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Chemical Modification of Tryptophan Residues and Stability Changes in Proteins

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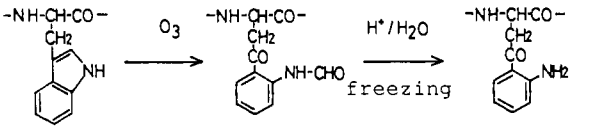
ABSTRACT: The role of tryptophan residues in the stability of proteins was studied by ozone oxidation, which causes a small change in the tryptophan side chain. Trp 187 of the constant fragment of a type λ immunoglobulin light chain, Trp 59 of ribonuclease T1, and Trp 62 of hen egg white lysozyme were oxidized specifically by ozone to *N'*-formylkynurenine or kynurenine. Judging from their circular dichroic and fluorescence spectra, these modified proteins were found to be the same as those of the respective intact proteins. However, even the slight modification of a single tryptophan residue produced a large decrease in the stability of these proteins to guanidine hydrochloride and heat. The smaller the extent of exposure of the tryptophan residue, the greater the effect of the modification on the stability. The formal kinetic mechanism of unfolding and refolding by guanidine hydrochloride of the C_L fragment was not altered by tryptophan oxidation, but the rate constants for unfolding and refolding changed. The thermal unfolding transitions were analyzed to obtain the thermodynamic parameters. The enthalpy and entropy changes for the modified proteins were larger than the respective values for the intact proteins.

Site-specific modification by synthetic and recombinant DNA technology has recently been used to clarify the structure, stability, and function of proteins. The chemical modification method has also been used so far mainly for identifying the functional groups of proteins. However, the structure and stability of such chemically modified proteins have not been studied in detail.

In the present studies, we examined the role of tryptophan residues in the structure and stability of proteins. For this purpose, we employed ozone oxidation to modify the tryptophan residue. No other amino acid closely resembles tryptophan. It is thus impossible to make a small change in a tryptophan side chain by site-directed mutagenesis, and it can only be achieved by using the chemical modification. Ozone oxidation modifies the tryptophan residue to *N'*-formylkynurenine (NFK),[1] which is converted to kynurenine (Kyn) by freezing in acid (Kuroda et al., 1975; Tamaoki et al., 1978; Yamasaki et al., 1979; Teshima et al., 1980; Fukunaga et al., 1982a,b):



Trp NFK Kyn

X-ray crystallographic analysis of *N*-acetylkynurenine crystals (Kennard et al., 1979) has shown that the carbonyl oxygen is hydrogen-bonded to the aromatic amino group at

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[1] Abbreviations: CD, circular dichroism; Gdn-HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; HPLC, high-performance liquid chromatography; C_L-(109-212), C_L fragment corresponding to sequence 109-212; NFK, *N'*-formylkynurenine; Kyn, kynurenine; NFK 187-C_L, C_L fragment in which Trp 187 is modified to NFK; Kyn 187-C_L, C_L fragment in which Trp 187 is modified to Kyn; NFK (150, 187)-C_L, C_L fragment in which Trp 150 and Trp 187 are modified to NFK; Kyn (150, 187)-C_L, C_L fragment in which Trp 150 and Trp 187 are modified to Kyn; NFK 59-RNase T1, ribonuclease T1 in which Trp 59 is modified to NFK; Kyn 59-RNase T1, ribonuclease T1 in which Trp 59 is modified to Kyn; NFK 62-lysozyme, hen egg white lysozyme in which Trp 62 is modified to NFK; Kyn 62-lysozyme, hen egg white lysozyme in which Trp 62 is modified to Kyn.

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the ortho position, that this hydrogen-bonded ring is coplanar within 3° to the benzene ring in the kynurenine molecule, and that this structure is similar to the structure of tryptophan.

In the present experiments, the constant (C_L) fragment of a type λ immunoglobulin light chain, ribonuclease T1 (RNase T1), and hen egg white lysozyme were used. The C_L fragment has two tryptophan residues at positions 150[2] and 187. Trp 150 is buried completely in the interior and Trp 187 is located near the surface of the molecule (Beale & Feinstein, 1976; Amzel & Poljak, 1979; Kawata et al., 1988). The former is conserved in all the domains of the immunoglobulin molecule but the latter is not. RNase T1 has only one tryptophan residue at position 59. Hen egg white lysozyme has six tryptophan residues and the degree of exposure of Trp 62 is the greatest. The degree of exposure of these tryptophan residues increases in the order Trp 150 (C_L) < Trp 59 (RNase T1) < Trp 187 (C_L) < Trp 62 (lysozyme). In spite of the small change in the structure of the tryptophan residue, ozone oxidation decreased the stability of these proteins to both heat and guanidine hydrochloride. We also found that the decrease in stability is closely related to the degree of exposure of the modified tryptophan residue; the smaller the degree of exposure, the greater the decrease in the stability.

Materials and Methods

Materials. Immunoglobulin light chain Nag (type λ) was prepared from urine of a multiple myeloma patient, as described previously (Goto et al., 1979). *Achromobacter lyticus* protease I and papain were obtained from Wako Pure Chemicals and Sigma, respectively. Gdn-HCl (specially purified grade) was obtained from Nacalai Tesque Inc. Other reagents were of the highest grade commercially available and were used without further purification.

Preparation of C_L(109–212). The C_L fragments so far used in our laboratory were obtained by digestion with papain or trypsin of Nag protein in which the penultimate cysteine residue had been alkylated with iodoacetamide. The C_L fragments thus obtained [C_L(109–214), C_L(105–214), and C_L(113–214) (Goto & Hamaguchi, 1987)] each contained an *S*-(carbamylmethyl)cysteine residue at position 213. These samples of the C_L fragment could not be used in the present ozone oxidation experiments, because the *S*-(carbamylmethyl)cysteine residue is easily oxidized by ozone. Therefore, we used the C_L(109–212) fragment, which lacks the cysteine residue at position 213. The C_L(109–212) fragment was prepared as follows. Nag protein was digested with papain in 0.1 M potassium phosphate buffer at pH 7.0 containing 20 mM dithiothreitol for 30 min at 37 °C at a substrate-to-enzyme ratio of 250:1 (w/w). In this reaction process, the interchain disulfide bond between the Cys 213 residues of the Nag protein dimer was reduced. The digestion product was subjected to gel filtration at 4 °C on a Sephadex G-75 column (3.6 × 109 cm) equilibrated with 10 mM Tris-HCl buffer at pH 8.6. Three peaks were separated. On the basis of the molecular weights estimated by SDS–polyacrylamide gel electrophoresis, it was found that the first and second peaks corresponded to the undigested protein and the C_L fragment, respectively. The third peak corresponded to a mixture of small peptides. On the basis of the results of the peptide mapping by digestion of Nag protein with *Achromobacter lyticus* protease I and amino acid analysis, the proteins in the second peak were found to be a mixture of C_L(109–212) and C_L(109–214). The C_L(109–212) was obtained as follows. 5,5′-Dithiobis(2-nitrobenzoic acid) was added to the pooled fractions of the second peak to complete the formation of the interchain disulfide bond between the Cys 213 residues of the C_L(109–214) dimer. The dimerization reaction was followed with a Gilson HPLC system on a TSK DEAE-NPR column (4.6 × 35 mm) with a linear gradient from 0 to 0.5 M NaCl in 10 mM Tris-HCl buffer at pH 7.5. A small amount of 5,5′-dithiobis(2-nitrobenzoic acid) was added several times until the peak of the C_L(109–214) monomer disappeared completely.

