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Conformational Stability of Ribosomal Protein L7/L12: Effects of pH, Temperature, and Guanidinium Chloride†

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ABSTRACT: The effects of pH, temperature, and guanidinium chloride on the conformation of ribosomal protein L7/L12 have been investigated in order to understand the stability of this protein dimer. The results indicate that many of the molecular forces stabilizing the conformation of the dimer are disrupted at low pH or high temperature. These acid- and thermal-denatured states, however, still retain considerable secondary structure. Approximately half of the α-helical content present in the native protein remains intact at pH below 2 and at temperatures above 90 °C. Further denaturation of the acid-denatured protein by 6 M guanidinium chloride results in a state which still contains ~20% α helix. Similar amounts of residual conformation remain when the native L7/L12 dimer is denatured with guanidinium chloride. Thermodynamic analysis of the conformational transitions studied indicates that none is compatible with a simple two-state process. The complexity of these denaturation data and the structural characterizations of the various denatured states are consistent with the possible existence of structural domains in the protein molecule possessing different conformational stabilities.

Protein L7/L12, from the large subunit of *Escherichia coli* ribosomes, is an extremely elongated (Wong & Paradies, 1974; Österberg et al., 1976; Luer & Wong, 1979), highly α-helical protein (Möller et al., 1970; Dzionara, 1970; Boublik et al., 1973; Gudkov et al., 1978a,b; Luer & Wong, 1979) which is known to exist as a dimer in solution (Möller et al., 1972; Wong & Paradies, 1974). Evidence is accumulating from reconstitution experiments (Caldwell et al., 1978; Koteliansky et al., 1978) and electron microscopic studies (Boublik et al., 1976; Lake, 1976; Strycharz et al., 1978) that the elongated dimer may exist in the functionally active ribosome. This suggests that studies on the L7/L12 dimer in solution may provide relevant structural information which can be applied to the growing knowledge of the biomolecular components of the ribosome.

† From the Department of Biochemistry, University of Kansas Medical Center, Kansas City, Kansas 66103. *Received April 24, 1979.* This investigation was supported by Research Grant GM 22962 and in part by Grant HL 18905 from the National Institutes of Health, U.S. Public Health Service, Bethesda, MD.

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0006-2960/80/0419-0176$01.00/0

The information required for ribosome assembly appears to reside in the individual proteins and RNAs, since reconstituted ribosomal subunits have been successfully assembled in vitro solely from their respective purified constituents (Traub & Nomura, 1968; Maruta et al., 1971; Held et al., 1973; Nierhaus et al., 1973; Cohlberg & Nomura, 1976). Furthermore, the recent demonstrations that the constituent proteins and RNA do possess unique three-dimensional structures under the conditions of reconstitution (Allen & Wong, 1978a–c, 1979; Luer & Wong, 1979) suggest that the self-assembly probably proceeds by specific molecular recognitions of the protein and RNA components during their interactions to form the 30S ribosomal subunit. These interactions also involve the complex conformational changes of the 16S RNA (Hochkeppel & Craven, 1977; Dunn & Wong, 1979a) and those of the proteins (Dunn & Wong, 1979b) and the resulting reconstituted intermediates (RI and RI*) (Dunn & Wong, 1979c).

With the ultimate goal of deciphering the molecular mechanism of ribosome assembly, further information must be obtained concerning the molecular forces which stabilize the unique three-dimensional structures of the component proteins and RNAs, so as to understand how they interact via molecular recognition during this complex assembly process.

Studies of protein denaturation leading to various denatured conformational states have been used to understand the conformations and to decipher the forces responsible for the stabilization of their unique functional structures [for reviews, see Kauzmann (1959), Hermans (1965), Tanford (1968), Lumry & Biltonen (1969), Brandts (1969), and Baldwin (1975)]. Most of the studies have dealt with denaturation by extreme pH or increased concentrations of denaturing solvents under isothermal conditions or with denaturation purely as a function of temperature. The most commonly employed denaturing reagents have been urea and guanidinium chloride. More recently, various combinations of denaturing conditions have been used simultaneously to understand better the nature of the denaturing process. As a result, considerable effort has been spent characterizing the products of denaturation (Tanford et al., 1967a,b; Nozaki & Tanford, 1967; Aune et al., 1967; Wong & Tanford, 1970) and studying the transition from native to denatured states in terms of kinetic and thermodynamic parameters (Hermans & Scheraga, 1961; Scott & Scheraga, 1963; Brandts, 1964a,b; Hermans & Acampora, 1967; Wong & Tanford, 1973). The information about denaturation gained from these early studies has been compiled almost exclusively from globular proteins. Very little work has been done with proteins which are not considered to be globular (Byers & Verpoorte, 1978).

