAEDANS as the standard run, as described in the text. In panel B, the free sulfhydryls of the protein were alkylated by IAM, and the disulfide-forming cysteine residues were identified by selective IAEDANS alkylation and subsequent peptide mapping analysis. The numbers above the peaks of 1, 2, 3, 4, 5, and 6 represent the elution of cysteine peptides for Cys11, Cys30, Cys73, Cys120, Cys367, and Cys382, respectively.

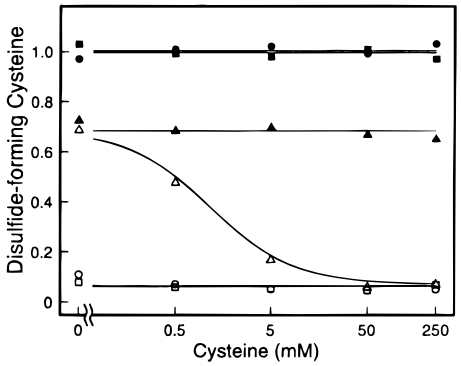

FIGURE 4: Quenching the intrachain sulfhydryl/mixed disulfide exchanges by bimolecular L-cysteine. OVA-SS-Pyr was incubated at 1.0 mg/mL in buffer A containing different concentrations of L-cysteine and 6 M urea and allowed to stand for 10 min at 25 °C, so that OVA-SS-Pyr was transferred to OVA-SS-Cys or OVA-SS. After urea and excess L-cysteine were removed by gel filtration, the protein was alkylated with 0.2 M IAM in the neutral/urea solution as described in the text. The disulfide-forming cysteines for Cys11 (□), Cys30 (○), Cys73 (●), Cys120 (■), Cys367 (▲), and Cys382 (△) were determined by peptide mapping analysis as described in the text. The number of the modified Cys367 was 0.69 in the original OVA-SS-Pyr.

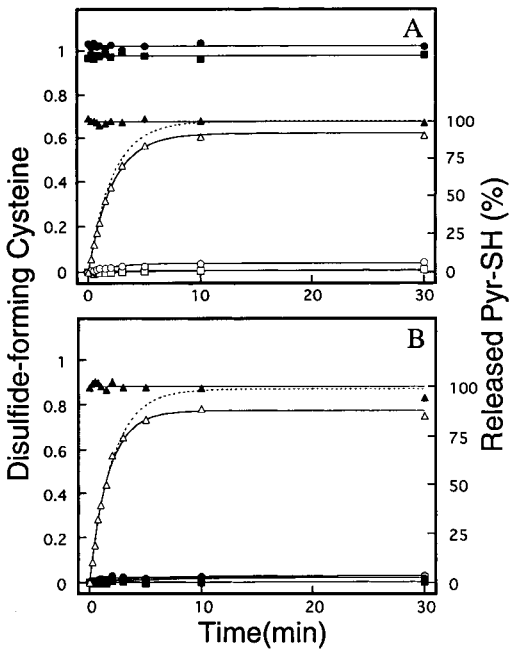

FIGURE 5: Time course for the exchange reactions in acid/urea solution. OVA-SS-Pyr (A) and $_{R}$OVA-SS-Pyr (B) were incubated at 1.0 mg/mL in buffer A containing 6 M urea for varying times at 25 °C, and the sulfhydryl/mixed disulfide exchanges were quenched by incubation at 25 °C for 10 min with 50 mM L-cysteine. The samples were passed through a gel filtration column and then alkylated with 0.2 M IAM in the neutral/urea solution. The disulfide-forming cysteines for Cys11 (□), Cys30 (○), Cys73 (●), Cys120 (■), Cys367 (▲), and Cys382 (△) and the percentage of the released Pyr-SH (···) were determined as described in the text. The number of the modified Cys367 was 0.69 in OVA-SS-Pyr and 0.88 in $_{R}$OVA-SS-Pyr as determined by absorption of the released Pyr-SH in step 1 in Figure 2.