The mixture of the C_L(109–212) monomer and the C_L(109–214) dimer was subjected to ion-exchange chromatography on a DEAE-cellulose column (2.6 × 43 cm) equilibrated with 10 mM Tris-HCl buffer at pH 8.6. Two peaks were eluted with a linear gradient from 0 to 0.1 M KCl in the same buffer. Results of peptide mapping and amino acid analysis for these peaks indicated that the first and second peaks consisted of the C_L(109–212) fragment and the dimer of the C_L(109–214) fragment, respectively. The pooled fractions from the first peak were dialyzed against distilled water and then lyophilized. Sixty-three milligrams of the C_L(109–212) fragment was obtained from 1 g of the light chain. The C_L(109–212) fragment was used in the present experiments.

Preparation of NFK 187-C_L and Kyn 187-C_L Fragments. Ozone oxidation of the C_L fragment was performed according to the method of Kuroda et al. (1975) with a minor modification. Trp 187, which is located near the surface of the C_L fragment molecule, was oxidized in 0.1 M potassium phosphate buffer, pH 6.0, at 0 °C. The other tryptophan residue (Trp 150) of the C_L fragment, which is located in the interior of the molecule, was not oxidized under these conditions. Ozone at a delivery rate of 8×10^{-2} μmol/min, generated with an ozonizer (type 0-3-2, Nihon Ozone Co., Tokyo), was introduced into the reaction solution. The concentration of ozone was estimated by KI/$Na_2S_2O_3$ titration. The reaction was monitored by absorption measurement of the reaction solution diluted 30-fold with distilled water. The NFK content introduced was estimated by using a molar extinction coefficient for NFK of 3.75×10^3 M^{-1} cm^{-1} at 322 nm (Fukunaga et al., 1982a). When about 0.6 mol of NFK had been introduced, the oxidation was stopped and the reaction mixture was immediately lyophilized.

The crude oxidized C_L fragment was dissolved in 50 mM NH_4HCO_3, and the solution was subjected to gel filtration on a Sephadex G-50 superfine column (3.0 × 145 cm) equilibrated with the same buffer. Three peaks were separated. On the basis of the results of amino acid analysis, it was found that the first and second peaks corresponded to the proteins denatured owing to excess oxidation of one or two Tyr residues in addition to the Trp residue. The results of amino acid analysis and absorption measurement of the third peak showed that about 0.5 mol of Trp per mole of C_L was oxizied to NFK. This finding showed that the third peak consisted of a mixture of the NFK 187-C_L and intact C_L fragments.

The NFK 187-C_L fragment was easily separated from the unmodified C_L fragment by gel filtration in the presence of Gdn-HCl, because, as will be described under Results, the NFK 187-C_L fragment is less stable than the unmodified C_L fragment to Gdn-HCl (see Figure 2). The fractions of the third peak were collected and lyophilized. This sample was dissolved in 50 mM Tris-HCl buffer containing 0.6 M Gdn-HCl at pH 7.5 and subjected to gel filtration on a Sephadex G-50 superfine column (2.8 × 98 cm) equilibrated with the same buffer. In 0.6 M Gdn-HCl at pH 7.5, the unmodified protein is intact but the modified protein is partially denatured.

[2] The numbering system used in this paper is based on type λ light chain (Kol) (Marquart et al., 1980).

Two peaks were separated by the gel filtration. The modified protein eluted earlier than the intact protein according to apparent molecular size. The first peak was dialyzed against distilled water at 4 °C and then lyophilized. The NFK 187-C_L fragment thus obtained showed a single band on polyacrylamide gel electrophoresis (15% gel) in the presence or absence of 0.1% SDS at pH 9.5 and 4.3. Peptide mapping with *Achromobacter lyticus* protease I and amino acid analysis showed that Trp 187 was specifically modified to NFK and no other residues were oxidized.[3]

Preparation of Kyn 187-C_L. The NFK at position 187 of the C_L fragment was converted to Kyn according to the method of Yamasaki et al. (1979). The NFK 187-C_L fragment was dissolved in 0.1 N HCl and frozen at −10 °C for 24 h. By this treatment the NFK was converted to Kyn. After the reaction, the frozen reaction solution was thawed, dialyzed against distilled water at 4 °C, and then lyophilized.

Modified RNase T1 and Hen Lysozyme. RNase T1, NFK 59-RNase T1, and Kyn 59-RNase T1 prepared as described by Fukunaga et al. (1982b) were kindly donated by Prof. F. Sakiyama (Institute for Protein Research, Osaka University). RNase T1 contains no Met residue, and Tyr and His residues were not modified by ozone oxidation (Fukunaga et al., 1982b). NFK 62-lysozyme was prepared as described by Kuroda et al. (1975) and was purified by CM-Sepharose CL-6B chromatography (T. Nakazawa and F. Sakiyama, unpublished). Kyn 62-lysozyme was prepared as described by Yamasaki et al. (1979). Spectroscopic and amino acid analyses showed that a single tryptophan residue is specifically modified.

Peptide Mapping. A protein solution (0.1%) was digested with *Achromobacter lyticus* protease I with a substrate-to-enzyme molar ratio of 400:1 in 50 mM Tris-HCl buffer, pH 9.0, at 37 °C for 4 h. The digested product was chromatographed on a Cosmosil 5C18 (Nacalai Tesque Inc.) reverse-phase column (4.6 × 250 mm) with a Gilson HPLC system (0% acetonitrile–50% acetonitrile in 0.05% trifluoroacetic acid).