The conformational stability of the L7/L12 dimer was first studied in a preliminary manner by Boublik et al. (1973). In this study, salt concentrations varying from 1 mM to 1 M were shown to cause essentially no change in α-helical content compared to that of the native structure. It is important to note that the pH values of the buffered solutions did not deviate from a range of 6.2–7.8 and that the estimations of α-helical content were made from ellipticities at a single wavelength obtained from circular dichroism spectra. Reversible decreases in α-helical content were achieved, however, when the dimer was exposed to high concentrations of urea or guanidinium chloride. Estimates of α-helical content were 18 and 14% in 7.2 M urea and 5.5 M guanidinium chloride, respectively. No attempt was made in this study to characterize any of the transitions to the denatured states. More recently, thermal denaturation of the L7/L12 dimer has been investigated by using microcalorimetry (Gudkov et al., 1978a,b). These studies report a transition temperature of 68–70 °C and a calorimetric enthalpy of thermal unfolding, ΔH_{cal}, of 136 kcal/mol for the L7/L12 dimer, but they provide no information about the changes in secondary structure associated with the thermal transition or the thermal-denatured state.

The present study describes work aimed at investigating the conformational stability of protein L7/L12 as a function of pH, temperature, and guanidinium chloride concentrations. Attempts are made to characterize the residual secondary structures of the various denatured states and to analyze the various thermodynamic parameters leading to such states. Ultimately, the information obtained can contribute to a better understanding of the inter- and intramolecular forces which stabilize the unique conformation of the L7/L12 dimer.

Experimental Procedures

Isolation of Protein L7/L12. *E. coli* strain MRE 600 (RNase I$^-$) was purchased from Microbiological Research Establishment, Porton, England, and stored at −70 °C until needed. The 70S ribosomes were isolated according to the procedure of Traub et al. (1971) with slight modifications (Allen & Wong, 1979). Protein L7/L12 was extracted from 70S ribosomes according to the procedure of Hamel et al. (1972) and purified further as previously described (Luer & Wong, 1979).

Ionic Strength Conditions. A nonbuffered salt solution, referred to as MK_{360} medium (20 mM $MgCl_2$ and 360 mM KCl), was employed in the denaturation studies. This solution was selected in an effort to simulate the high ionic strength environment necessary for optimal ribosome assembly (Traub & Nomura, 1969).

Circular Dichroism. CD measurements were obtained from a JASCO Model J-20 spectropolarimeter. The temperature in the sample cell was controlled by a water-jacketed aluminum cell holder with constant-temperature water circulated from a Lauda K-2/R water bath. The temperature of the sample was measured directly with a telethermometer, Model YSI 425C. The results were expressed in terms of mean residue weight ellipticity, $[\Theta]_{MRW}$, according to eq 1

$$[\Theta]_{MRW} = \frac{\Theta(MRW)}{dc} \quad (1)$$

where Θ is observed ellipticity in degrees, MRW is the mean residue weight of L7/L12 which was calculated to be 102, d is cell path length in decimeters, and c is protein concentration in grams per milliliter. The instrument was calibrated routinely with d-10-camphorsulfonic acid according to Cassim & Yang (1969). During all measurements the spectropolarimeter was continuously purged with pure nitrogen gas.

Acid Denaturation. In preparation for the pH-dependent studies, a 2-mL sample of known concentration of the L7/L12 dimer was prepared in the MK_{360} medium. From stock solutions of 25 mM, 50 mM, and 1 M HCl, known volumes were added to the protein samples and the pH values were measured. The change in ionic strength of the sample solutions as a result of HCl addition was not significant when this protocol was followed. Following a 15-min incubation at each pH value, the observed ellipticities at 222 nm were recorded. After each measurement, the pH was redetermined before the next adjustment was made. All pH measurements were performed in the CD cuvette to avoid a possible change in volume or loss of protein as a result of sample transfer. A complete CD spectrum was obtained for protein L7/L12 from 250 to 200 nm for both the native and acid-denatured samples.

After reaching the acid-denatured states in this ionic condition, the pH values of the protein samples were readjusted to the neutral range where the extent of reversibility to the native ellipticity was measured. The temperature of the sample cell compartment in the spectropolarimeter was maintained at 25 °C for all incubations and CD measurements.