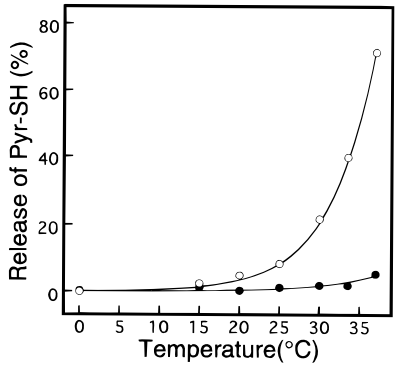

FIGURE 6: Bulk exchange reactions at different temperatures in the absence of denaturant as monitored by the release of Pyr-SH. OVA-SS-Pyr (●) and $_{R}$OVA-SS-Pyr (○) were incubated at 1.0 mg/mL in buffer A for 2 h at varying temperatures, and the released Pyr-SH was determined by absorption at 343 nm. The number of the modified Cys367 was 1.0 in OVA-SS-Pyr and 0.90 in $_{R}$OVA-SS-Pyr.

If the protein sulfhydryls can contact the Cys367-mixed disulfide in a non-native state, sulfhydryl/mixed disulfide exchange reactions will be induced by reaction 2 in step 2 thereby releasing Pyr-SH and at the same time generating cystine disulfide isomers. As the disulfide isomers, three molecular species of [73-120/11-367], [73-120/30-367], and [73-120/367-382] for OVA-SS-Pyr and five species of [11-367], [30-367], [73-367], [120-367], and [367-382] for $_{R}$-OVA-SS-Pyr should be possible. Bulk exchange reactions can be monitored by absorption of released Pyr-SH. Quantitative analyses for the disulfide-forming cysteines may provide information about the accessibilities of the free sulfhydryls relative to the Cys367-mixed disulfide. For the determination of disulfide-forming cysteines, however, the blocking of free protein sulfhydryls by alkylation in a high concentration of urea at near-neutral pH is a prerequisite. In a preliminary experiment, we found that IAM at a concentration as high as 0.2 M was unable to quench the sulfhydryl/mixed disulfide exchanges because of quite a rapid rate for reaction 2 at near-neutral pH, when OVA-SS-Pyr was directly transferred to a neutral IAM/urea mixture. We have, however, found that alkylation with the same concentration of IAM in a neutral/urea solution is sufficient to quench the sulfhydryl/disulfide exchange in OVA-SS-Cys (*18*). Thus, we first replaced as step 3 the mixed disulfide group of thiopyridine by L-cysteine at pH 2.2 in buffer A containing 6 M urea according to reaction 3. This reaction should be competitive with reaction 2 in the acid/urea solution. The experiments were, therefore, carried out with varying L-cysteine concentrations. As shown in Figure 4, when OVA-SS-Pyr was incubated with varying concentrations of L-cysteine under the acid/urea conditions and then alkylated with 0.2 M IAM in neutral/urea solution, the levels of disulfide-forming cysteines showed almost invariable data of about

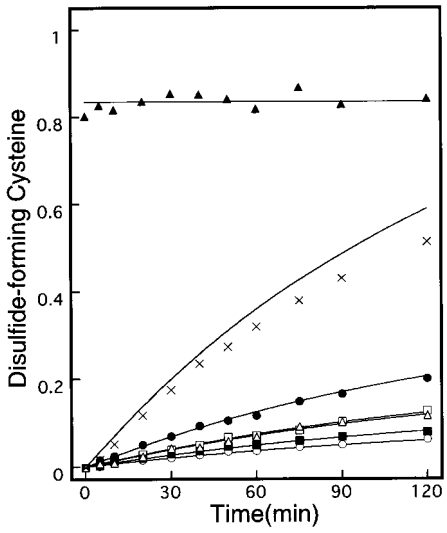

FIGURE 7: Time course of sulfhydryl/mixed disulfide exchanges in the absence of denaturant. $_{R}$OVA-SS-Pyr (the number of the modified Cys367, 0.88) was incubated at 1.0 mg/mL and 37 °C in buffer A, and the released Pyr-SH (×) was determined at different incubation times by absorption at 343 nm. The mixtures were vigorously mixed with 1.5 volumes of buffer A containing 83.3 mM L-cysteine and 10 M urea and incubated at 25 °C for 10 min. The disulfide-forming cysteines for Cys11 (□), Cys30 (○), Cys73 (●), Cys120 (■), Cys367 (▲), and Cys382 (△) were determined as described in the text. The rate constants for the intrachain sulfhydryl/mixed disulfide exchange reactions, $k_{\text{intra(Cys}-i\text{)}}$ (i = 11, 30, 73, 120, 367, or 382), were estimated by nonlinear least-squares fitting using the equations:

$$[N_{\text{Cys}-i}] = \frac{k_{\text{intra(Cys}-i\text{)}}}{k_{\text{B}}}\{1 - \exp(-k_{\text{B}}t)\}$$

$$k_{\text{B}} = k_{\text{intra(Cys11)}} + k_{\text{intra(Cys30)}} + k_{\text{intra(Cys73)}} + k_{\text{intra(Cys120)}} + k_{\text{intra(Cys382)}}$$

$$[\text{Pyr-SH}] = 1 - \exp(-k_{\text{B}}t)$$

where $N_{\text{Cys}-i}$ is the disulfide-forming number for each of the five cysteine residues (see Experimental Procedures) at reaction time t. The constant k_{B} is the first-order rate constant for the bulk exchange reactions.

1.0 in all tested L-cysteine concentrations for the native cystine-forming Cys73 and Cys120. For Cys11 and Cys30, disulfide-forming cysteine levels were very low at all the L-cysteine concentrations. The disulfide-forming level for Cys382 was almost the same as that for Cys367 in the absence of L-cysteine but markedly decreased with increasing L-cysteine concentrations to a minimum level at L-cysteine concentrations higher than 50 mM. These results indicated that the presence of 50 mM L-cysteine is sufficient to quench reaction 2 in the acid/urea solution as step 3. The disulfide-forming cysteines in the protein samples that contain the cystine disulfide isomers and the cysteine-mixed disulfide derivative can be determined in step 4 by the peptide mapping analysis established previously (*8*).

For the present strategy to correctly work, it is prerequisite that exchange reactions in step 2 are practically restricted to reaction 2. The sulfhydryl/disulfide exchange reaction proceeds in such a way that an ionized sulfhydryl attacks the nucleophile to the central sulfur of a disulfide, thereby leaving the other group sulfur:

$$R_{nuc}\text{-}S^- + R_c\text{-}S\text{-}S\text{-}R_{lg} \rightarrow R_{nuc}\text{-}S\text{-}S\text{-}R_c + R_{lg}\text{-}S^- \quad (4)$$

where the subscripts of nuc, c, and lg represent nucleophile, central group, and leaving group, respectively. As sulfhydryl/disulfide exchange reactions other than reaction 2, two side reactions by cysteine nucleophile (sides reaction a and b) are possible. Side reaction a is the nucleophile attack of a cysteine sulfhydryl (Cys_{nuc}-S^-) to a cystine disulfide (Cys_c-S-S-Cys_{lg}) producing the new cystine disulfide (Cys_{nuc}-S-S-Cys_c) and cysteine sulfhydryl (Cys_{lg}-S^-) in a protein molecule. The other (side reaction b) is the nucleophile attack of a cysteine sulfhydryl (Cys_{nuc}-S^-) to the thiopyridine-mixed disulfide (Pyr_c-S-S-Cys_{lg}) producing the new mixed disulfide (Cys_{nuc}-S-S-Pyr_c) and cysteine sulfhydryl (Cys_{lg}-S^-). In addition, Pyr-SH released in a first exchange reaction may make the nucleophile attack on a protein disulfide as bimolecular side reactions (side reactions c and d). Pyr-SH (Pyr_{nuc}-S^-) attacks, in side reaction c, a cystine disulfide (Cys_c-S-S-Cys_{lg}) producing the new protein-mixed disulfides (Pyr_{nuc}-S-S-Cys_c) and cysteine sulfhydryl (Cys_{lg}-S^-). In side reaction d, Pyr-SH attacks the protein thiopyridine-mixed disulfide (Pyr_c-S-S-Cys_{lg}) generating free Pyr_{nuc}-S-S-Pyr_c and the protein sulfhydryl (Cys_{lg}-S^-).