CD Measurement. Measurements were carried out with a Jasco spectropolarimeter, Model J-500A, equipped with a data processor (DP-501). The CD instrument was calibrated with *d*-10-camphorsulfonic acid. The results are expressed as mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = (100 \times \theta_{obs})/(l\,c)$, where θ_{obs} is the observed ellipticity in degrees, c is the residue molar concentration of the protein, and l is the length of the light path in centimeters. In the calculation of c, a value of 108 was used as the mean residue molecular weight. The CD spectra below and above 250 nm were measured in 0.1-cm and 2.0-cm cells, respectively. The temperature was controlled with a thermostatically controlled cell holder.

Fluorescence Measurement. Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer, Model MPF-4, equipped with a spectral corrector. The temperature was controlled at 25 °C with a thermostatically controlled cell holder. The tryptophanyl and kynurenyl fluorescences were measured with 295-nm and 375-nm light, respectively, for excitation.

Thermal Unfolding. Thermal unfolding curves were measured in terms of the ellipticity at an appropriate wavelength chosen for each protein. The temperature was increased continuously at a rate of 0.5 °C/min and was monitored with a Sensortek Model BAT-12 thermometer. The reversibility was examined by measuring the ellipticity by lowering the temperature at a rate of 2 °C/min.

Unfolding by Gdn-HCl. All the measurements for the C_L and modified C_L fragments were carried out in 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl at 25 °C. Unfolding equilibria by Gdn-HCl were measured by CD at 218 nm and tryptophanyl fluorescence at 350 nm. The protein solutions at different concentrations of Gdn-HCl were allowed to stand overnight at room temperature before measurement. The protein concentration was 0.025 mg/mL for both CD and fluorescence measurements.

Fast unfolding and refolding reactions were measured on a Union Giken stopped-flow spectrophotometer, Model RA-401, with fluorescence detection. The excitation wavelength was 280 nm and the emission above 330 nm was measured. The details of the apparatus have been described previously (Goto & Hamaguchi, 1982). The unfolding was initiated by mixing a protein solution at pH 7.5 with Gdn-HCl solution at a given concentration at pH 7.5 in a 1:1 ratio. The refolding was initiated by mixing a denatured protein solution in 1.8 M Gdn-HCl with Gdn-HCl solution at a given concentration at pH 7.5 in a 1:5 ratio.

Slow unfolding and refolding reactions were measured with the Hitachi fluorescence spectrophotometer. The excitation wavelength was 295 nm and the emission was measured at 350 nm. The unfolding was initiated by manual mixing of 100 μL of protein solution in 0 M Gdn-HCl at pH 7.5 with 3 mL of Gdn-HCl solution at a given concentration at pH 7.5. For the measurements of refolding reactions, the proteins were initially denatured in 1.8 M Gdn-HCl at pH 7.5. The refolding was initiated by mixing 100 μL of the protein solution with 3 mL of Gdn-HCl solution at a given concentration at pH 7.5. The final protein concentration was about 0.02 mg/mL. All the kinetic data were analyzed as described previously (Goto & Hamaguchi, 1982).

Protein Concentration. Protein concentrations were determined spectrophotometrically. The absorption coefficients of the intact C_L, NFK 187-C_L, and Kyn 187-C_L fragments were 16 800 M^{-1} cm^{-1} at 280 nm, 3700 M^{-1} cm^{-1} at 325 nm, and 4300 M^{-1} cm^{-1} at 372 nm, respectively. These values were determined by amino acid analysis of a protein solution with known absorbance. The absorption coefficients used for hen lysozyme, NFK 62-lysozyme, and Kyn 62-lysozyme were 39 000 M^{-1} cm^{-1} at 280 nm (Imai et al., 1963), 3750 M^{-1} cm^{-1} at 322 nm, and 4700 M^{-1} cm^{-1} at 360 nm (Teshima et al., 1980), respectively. The absorption coefficients used for RNase T1, NFK 59-RNase T1, and Kyn 59-RNase T1 were 20 500 M^{-1} cm^{-1} at 280 nm, 3750 M^{-1} cm^{-1} at 322 nm, and 4720 M^{-1} cm^{-1} at 380 nm (Fukunaga et al., 1982a), respectively.

Amino Acid Analysis. The amino acid compositions of the proteins used were determined with an Irica amino acid analyzer (Model A-5000) or a Hitachi amino acid analyzer (Model 835-S). The samples were hydrolyzed in evacuated, sealed tubes with 6 N HCl or 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Simpson et al., 1976) at 110 °C for 24 h.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of SDS was carried out according to the method of Weber and Osborn (1969). Polyacrylamide gel electrophoresis in the absence of SDS was carried out at pH 9.5 and 4.3 according to the method of Davis (1964) and that of Reisfeld et al. (1962), respectively.

pH Measurement. pH was measured with a Radiometer PHM 26c meter at 25 °C.

[3] The C_L fragment contains no methionine residue.

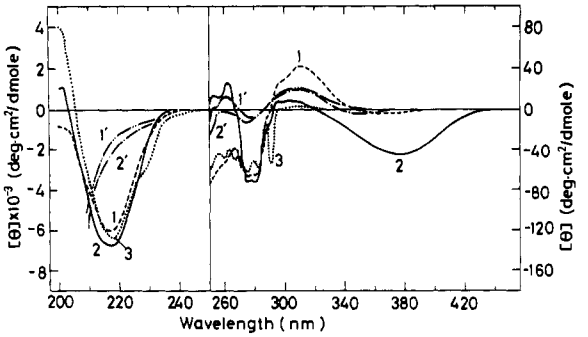

FIGURE 1: CD spectra of NFK 187-C_L (1), Kyn 187-C_L (2), and intact C_L (3) fragments in 50 mM Tris-HCl buffer containing 0.15 M NaCl at pH 7.5 and 25 °C and those of denatured NFK 187-C_L (1′) and Kyn 187-C_L (2′) fragments in 4 M Gdn-HCl at pH 7.5 and 25 °C.

RESULTS

CD and Fluorescence Spectra. The absorption spectra of the NFK 187-C_L and Kyn 187-C_L fragments had maxima at 322 nm due to the NFK group and at 375 nm due to the Kyn group, respectively.

Figure 1 shows the CD spectra of the NFK 187-C_L and Kyn 187-C_L fragments together with that of the intact C_L fragment. The CD spectrum of the intact C_L fragment below 250 nm had a negative maximum at 218 nm, which is characteristic of the β-structure, and a shoulder at around 230 nm. On modification of Trp 187 to NFK or Kyn, the ellipticity at 218 nm changed slightly and the shoulder disappeared. This is due to the optical activity of the NFK or Kyn group (Teshima et al., 1980).