Thermal Denaturation. As in the acid denaturation studies, 2-mL volumes of known concentration of protein L7/L12 were prepared in the MK_{360} medium. By use of the same stock HCl solutions, known volumes were added until pH values corresponding to representative portions of the acid-denaturation profiles were obtained. The samples were placed in a water-jacketed aluminum cell holder for which a temperature-difference calibration curve, relating the water bath and sample temperatures, had been previously determined. A drop of silicone oil was layered over each sample to prevent evaporation. Each sample was allowed to incubate for 15 min at each temperature along the denaturation profile, followed by the recording of the observed ellipticity value at 222 nm. Complete CD spectra from 250 to 200 nm were obtained for each initial sample at 25 °C and each thermally denatured sample at 90 °C. After the heat-denatured spectrum was recorded, the temperature was cooled to 25 °C where the extent of reversibility to the native state was measured. The observed ellipticity at 222 nm was measured periodically during the cooling process to follow the path of renaturation.

Denaturation by Guanidinium Chloride. Samples for exposure to increasing concentrations of guanidinium chloride (GdmCl) were prepared in two ways. In one procedure, the acid-denatured states of the dimer L7/L12 were generated by addition of known volumes of stock HCl solutions to known volumes of known concentration of protein in the MK_{360} medium. Alternatively, native L7/L12 dimer was prepared at a known concentration. Following preparation by either method, aliquots of the protein sample were then added to appropriate volumes of increasing GdmCl concentrations. Each sample was allowed to incubate for at least 2 h at 25 °C after which CD spectra were recorded. The high absorbance of GdmCl at low wavelengths prevented the recording of circular dichroic signals much below 212 nm. Following the CD measurements, final concentrations of GdmCl concentrations were determined by obtaining the refractive index of each sample. The sample compartment was maintained at 25 °C during the recording of all CD spectra.

Thermodynamic Analysis. The data are treated by analytical methods appropriate to reversible denaturation of proteins as described by Tanford (1968) and Hermans (1965). If a two-state transition is assumed, the native state (N) is thought to be in equilibrium with the denatured state (D) as a result of exposure of the protein to different denaturing conditions. The transition, $N \rightleftharpoons D$, can be characterized by the single variable, f_D, defined as the fraction of the protein molecule in the denatured state. By use of mean residue weight ellipticity as the experimental variable to follow the transition, $[\Theta]_N$ and $[\Theta]_D$ are the characteristic values for the initial and final states. Therefore, at any point along the transition, the observed mean residue weight ellipticity can be expressed as shown in eq 2

$$[\Theta]_{obsd} = [\Theta]_N + f_D([\Theta]_D - [\Theta]_N) \quad (2)$$

and the fraction of the protein molecule in the denatured state, f_D, can be measured experimentally as shown in eq 3

$$f_D = \frac{[\Theta]_{obsd} - [\Theta]_N}{[\Theta]_D - [\Theta]_N} \quad (3)$$

Since each experimental value along the transition will yield a unique value for f_D, the equilibrium constant, K_D, for the reaction $N \rightleftharpoons D$ can be represented as eq 4 where

$$K_D = \frac{f_D}{(1 - f_D)} \quad (4)$$

Only experimental values for f_D greater than 0.1 but which did not exceed 0.9 were considered to yield reliable results. With these restrictions on f_D, subsequent values for K_D necessarily fell between 0.1 and 10.

Where the transitions arise from reversible thermal denaturation, the data can be represented in a van't Hoff plot in which the ln K_D is plotted as a function of the reciprocal of the absolute temperature. The slopes of the resulting plots can be related to the standard enthalpy of the unfolding reaction ($\Delta H°_{vH}$) as defined by the van't Hoff equation (eq 5)

$$\Delta H°_{vH} = -R\frac{\partial \ln K_D}{\partial(1/T)} \quad (5)$$

The T_m values for each thermal transition can also be obtained from these plots. At the temperature where the fraction of denatured material is 0.5, the equilibrium constant will necessarily be 1. The intersection of the straight-line plots of the data with the line representing ln $K_D = 0$ provides T_m values which are seen to decrease with decreasing pH.