The rate of the exchange reaction follows a Brønsted relation; the rate constant, *k*, for the reaction can be expressed in units of s^{-1} M^{-1} with inclusion of the ionization term of nucleophile as follows:

$$\log k = C + \beta_{nuc}pK_{nuc} + \beta_c pK_c + \beta_{lg}pK_{lg} - \log(1 + 10^{pK_{nuc}-pH}) \quad (5)$$

The Brønsted coefficients estimated using many combinations of model sulfhydryl and disulfide compounds (*21*, *22*, *27*) are 0.5, −0.27, and −0.73 for β_{nuc}, β_c, and β_{lg}, respectively. The p*K* value for leaving Pyr-SH is −1.1 (*20*), while the p*K* values of protein sulfhydryls vary from 8.5 to 9.1 (*21*). If a p*K* value of 8.8 is taken for L-cysteine sulfhydryl and for a typical protein sulfhydryl, the rate constant for reaction 2 should be greater at pH 2.2 by 1.7×10^7-, 3.6×10^4-, 3.8×10^5-, and 8.0×10^2-fold than the rate constants for side reactions a, b, c, and d, respectively. These values indicate that reaction 2 is the highly predominant one as a sulfhydryl/disulfide exchange reaction under the acidic conditions. Such highly predominant exchange reaction of reaction 2 was confirmed by experimental observations.

We examined the mode of the exchanges using the acid/urea denaturing conditions. In a previous report (*8*), the effective concentration of a disulfide relative to cysteine sulfhydryls in urea-denatured OVA at near-neutral pH has been shown to depend on the number of amino acid residues separating the two cysteines to a power of about −2. The numbers of amino acid residues separating Cys367 from Cys11, Cys30, Cys73, Cys120, and Cys382 are 356, 337, 294, 247, and 15, respectively. If reaction 2 is the highly predominant exchange reaction at the acidic pH, the produced disulfide isomers should consist almost totally of [73-120/367-382] for OVA-SS-Pyr and of [367-382] for $_R$OVA-SS-Pyr. Figure 5 (panels A and B) demonstrates that this is indeed the case. The release of Pyr-SH was almost completed within 10 min of the incubation for either OVA-SS-Pyr or $_R$OVA-SS-Pyr. This increase should be largely accounted for by the exchange reaction between Cys367-mixed disulfide and Cys382 sulfhydryl, since marked and concomitant increase in the disulfide-forming cysteine was observed only for Cys382.

Furthermore, the data for the disulfide-forming Cys73, Cys120, and Cys367 exclude the possibility for involvement of the four side reactions. A cysteine sulfhydryl in side reaction a and free Pyr-SH in side reaction c attack the native cystine disulfide Cys73-Cys120. The amounts of disulfide-forming cysteines for Cys73 and Cys120, therefore, should be decreased during the incubation time, if side reaction a or c is involved in the exchange reactions in the acid/urea condition. Likewise, the involvement of side reaction b or d should result in decrease in the amount of disulfide-forming Cys367, since the Cys367-mixed disulfide is attacked by a cysteine sulfhydryl in side reaction b and by free Pyr-SH in side reaction d. The data in Figure 5, however, clearly demonstrate that no significant decrease was detected for the disulfide-forming Cys73, Cys120, or Cys367 during the sulfhydryl/mixed disulfide exchange reactions. The retain of initial level of disulfide-forming Cys367 was also confirmed during the intrachain exchange reactions in the absence of a denaturant (see Figure 7).

To examine the possibility for the involvement of the side exchange reactions by an alternative way, we first incubated OVA-SS-Pyr with 50 mM L-cysteine in the acid/urea solution thereby generating OVA-SS-Cys and Pyr-SH by reaction 3 and then let the mixture stand for various times under the same acid/urea conditions. We found that the initial levels of disulfide-forming Cys73, Cys120, and Cys367 were retained during the incubation time. In addition, no exchange reaction was detected in nonmodified OVA between the four cysteine sulfhydryls (Cys11, Cys30, Cys367, Cys382) and the native cystine disulfide Cys73-Cys120 under the same acid/urea-denaturing conditions as in Figure 5. These results reinforce the practical absence of the nucleophile attack reaction by a cysteine sulfhydryl (side reaction a) or by free Pyr-SH (side reaction c) on the cysteine-mixed disulfide or on the native cystine Cys73-Cys120.