The CD spectrum of the NFK 187-C_L fragment had a positive maximum at 325 nm due to the NFK group. The CD spectrum of the Kyn 187-C_L fragment had a broad negative maximum at 380 nm due to the Kyn group. The CD bands were decreased greatly on denaturation with 4 M Gdn-HCl.

As reported previously from our laboratory (Kikuchi et al., 1987; Tsunenaga et al., 1987), almost all the fluorescence of Trp 150 of the C_L fragment (type λ) is strongly quenched by the intrachain disulfide bond. The fluorescence intensities of the NFK 187-C_L and Kyn 187-C_L fragments at pH 7.5 in the absence of denaturant were greatly quenched and were less than 5% of the fluorescence in 4 M Gdn-HCl. These fluorescence spectra were very similar to that of a type κ C_L fragment, in which only one tryptophan residue at position 148 is strongly quenched by the closely located intrachain disulfide bond. In 4 M Gdn-HCl, the fluorescence spectra of the modified fragments had a maximum at 350 nm and the fluorescence intensity was about 50% of that of the intact C_L fragment. These findings indicate that Trp 187 is specifically modified and that this modification does not produce any perturbation of the conformation of the fragment molecule.

The fluorescence spectra of the Kyn group in the Kyn 187-C_L fragment, Kyn 59-RNase T1, and Kyn 62-lysozyme were measured at pH 7.5 and 25 °C. The maximum wavelengths for the Kyn 187-C_L, Kyn 59-RNase T1, and Kyn 62-lysozyme were 473, 464, and 475 nm, respectively, and the fluorescence intensities at the maximum wavelengths of Kyn 59-RNase T1 and Kyn 62-lysozyme relative to that of the Kyn 187-C_L fragment were 0.93 and 0.24, respectively.

Unfolding Equilibria of the Modified C_L Fragment by Gdn-HCl. Figure 2 shows the unfolding curves obtained with Gdn-HCl of the intact C_L, NFK 187-C_L, and Kyn 187-C_L fragments. The unfolding curve of C_L(109–212) was found to be identical with that of C_L(109–214) reported by Goto and Hamaguchi (1979) (A. Ishiwata et al., unpublished). These unfolding transitions were found to be reversible. The transition curves were normalized by assuming that ellipticities or fluorescence for the native and unfolded proteins which were observed before and after the transition zone, respectively, can be extrapolated linearly into the transition zone. The unfolding transition curve measured by CD agreed well with the curve measured by fluorescence. The Gdn-HCl concentrations at the midpoints of the unfolding curves for the intact C_L, NFK 187-C_L, and Kyn 187-C_L fragments were 1.2, 0.55, and 0.60 M, respectively.

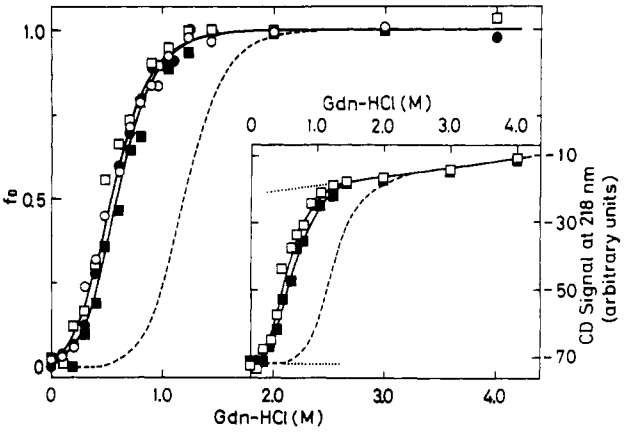

FIGURE 2: Unfolding transitions by Gdn-HCl of NFK 187-C_L (○, □), Kyn 187-C_L (●, ■), and intact C_L (broken line) fragments at pH 7.5 and 25 °C. Unfolding transitions were measured in terms of the change in CD at 218 nm (□, ■) and fluorescence intensity at 350 nm (○, ●). The fluorescence was measured with 295-nm light for excitation. The solid lines indicate the theoretical curves calculated from the values of $\Delta G_D^{H_2O}$ and Δn (Gdn-HCl binding model; see Table II). The broken line indicates the transition curve of the intact C_L fragment cited from the previous paper (Goto & Hamaguchi, 1987). The ordinate represents the fraction of unfolded protein (f_D). The inset shows the changes with the concentration of Gdn-HCl in the CD signal at 218 nm for NFK 187-C_L (□) and Kyn 187-C_L (■) fragments. The solid lines in the transition region are theoretical curves based on the parameters obtained from the Gdn-HCl model (see Table III). The broken line indicates the transition curve of the intact C_L fragment cited from the previous paper (Goto & Hamaguchi, 1987).

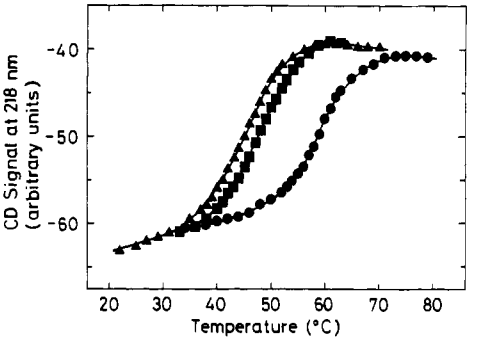

FIGURE 3: Thermal unfolding transitions of NFK 187-C_L (▲), Kyn 187-C_L (■), and intact C_L (●) fragments in 0.01 M sodium phosphate buffer containing 0.15 M NaCl at pH 7.5. The transitions were measured in terms of the change in CD at 218 nm. The solid lines in the transition region are theoretical curves based on the parameters given in Table I.

Thermal Unfolding. Figure 3 shows the thermal unfolding curves of the intact C_L, NFK 187-C_L, and Kyn 187-C_L fragments measured in terms of the ellipticity at 218 nm. These thermal unfolding transitions were almost completely reversible. The midpoints of the transition curves (T_m) of the intact C_L, NFK 187-C_L, and Kyn 187-C_L were 61.0, 46.1, and 48.8 °C, respectively.