On the basis of the theoretical and experimental results, we concluded that the intrachain exchange reactions in OVA-

Table 1: Effective Concentrations of the Five Cysteine Sulfhydryls Relative to Cys367-Mixed Disulfide[a]

	kinetic constants			
cysteine	k_{bi-3} (M^{-1} min^{-1})	k_{bi} (M^{-1} min^{-1})	k_{intra} (min^{-1})	C_{eff} (μM)
Cys11	479	136	0.00154	11.4
Cys30	578	164	0.00076	4.6
Cys73	599	170	0.00259	15.2
Cys120	618	175	0.00103	5.9
Cys382	591	168	0.00149	8.9

[a] The kinetic constants, k_{bi-3}, k_{bi}, and k_{intra}, were determined as described in the text. The effective concentrations of the five cysteine residues relative to Cys367-mixed disulfide were calculated using the k_{bi} and k_{intra} values according to eq 6 in the text.

SS-Pyr and $_R$OVA-SS-Pyr are practically restricted to reaction 2.

Sulfhydryl/Mixed Disulfide Exchanges in the Absence of a Denaturant. The bulk exchange reactions in OVA-SS-Pyr and $_R$OVA-SS-Pyr were investigated in the absence of a denaturant at the acidic pH by monitoring the release of Pyr-SH. As shown in Figure 6, at a temperature lower than 15 °C, no release of Pyr-SH was observed either for OVA-SS-Pyr or for $_R$OVA-SS-Pyr at a prolonged incubation time of 2 h. In $_R$OVA-SS-Pyr, significant exchange reactions were detected at physiological temperatures from 20 to 37 °C; at 37 °C, more than 70% of the thiopyridine-mixed disulfide underwent the exchange reactions during the incubation time of 2 h. In the same temperature range, however, very few exchange reactions occurred in OVA-SS-Pyr; only 5.4% of the thiopyridine-mixed disulfide underwent the exchange reactions at 37 °C. These data strongly suggest that $_R$OVA-SS-Pyr has more fluctuating nature than OVA-SS-Pyr.

To investigate the mode of conformational fluctuation in $_R$OVA-SS-Pyr by the sulfhydryl/mixed disulfide exchange reactions, we determined the rates for the formation of different disulfide isomers ($_R$OVA-SS) produced at 37 °C. Figure 7 represents the time courses for the release of Pyr-SH and for the production of the disulfide-forming Cys11, Cys30, Cys73, Cys120, and Cys382. The data in Figure 7 provide important information. First, unlike the urea denaturation (Figure 5B), Cys11, Cys30, Cys73, Cys120, and Cys382 all increased as disulfide-forming cysteines with different kinetics, suggesting that the kinetic data can be related to the accessibilities of the five exchange-competent sulfhydryls to the Cys367-mixed disulfide in the absence of urea at pH 2.2. Second, the time course for the sum of the levels of disulfide-forming Cys11, Cys30, Cys73, Cys120, and Cys382 was almost exactly the same as that for the release of Pyr-SH. Last, the level of disulfide-forming Cys367 was almost constant during the time of the exchanges. These data reinforce that the sulfhydryl/mixed disulfide exchange approach works for analyzing the mode of protein chain fluctuation at acidic pH.

The first-order rate constants for the intrachain sulfhydryl/mixed disulfide exchanges according to reaction 2 (k_{intra}) were obtained for each of cysteine sulfhydryls by fitting the data in Figure 7, as summarized in Table 1.

Effective Concentrations. The first-order rate constant, k_{intra}, for the exchange reaction should depend on the accessibility, hence the effective concentration, of each of the five cysteine sulfhydryls relative to the Cys367-mixed disulfide. The effective concentration, C_{eff}, is related to k_{intra} by the following equation (*21*):

$$C_{eff} = k_{intra}/k_{bi} \quad (6)$$

where k_{bi} represents the second-order rate constant for the bimolecular reaction of the nucleophile attack of each cysteine sulfhydryl to the mixed disulfide. The k_{bi} value can be determined as the second-order rate constant for a bimolecular exchange reaction between freely accessible protein sulfhydryl in one protein molecule and mixed disulfide in another protein molecule. The rate for the bimolecular reaction is, however, practically difficult to be determined as a reaction between freely accessible amino acid residues in the bimolecular protein−protein system, because of the polymer chain's nature.

In a previous report (*28*), the effective concentrations for the intrachain disulfide formation in bovine pancreatic trypsin inhibitor have been estimated. The wild type of the inhibitor contains six cysteine residues. Using 15 possible two-cysteine mutants, the rate constant (k_{intra}) for the intrachain cystine disulfide formation by nucleophile attack of a cysteine sulfhydryl to the glutathione-mixed disulfide of the other cysteine residue has been determined. For the determination of bimolecular rate constant (k_{bi}) for each of the disulfide formations, a set of two corresponding short cysteine peptides has been employed. This procedure appears to provide a direct way for estimating the bimolecular rate constants. The rate for a sulfhydryl/disulfide exchange reaction, however, depends on the p*K* values of the nucleophile and central and leaving sulfhydryl groups (see eq 5). It is not clear whether the p*K* values of the cysteine sulfhydryls are invariable in whole protein and small peptide systems.

In the present study, we employed an alternative procedure by which the accessibility problem in a bimolecular protein−protein system can be circumvented by utilizing three model bimolecular reactions, each of which includes reactant(s) with small molecular size(s) of L-cysteine and/or Pyr-S-S-Cys:

$$\text{L-Cys} + {}_R\text{OVA-SS-Pyr} \xrightarrow{k_{bi-1}} {}_R\text{OVA-SS-Cys} + \text{Pyr-SH} \quad (7)$$

$$\text{L-Cys} + \text{Pyr-S-S-Cys} \xrightarrow{k_{bi-2}} \text{Cys-S-S-Cys} + \text{Pyr-SH} \quad (8)$$

$${}_R\text{OVA} + \text{Pyr-S-S-Cys} \xrightarrow{k_{bi-3}} {}_R\text{OVA-SS-Cys} + \text{Pyr-SH} \quad (9)$$

The reaction of eq 7 is essentially the same as reaction 3 except that the cystine-intact form is replaced by the cystine-reduced protein form; L-cysteine makes a nucleophile attack on the central cysteine residue of the Cys367-mixed disulfide by leaving Pyr-SH. In the reaction of eq 8, nucleophile L-cysteine attacks on the central cysteine residue of Cys-S-S-Pyr, thereby leaving Pyr-SH. In the reaction of eq 9, protein sulfhydryls make nucleophile attacks on the central cysteine residue of Cys-S-S-Pyr, thereby leaving Pyr-SH. The constants k_{bi-1}, k_{bi-2}, and k_{bi-3} are the second-order rate constants for the reactions of eqs 7, 8, and 9, respectively. Using the Brønsted equation (eq 5), the rate constant k_{bi} can be related to these rate constants by a simple equation:

$$k_{bi} = k_{bi-1}k_{bi-3}/k_{bi-2} \quad (10)$$

The rates for the three reactions were measured at pH 2.2 and 37 °C in the presence of 6 M urea. Under the conditions for estimating k_{bi-1}, the attacks on the thiopyridine-mixed disulfide by bimolecular L-cysteine and by an intrachain cysteine sulfhydryl are competitive with each other (see Figures 4 and 5). For the reaction of eq 7, therefore, pseudo-first-order rate constants were measured using 22 μM $_R$OVA-SS-Pyr and various higher concentrations of L-cysteine by monitoring the release of Pyr-SH, and the obtained rate constants were plotted as a function of L-cysteine concentrations, as shown in Figure 8. The k_{bi-1} value determined from the slope of the plot was 487 M^{-1} min^{-1}.

For the reaction of eq 8, the pseudo-first-order rate constant was measured using 22 μM L-cysteine and 0.11−1.1 mM Pyr-S-S-Cys by monitoring the release of Pyr-SH, and the obtained k_{bi-2} value was 1720 M^{-1} min^{-1}.

For the determination of k_{bi-3} values, the fully disulfide-reduced protein was incubated at 22 μM with 2.2 mM Pyr-S-S-Cys for various times, and disulfide-forming cysteines were quantified by the peptide mapping procedure in the same way as in Figure 5. The pseudo-first-order rate constant was obtained from the time course of the disulfide formation of each of the cysteine residues. For the determination of each k_{bi-3} value, the pseudo-first-order rate constant was divided by the concentration of Pyr-S-S-Cys.