We measured the thermal transition curves of the intact C_L and Kyn 187-C_L fragments at various pH values. Assuming a two-state transition, the changes in enthalpy (ΔH) at various

Table I: Thermodynamic Parameters and Transition Midpoints for the Thermal Unfolding of the C_L Fragment, RNase T1, Lysozyme, and Their Modified Proteins at pH 7.5

	parameters at T_m			parameters at 25 °C		
	T_m[c] (°C)	ΔH_D (kcal/mol)	ΔS_D (eu)	ΔH_D (kcal/mol)	ΔS_D (eu)	ΔG_D (kcal/mol)
intact C_L[a]	61.0 ± 0.5	66.5 ± 0.9	199 ± 2	1.7 ± 1.8	−6 ± 6	3.6 ± 1.7
NFK 187-C_L[a]	46.1 ± 0.2	54.9 ± 0.5	172 ± 3	10.6 ± 0.9	28 ± 3	2.1 ± 0.8
Kyn 187-C_L[a]	48.8 ± 0.3	55.6 ± 0.5	173 ± 2	5.6 ± 1.1	11 ± 4	2.2 ± 1.2
intact RNase T1[a]	48.7 ± 0.7	87.9 ± 1.5	268 ± 1	48.8 ± 2.7	147 ± 9	5.0 ± 2.2
NFK 59-RNase T1[a]	25.3 ± 0.1	82.2 ± 2.1	275 ± 7	81.7 ± 2.3	274 ± 8	0.1 ± 0.3
Kyn 59-RNase T1[a]	33.9 ± 0.6	82.4 ± 1.4	268 ± 4	67.7 ± 2.4	219 ± 8	2.2 ± 1.8
intact lysozyme[b]	57.9 ± 0.1	(73.7 ± 0.8)	(222 ± 2)	(24.4 ± 0.8)	(66 ± 2)	(4.8 ± 0.1)
NFK 62-lysozyme[b]	54.8 ± 0.4	(77.5 ± 1.3)	(236 ± 3)	(32.8 ± 1.9)	(93 ± 6)	(4.9 ± 1.2)
Kyn 62-lysozyme[b]	57.6 ± 0.6	(75.0 ± 2.0)	(227 ± 6)	(26.1 ± 2.9)	(71 ± 9)	(4.9 ± 1.8)

[a] In 0.01 M sodium phosphate buffer containing 0.15 M NaCl. [b] In the presence of 1.5 M Gdn-HCl at pH 7.5. [c] T_m is the temperature at the midpoint of the unfolding transition. The thermal transition curve was analyzed by assuming the two-state approximation of N(native) ⇌ D(unfolded), and the equilibrium constant of unfolding (K_D = [D]/[N]) was determined from the equation $K_D = f_D/(1 - f_D)$, where f_D is the fraction of the unfolded molecule at each temperature. ΔH at T_m was determined from the van't Hoff plot. The parameters at 25 °C were calculated by using eqs 2–4 (see text). The values in parentheses were estimated from the thermal unfolding transitions of about 80% reversibility.

T_m values on unfolding were determined by using a van't Hoff plot. The dependence of ΔH at T_m on T_m was linear for each protein (data not shown). From the slopes of the straight lines, the changes in heat capacity (ΔC_p) on unfolding of the intact C_L and Kyn 187-C_L fragments were estimated to be 1.8 and 2.1 kcal deg^{-1} mol^{-1}, respectively.

Figure 4a shows the thermal unfolding curves of the intact RNase T1, NFK 59-RNase T1, and Kyn 59-RNase T1 measured in terms of the ellipticity at 218 nm. The transitions for the three proteins were completely reversible. The midpoints of the transition curves of the intact RNase T1, NFK 59-RNase T1, and Kyn 59-RNase T1 were 48.7, 25.3, and 33.9 °C, respectively.

Figure 4b shows the thermal unfolding curves of the intact lysozyme, NFK 62-lysozyme, and Kyn 62-lysozyme, measured in terms of the ellipticities at 222, 229, and 229 nm, respectively. These transition curves were measured in the presence of 1.5 M Gdn-HCl at pH 7.5, because, in the absence of the denaturant, the transition midpoint is very high [75 °C (Ogasahara & Hamaguchi, 1967)] and the reversibility is very low. The transitions were about 80% reversible. The midpoints of the transition curves of the intact lysozyme, NFK 62-lysozyme, and Kyn 62-lysozyme were 57.9, 54.8, and 57.6 °C, respectively. The transition temperatures and the enthalpy and entropy changes for the thermal unfolding of the proteins studied are summarized in Table I.

The effect of oxidation of the tryptophan residue on the stability was thus the largest for RNase T1 and the least for hen lysozyme. For the three proteins studied, the NFK protein was less stable than the Kyn protein.

Kinetics of Unfolding and Refolding. In order to understand the cause of the lower stability of the Kyn 187-C_L fragment relative to the intact C_L fragment, we measured the kinetics of unfolding and refolding by Gdn-HCl of the Kyn 187-C_L fragment, and the results were compared with those for the intact C_L fragment reported previously (Goto & Hamaguchi, 1982).

The unfolding kinetics inside the transition zone were described by two exponential decay terms:

$$F(t) - F(\infty) = F_1 \exp(-\lambda_1 t) + F_2 \exp(-\lambda_2 t) \quad (1)$$

where λ_1 and λ_2 are the apparent rate constants of the slow and fast phases, respectively, and F_1 and F_2 are the amplitudes of the respective phases. The amplitudes of the slow and fast phases relative to the total change in fluorescence are described by α_1 and α_2, respectively, where $\alpha_1 + \alpha_2 = 1$. In the Gdn-HCl concentration range above 1.4 M, the relative amplitude α_2 was 1, and the total fluorescence change could be expressed as a first-order process. The refolding kinetics measured in the concentration range of Gdn-HCl from 0.10 to 0.63 M were described as two exponential decay terms, as shown in eq 1.

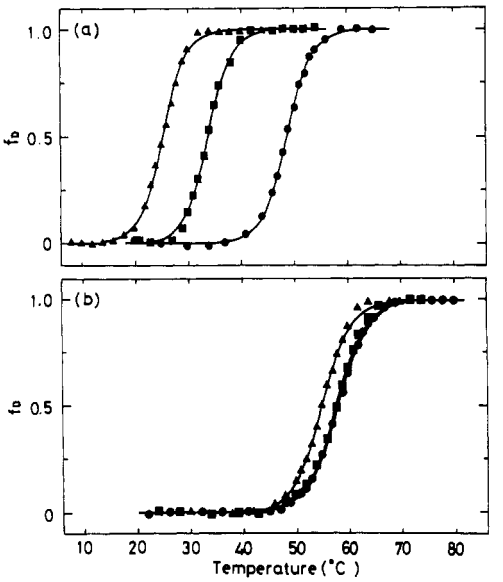

FIGURE 4: (a) Thermal unfolding transitions of NFK 59-RNase T1 (▲), Kyn 59-RNase T1 (■), and intact RNase T1 (●) in 0.01 M sodium phosphate buffer containing 0.15 M NaCl at pH 7.5. (b) Thermal unfolding transitions of NFK 62-lysozyme (▲), Kyn 62-lysozyme (■), and intact lysozyme (●) in the presence of 1.5 M Gdn-HCl at pH 7.5. The transitions were measured in terms of the change in CD at 218 nm for the intact and modified RNase T1's, at 222 nm for the intact lysozyme, and at 229 nm for the modified lysozymes. The ordinate represents the fraction of unfolded protein (f_D).