Table 1 summarizes the rate constants and the effective concentrations for the five cysteine residues. The values for k_{bi} were not greatly variable for the five cysteine residues. This was in great contrast to the bovine pancreatic trypsin inhibitor system in which highly variable k_{bi} values were obtained for 15 different combinations of six cysteine peptides (*28*). The effective concentrations were, however, significantly variable; the value for Cys73 was more than 3 times that for Cys30.

Implications of the Results from the Exchange Analyses for Protein Conformational State. According to the X-ray crystallographic data of native OVA (*14*), the distances of the sulfur atoms of Cys11, Cys30, Cys73, Cys120, and Cys382 from the sulfur atom of Cys367 are 6.6, 11.0, 29.7, 29.7, and 14.5 Å, respectively (Figure 1). If the accessibilities of the five cysteine residues to the mixed disulfide were simply dependent on the sulfur-atom-to-sulfur-atom distances in the native protein topology, the effective concentrations would have been in the order: Cys11 > Cys30 > Cys382 > Cys73/Cys120. The data in Table 1, however, demonstrate that this was not the case: the effective concentration was in the order of Cys73 > Cys11 > Cys382 > Cys120 > Cys30. The effective concentration data also exclude the possibility for random coiled conformation in the absence of a denaturant. First, Cys382, the nearest cysteine residue from Cys367 in the primary structure, is the predominant cysteine nucleophile for the exchange reaction (Figure 5), indicating its highest effective concentration in the presence of a high concentration of urea. Second, the k_{intra} value for this exchange reaction is 0.901 min^{-1} at 37 °C in the presence of 6 M urea, according to the data in Figure 8 (in the absence of L-cysteine); this value corresponds to C_{eff} of 5.4 mM, which is about 600-fold of the C_{eff} for Cys382 in the absence of urea. These results strongly suggest that $_R$OVA assumes a partially denatured, non-native conformation at the acidic pH.

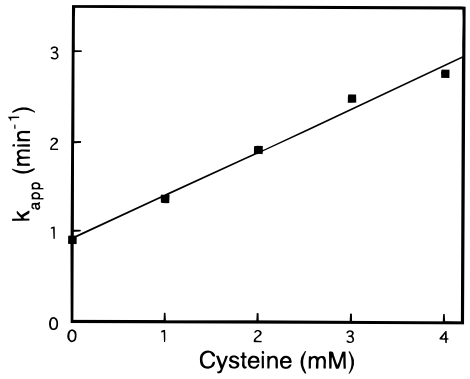

FIGURE 8: Determination of the rate for the exchange reaction between bimolecular L-cysteine and the thiopyridine-mixed disulfide. $_R$OVA-SS-Pyr was incubated at 22 μM with various higher concentrations of L-cysteine at 37 °C in buffer A containing 6 M urea, and the release of Pyr-SH was measured by absorption at 343 nm. The pseudo-first-order rate constants, k_{app}, obtained from the time course of Pyr-SH release were plotted as a function of L-cysteine concentrations, and the second-order rate constant for the reaction was obtained from the slope of the plot. The number of the modified Cys367 was 0.89 in the original $_R$OVA-SS-Pyr.

Several lines of evidence have shown that OVA (with the native cystine disulfide) assumes a unique molten globule-like conformation at pH 2.2. CD spectra represent that the secondary structure content is almost exactly the same at pH 2.2 and 7.0 but that the native tertiary interactions are almost completely disrupted at the acidic pH (*17*). The overall conformation of acid OVA as evaluated by binding of a hydrophobic probe, anilino-1-naphthalene-8-sulfonate, is also consistent with a molten globule-like state (*18*). However, the protein hydrodynamic volume estimated by intrinsic viscosity and size-exclusion chromatography is essentially the same at the two pH values (*17*). Furthermore, the protein exhibits a large endothermic transition during the heat denaturation at the acidic pH (*29*). These results are consistent with a highly ordered molten globule-like state for OVA at pH 2.2. We observe that the structural characteristics of $_R$OVA (disulfide-reduced form) are almost indistinguishable from those of the disulfide-bonded form as evaluated by CD, size-exclusion chromatography, and hydrophobic probe binding analyses at pH 2.2 (unpublished experiments).