Figure 5 shows the dependence on Gdn-HCl concentration of the apparent rate constants of unfolding and refolding, λ_1 and λ_2, and the relative amplitude of the fast phase α_2. When compared with the results for the intact C_L fragment (Goto & Hamaguchi, 1982), the changes in λ_1, λ_2, and α_2 produced by Gdn-HCl were all shifted parallel to lower concentrations of Gdn-HCl by about 0.7 M.

Double-Jump Experiment. Previously Goto and Hamaguchi (1982) showed that two forms of the unfolded C_L fragment exist in the unfolded state. In order to know whether two such forms also exist in the unfolded state of the Kyn 187-C_L fragment, we performed double-jump experiments (Brandts et al., 1975; Nall et al., 1978). The Kyn 187-C_L fragment was first unfolded by 3 M Gdn-HCl, and then refolding was initiated at 0.1 M Gdn-HCl after various intervals under the unfolding conditions. If there is a slow equilibrium between the two forms in the unfolded state ($U_2 \rightleftharpoons U_1$), the

Table II: Parameters for Unfolding Transitions by Gdn-HCl of the Intact C_L, NFK 187-C_L, and Kyn 187-C_L Fragments at pH 7.5 and 25 °C

		Gdn-HCl binding		linear extrapolation	
	C_m[a] (M)	$\Delta G_D^{H_2O}$ (kcal/mol)	Δn	$\Delta G_D^{H_2O}$ (kcal/mol)	$-(d\Delta G_D/d[Gdn\text{-}HCl])$ (kcal mol^{-1} M^{-1})
intact C_L[b]	1.2	5.7	29.5	4.1 ± 0.1	3.5 ± 0.1
NFK 187-C_L	0.55 ± 0.06	2.4 ± 0.1	22.6 ± 1.0	1.9 ± 0.1	3.4 ± 0.1
Kyn 187-C_L	0.60 ± 0.07	2.7 ± 0.2	23.0 ± 1.3	2.1 ± 0.2	3.5 ± 0.2

[a] Concentration of Gdn-HCl at the midpoint of the curve for unfolding by Gdn-HCl. [b] Cited from Goto and Hamaguchi (1987). The transition curves were analyzed by assuming the two-state approximation of N(native) ⇌ D(unfolded). The equilibrium constant of unfolding (K_D) was determined by $K_D = f_D/(1 - f_D)$ at each concentration of Gdn-HCl. $\Delta D_D^{H_2O}$ is the free energy change of unfolding in the absence of Gdn-HCl and was obtained by two methods. In one method (linear extrapolation), the values of ΔG_D were plotted against the concentration of Gdn-HCl, and the value of $\Delta G_D^{H_2O}$ was obtained by linear extrapolation to zero concentration of Gdn-HCl. In the other method (Gdn-HCl binding model), we estimated $\Delta G_D^{H_2O}$ by using the equation proposed by Tanford (1970): $\Delta G_D = \Delta G_D^{H_2O} - \Delta nRT \ln (1 + ka_\pm)$, where Δn is the difference in the number of binding sites between the unfolded and folded states, k is the average binding constant of the sites, the $a_\pm$ is the mean activity of Gdn-HCl. We used 0.6 M^{-1} as the value of k [Pace & Vanderburg, 1979; see Goto and Hamaguchi (1979)].

refolding kinetics should depend on the amplitude of the slow phase (α_1) under the refolding conditions after various times of exposure of the Kyn 187-C_L fragment to 3 M Gdn-HCl. The dependence was expressed by one exponential decay process, and an apparent rate constant of 3.4×10^{-2} s^{-1} was obtained. This value is comparable to the value of 3.2×10^{-2} s^{-1} obtained for the intact C_L fragment (Goto & Hamaguchi, 1982).

Discussion

Conformations of the NFK 187-C_L and Kyn 187-C_L Fragments. As shown in Figure 1, oxidation of Trp 187 of the C_L fragment to NFK or Kyn did not change the secondary structure. The fluorescence spectra of the NFK 187-C_L and Kyn 187-C_L fragments were very similar to the spectrum of the type κ C_L fragment, which lacks a tryptophan residue at the position corresponding to Trp 187 of the type λ C_L fragment and has only one tryptophan residue (Trp 148) corresponding to Trp 150 of the type λ C_L fragment (Tsunenaga et al., 1987). The fluorescence of the type κ C_L fragment is strongly quenched owing to the proximity of Trp 148 to the intrachain disulfide bond, a strong quencher, and, at most, accounts for 5% of the fluorescence of the C_L fragment in 4 M Gdn-HCl. All these observations indicate that the overall structures of the NFK 187-C_L and Kyn 187-C_L fragments are the same as that of the intact C_L fragment.

Ozone oxidation of the C_L fragment denatured in 2 M Gdn-HCl yielded a fragment in which both Trp 187 and Trp 150 were modified to NFK [NFK (150, 187)-C_L fragment]. The Kyn (150, 187)-C_L fragment was obtained by treating the NFK (150, 187)-C_L fragment in 6 M Gdn-HCl with acid at −10 °C. Judging from the CD spectra, both the NFK (150, 187)-C_L and Kyn (150, 187)-C_L fragments were completely unfolded even in the absence of denaturant. This suggests that Trp 150 is very important for folding of the C_L fragment molecule. The structure of *N*-acetylkynurenine in crystals is similar to that of tryptophan (Kennard et al., 1979). Even such a small change in Trp 150 causes unfolding of the molecule. Thus residues other than Trp at position 150 cannot maintain the intact structure. This may explain why Trp 150 in the interior of the molecule is highly conserved in the other domains of the immunoglobulin molecule.

Stability of the NFK 187-C_L and Kyn 187-C_L Fragments. Conversion of Trp 187 to NFK or Kyn in the C_L fragment resulted in a protein less stable against Gdn-HCl and heat compared with the intact fragment (Figures 2 and 3).

The unfolding curves produced when Gdn-HCl was used (Figure 2) were analyzed by assuming a two-state approximation, and the free energy change of unfolding in the absence of Gdn-HCl, $\Delta G_D^{H_2O}$, was estimated by using two methods of extrapolation [see Pace et al. (1989)], a linear extrapolation

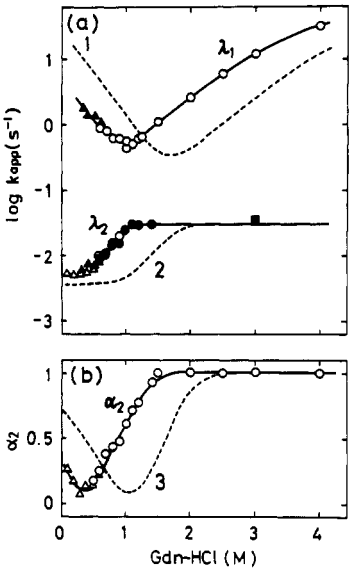

FIGURE 5: Dependence on Gdn-HCl concentration of (a) the apparent rate constants (λ_1 and λ_2) and (b) the relative amplitude (α_2) of the fast phase for unfolding and refolding kinetics of the Kyn 187-C_L fragment at pH 7.5 and 25 °C. (○) From unfolding kinetics obtained by stopped-flow measurements; (▲) from refolding kinetics obtained by stopped-flow measurements; (△) from unfolding kinetics obtained by manual mixing; (●) from refolding kinetics obtained by manual mixing; (■) the apparent rate constant of the isomerization process of U_2 to U_1 in mechanism 1 after unfolding measured by double-jump experiments. The initial concentrations of Gdn-HCl for unfolding and refolding measurements were 0 and 1.8 M, respectively. The final protein concentration was 0.025 mg/mL. The broken lines, 1, 2, and 3, represent the values of λ_1, λ_2, and α_2, respectively, for the intact C_L fragment cited from the previous paper (Goto & Hamaguchi, 1982).

method and a method based on the Gdn-HCl binding model. Table II summarizes the values of $\Delta G_D^{H_2O}$ obtained for the intact C_L, NFK 187-C_L, and Kyn 187-C_L fragments. Although the values obtained by the denaturant binding model were larger than the values obtained by linear extrapolation, it is shown that the stability of the C_L fragment is decreased upon modification of Trp 187. It is unknown at present which method is more appropriate for estimating the value of $\Delta G_D^{H_2O}$. However, the value obtained by the denaturant binding model is always larger than the value obtained by linear extrapolation (Pace et al., 1989).

The thermal unfolding curves were analyzed as follows. Assuming a two-state approximation, the enthalpy change (ΔH_D) upon unfolding was evaluated by using the van't Hoff equation. From the free energy change of unfolding, $\Delta G_D = 0$ at $T = T_m$, the entropy change (ΔS_D) is obtained. At a given temperature (T), $\Delta H_D(T)$, $\Delta S_D(T)$, and $\Delta G_D(T)$ can be obtained by using the equations

$$\Delta H_D(T) = \Delta H(T_m) + \Delta C_p(T - T_m) \quad (2)$$

$$\Delta S_D(T) = \Delta S(T_m) + \Delta C_p \ln (T/T_m) \quad (3)$$

$$\Delta G_D(T) = \Delta H(T_m) - T\Delta S(T_m) + \Delta C_p[T - T_m - T \ln (T/T_m)] \quad (4)$$

In these calculations, a value of 1.8 kcal deg^{-1} mol^{-1} for the intact C_L and a value of 2.1 kcal deg^{-1} mol^{-1} for the Kyn 187-C_L were used as the value of ΔC_p (see Results). The value of ΔC_p for the NFK 187-C_L fragment was assumed to be the same as the value for Kyn 187-C_L. The thermodynamic parameters for the thermal unfolding of the C_L fragment and its modified proteins are shown in Table I. The values of ΔG_D at 25 °C for the intact and modified C_L fragments thus obtained are in good agreement with the respective values estimated from the data for unfolding by Gdn-HCl (Table II). On oxidation of Trp 187 to NFK or Kyn, the stability of the C_L fragment is decreased.

The lower stability of the Kyn 187-C_L fragment compared with the intact C_L fragment may be explained on the basis of the kinetic data of unfolding and refolding by Gdn-HCl. As shown in Figure 5, the apparent rate constants, λ_1 and λ_2, and the relative amplitude of the fast phase, α_2, of the Kyn 187-C_L fragment are very similar in shape to those for the intact C_L fragment, except that they shift to lower concentrations of Gdn-HCl by about 0.7 M. This indicates that the unfolding and refolding of the Kyn 187-C_L fragment can be explained in terms of the same mechanism used for the folding kinetics of the intact C_L fragment. Goto and Hamaguchi (1982) showed that the unfolding and refolding kinetics of the C_L fragment at Gdn-HCl concentrations above 1.0 M can be explained in terms of the following three-species mechanism, which had been first proposed for the folding kinetics of ribonuclease A by Garel and Baldwin (1976) and Brandts et al. (1975):

mechanism 1

$$U_1 \underset{k_{21}}{\overset{k_{12},\ \text{slow}}{\rightleftharpoons}} U_2 \underset{k_{32}}{\overset{k_{23},\ \text{fast}}{\rightleftharpoons}} N$$

where N is the native protein, U_1 and U_2 are the slow-folding and fast-folding species, respectively, of unfolded protein, and k_{12}, k_{21}, k_{23}, and k_{32} are the microscopic rate constants for the respective processes. U_1 and U_2 are indistinguishable from one another on the basis of CD and fluorescence properties.

In the case of the intact C_L fragment, the value of α_2 increases below 1.0 M Gdn-HCl. This was explained by assuming the presence of an intermediate in the folding process in this concentration range of Gdn-HCl (Goto & Hamaguchi, 1982). In the case of the Kyn 187-C_L fragment, the value of α_2 increases below 0.2 M Gdn-HCl, and this may also reflect the presence of an intermediate. However, the folding kinetics in the concentration range above 0.2 M Gdn-HCl could be analyzed on the basis of mechanism 1. The value of ($k_{12} + k_{21}$) was estimated to be 3.37×10^{-2} s^{-1} by the double-jump experiments. Assuming that the equilibrium constant (K_{21}) between U_1 and U_2 is independent of the concentration of Gdn-HCl and that the value of λ_1 at low concentrations below the transition is equal to k_{12}, the value of K_{21} ($=k_{21}/k_{12}$) was estimated to be 6. The values of k_{12}, k_{21}, k_{23}, and k_{32} were then estimated from the experimentally determined values of λ_1, λ_2, and α_2. Figure 6 shows the microscopic rate constants thus obtained as a function of the concentration of Gdn-HCl. As can be seen, the rate constants for the interconversion between U_1 and U_2 below 1.2 M Gdn-HCl are essentially constant and agree with the value obtained by the double-jump experiment using 3 M Gdn-HCl. The changes in the unfolding and refolding rate constants for the intact C_L and the Kyn 187-C_L fragments with the concentration of Gdn-HCl show exactly the same shape except that the rate constants for the modified fragment are shifted to lower concentrations of Gdn-HCl by about 0.7 M. The kinetic data suggest a large effect on the transition state for folding and unfolding. When compared at 1 M Gdn-HCl, for example, the unfolding rate constant (k_{32}) and refolding rate constant (k_{23}) for the Kyn 187-C_L fragment are about 10 times larger and 7 times smaller, respectively, than those for the intact C_L fragment. This shows that the Kyn 187-C_L fragment is destabilized by both the increase in the unfolding rate and the decrease in the refolding rate.