Despite these macroscopic structural similarities, the cystine-reduced protein, but not the cystine-intact one, undergoes significant intrachain sulfhydryl/mixed disulfide exchanges at a physiological temperature of 37 °C (Figure 6). Such a microscopic difference may be related to the participation of Cys73 and Cys120 in the exchanges in $_R$-OVA-SS-Pyr but not in OVA-SS-Pyr. As evaluated by the k_{intra} values (Table 1), however, more than 50% of the exchanges are accounted for by the exchange reactions by the other three nucleophile cysteine sulfhydryls (Cys11, Cys30, and Cys382). More than 70% of the thiopyridine-mixed disulfide group undergoes the exchange reaction in the cystine-reduced protein during 2-h incubation at 37 °C, while the release of Pyr-SH from the cystine-intact form was only 5.4% (Figure 6). We therefore conclude that the occurrence of the intramolecular exchange reactions reflects largely the increased conformational fluctuation in the cystine-reduced form as compared to that in the cystine-intact protein.

In previous reports, we have also observed the macroscopic structural similarity and microscopic difference between the

cystine-intact and -reduced proteins at near-neutral pH. The native structural characteristics of the cystine-intact form are still retained in the cystine-reduced form as evaluated by CD spectrum and trypsin-resistance analyses (*25*, *30*). The results from protein fragmentation analysis by another protease, however, support a local structural difference. As a common property of a member of the serpin superfamily, OVA undergoes limited proteolysis at near-neutral pH with subtilisin (*31*) or elastase (*32*) at the canonical P1−P1′ site (Ala352-Ser353) located in helix R. In addition to this canonical cleavage, the N-terminal side of Cys73 is cleaved by subtilisin in $_R$OVA (*25*, *30*). This may be related to the highly fluctuating nature around Cys73 in the cystine-reduced protein; this cysteine residue is located in a loop region between helix C2 and D and anchored in the cystine-intact form to Cys120 included in helix E. Such fluctuating nature of Cys73 may be retained or even enhanced at the acidic pH, since the highest effective concentration is observed for Cys73 (Table 1) despite its farthest three-dimensional localization from Cys367 in the native structure (*14*).

CONCLUSION

The importance of partially denatured conformational state, including the molten globule state, as an equilibrium counterpart of a protein folding intermediate has been pointed out (*2*−*7*). For investigating such a non-native state, several methodological approaches including the conventional spectroscopic techniques (e.g., CD, infrared, fluorescence, and hydrophobic dye binding analyses), small-angle X-ray scattering, and hydrogen−deuterium exchange analysis by mass spectrometry have been employed. However, the type of obtainable information is different for the individual approaches, and most of them are usually poor for addressing protein conformation in terms of a specific amino acid residue. One of the best approaches, the hydrogen−deuterium exchange analysis by NMR, works only for a protein with a small mass range. Partially denatured conformation is most widely induced at acidic pH, and the protein conformation at acidic pH that is variable depending on protein species and solvent conditions has been one of the major subjects in protein chemistry (*33*−*36*).

The present study demonstrates that the sulfhydryl/mixed disulfide exchange approach provides unique information about the fluctuating nature of a protein under acidic conditions. The highly reactive nature of cysteine sulfhydryl/thiopyridine-mixed disulfide exchange allows us to directly determine the effective concentrations, hence the accessibilities, of cysteine sulfhydryls relative to the mixed disulfide at acidic pH. The present studies are still limited to the analysis of the non-native conformational state of OVA. The sulfhydryl/mixed disulfide exchange approach, however, may generally work as a useful method for analyzing the conformational state of sulfhydryl proteins at acidic pH, if a condition is selected to introduce thiopyridine-mixed disulfide into a single protein sulfhydryl. Furthermore, such a single sulfhydryl site can be designed to be introduced into a variety of sites in a protein, so that the effective concentrations of other cysteine sulfhydryls relative to the mixed disulfide site can be estimated in multiple combinations. We believe that the present study provides the chemical basis for such promising analysis for the partially folded conformational state at acidic pH.

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BI980353Z