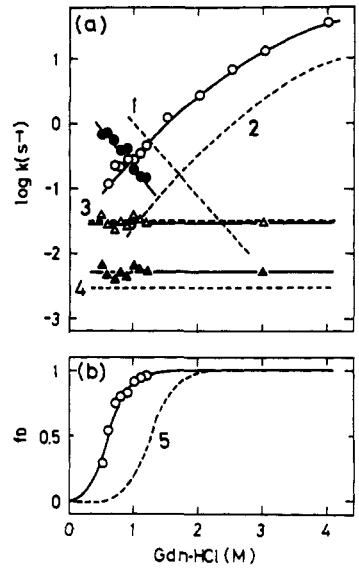

FIGURE 6: Dependence on Gdn-HCl concentration of the microscopic rate constants estimated on the basis of mechanism 1 for folding of the Kyn 187-C_L fragment (a) and the transition curve for unfolding calculated with the microscopic rate constants (b) at pH 7.5 and 25 °C. (●) k_{23}; (○) k_{32}; (△) k_{21}; (▲) k_{12}. The continuous line in (b) indicates the transition curve for unfolding obtained from equilibrium measurements. The broken curves 1–5 represent the values of k_{23}, k_{32}, k_{21}, k_{12}, and f_D for the intact C_L fragment cited from the previous paper (Goto & Hamaguchi, 1982).

Stability of Modified RNase T1's and Lysozymes. The thermal transition curve (Figure 4a) of intact RNase T1 was analyzed by using eqs 2–4, and the free energy change of unfolding at 25 °C was estimated (Table I). In this calculation, a value of 1.65 kcal deg^{-1} mol^{-1} was used as the value of ΔC_p (Pace & Laurents, 1989). As shown in Table I, the free energy change of unfolding of the intact RNase T1 in 0.01 M sodium phosphate buffer containing 0.15 M NaCl was found to be 5.0 kcal/mol. Although the value of the free energy change of unfolding of RNase T1 depends on the concentrations of the salt, this value may be compared with the value of 5.5 kcal/mol obtained in 0.03 M 3-(*N*-morpholino)propanesulfonic acid at pH 7 by linear extrapolation of the urea denaturation curve (Pace & Grimsley, 1988). The thermal unfolding curves for NFK 59-RNase T1 and Kyn 59-RNase T1 were analyzed on the assumption that the values of ΔC_p for these modified proteins are the same as that for the intact RNase T1. As can be seen in Table I, modification of Trp 59 of RNase T1 to NFK or Kyn resulted in a remarkable decrease in stability.

The thermal unfolding transitions of the intact and modified lysozymes (Figure 4b) were analyzed by using eqs 2–4. In this calculation, the value of ΔC_p for the intact lysozyme was assumed to be 1.5 kcal deg^{-1} mol^{-1} (Privalov, 1979). The same value was also assumed for the ΔC_p values of the modified

lysozymes. As can be seen in Table II, no significant change in the free energy change of unfolding at 25 °C occurred on modification of Trp 62 to NFK or Kyn.

Thermal Factors and Stability. As described above, the extent of the decrease in the stability of the proteins on modification of Trp increases in the order Kyn 62-lysozyme < Kyn 187-C_L fragment < Kyn 59-RNase T1. We found that this order is related to crystallographic thermal factors of the tryptophan residues. The thermal factors of Trp 59 of RNase T1, Trp 150 and Trp 187 of the C_L fragment, and Trp 62 of hen lysozyme were obtained from the data of Sugio et al. (1988), Marquart et al. (1980), and Rao et al. (1983), respectively. We calculated the thermal factor of each tryptophan residue relative to the average values of whole atoms in the protein. The relative thermal factors of the tryptophan side chains are as follows: 0.77 for Trp 59 of RNase T1, 0.84 for Trp 187 of the C_L fragment, 0.54 for Trp 150 of the C_L fragment, and 1.97 for Trp 62 of lysozyme.

The polarity of the microenvironment of the Kyn residue may also be estimated from the kynurenyl fluorescence spectra. Fukunaga et al. (1982a) found that the fluorescence intensity and the maximum wavelength of *N*-acetylkynurenineamide depended linearly on the polarity of the solvent. On the basis of their result, the polarity of the microenvironment of the Kyn residue is estimated to correspond to 53% dioxane for Kyn 59-RNase T1, 25% for the Kyn 187-C_L fragment, and 18% for Kyn 62-lysozyme. These values of polarity are in accord with the mobility estimated from the crystallographic thermal factors. These findings indicate that the lower the mobility or solvent accessibility of the tryptophan residue, the greater the extent of the decrease in stability upon modification. Alber et al. (1987) found that all of the temperature-sensitive mutations in bacteriophage T4 lysozyme occur at sites with low mobility and low solvent accessibility in the folded protein.

Finally, with regard to the changes in the thermodynamic parameters for thermal unfolding upon modification (Table I), we observed that the enthalpy change and entropy change of the modified proteins were larger than the respective values for the intact proteins. As the contribution of the entropy term is larger than the enthalpy term, the free energy changes for the unfolding of the proteins become smaller, the stability being decreased. The reason why the enthalpy and entropy changes for the unfolding of the modified proteins are larger than those for the unfolding of the intact proteins is not clear at present. Recently, Shortle et al. (1988) studied the changes in stability of *Staphylococcus* nuclease with amino acid substitutions of a buried hydrophobic residue with a bulkier and more polar residue. They observed that the enthalpy and entropy changes for the thermal unfolding are both larger and the free energy change is smaller for the mutant protein compared with the values for the wild protein. They explained these findings in terms of greater disruption of intrachain interactions in the unfolded state of the mutant protein.

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Registry No. L-Trp, 73-22-3; RNase T1, 9026-12-4; lysozyme, 9001-63-2.

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