With experimental values for the standard enthalpies and T_m values, the standard entropies for the thermal unfolding can also be determined. The standard entropy of unfolding, $\Delta S°_u$, can be related to the standard enthalpy of unfolding and the standard free energy of unfolding, $\Delta G°_u$, as shown in eq 6, where

$$\Delta S°_u = \frac{(\Delta H°_{vH} - \Delta G°_u)}{T} \quad (6)$$

Since $K_D = 1$ and, therefore, $\Delta G° = 0$ at the T_m, eq 6 can be rewritten as shown in eq 7, where

$$\Delta S°_u = \frac{\Delta H°_{vH}}{T_m} \quad (7)$$

The GdmCl denaturation data are analyzed in a similar fashion. With the assumption that the unfolding of the acid-denatured state and the native state can be approximated by a two-state process, initial and final states as determined by CD can be related to an apparent equilibrium constant, K_{app}, as defined in eq 8

$$K_{app} = \frac{[\Theta]_{obsd} - [\Theta]_i}{[\Theta]_{GD} - [\Theta]_{obsd}} \quad (8)$$

where $[\Theta]_{obsd}$, $[\Theta]_i$, and $[\Theta]_{GD}$ are the mean residue weight ellipticity values at 222 nm for the observed sample at a known GdmCl concentration, the initial state (acid denatured or native), and the GdmCl-denatured state (final state), respectively. Information can be obtained from a plot of the equilibrium constant as a function of the log of the denaturant concentration. This equilibrium constant varies with the GdmCl concentration near the midpoint of the transition according to eq 9

$$K_{app} = A[\text{GdmCl}]^n \quad (9)$$

where A and n are constants. The slope of the line provides a value for the constant n. From this value, which reflects the steepness of the experimental transition, an indication of the relative degree of cooperativity for the denaturation process can be assessed.

Miscellaneous. All pH measurements were made with a Radiometer Model PHM 64 Research pH meter equipped with a combined glass electrode (GK2303C). Sample tem-

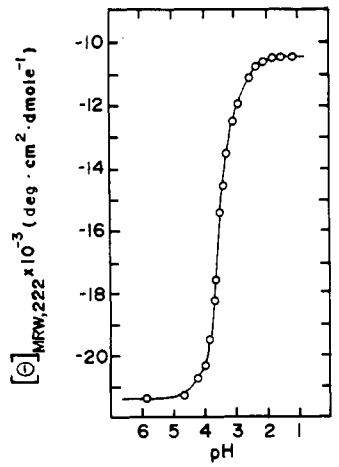

FIGURE 1: Acid-denaturation profile for protein L7/L12. Conditions: MK_{360} medium; 25 °C. Identical profiles were obtained at protein concentrations of 0.015 and 0.018 mg/mL.

peratures were controlled by a Lauda K-2/R circulating water bath with antifreeze as the circulating solution. GdmCl was ultrapure quality purchased from Schwarz/Mann. Refractive indices were determined by using a Bausch & Lomb refractometer. Analyses of secondary structural information contained in far-ultraviolet circular dichroism spectra were carried out by the method of Yang and co-workers (Chen et al., 1974) using a BMD073 computer program (*Biomedical Computer Programs*, 1973) and an IBM 270/145 computer as described previously (Luer & Wong, 1978). A slight modification of the analysis was required in the analysis of the spectra where the presence of guanidinium chloride prevented recording below 212 nm. For generation of the best fit from the nonlinear least-squares analysis, only those portions of the reference spectra for 100% α helix, β structure, and unordered structure from 240 to 212 nm were entered into the computer program. Protein concentrations were determined by using the modification by Hartree (1972) of the Lowry procedure and using bovine serum albumin as a standard. Dialysis tubing was treated by boiling in 1% Na_2CO_3 and 1 mM EDTA for 30 min and washed thoroughly with distilled water.

Results

Acid Denaturation. Addition of the L7/L12 dimer to the unbuffered MK_{360} salt solution results in samples whose pH values are around 6. Analyses of the corresponding CD spectra reveal that the secondary structure of protein L7/L12 under these conditions is comparable to that determined for the native protein dimer (Luer & Wong, 1979). The acid-denaturation profile is shown in Figure 1. There is no change in the mean residue weight ellipticity value at 222 nm, $[\Theta]_{MRW,222}$, until below pH 5, after which a sharp decrease is apparent. The midpoint of transition for the acid denaturation occurs at about 3.5, while the $[\Theta]_{MRW,222}$ begins to plateau at pH values of about 2. No further decrease in CD is observed as the pH is lowered to about 1.5. The ellipticity value apparent for the acid-denatured state indicates that much of the native secondary structure remains intact. This observation is verified by computer analysis of the entire CD spectrum of the acid-denatured state which suggests that approximately 33% α helix is present at pH 1.61 (Table I).

Hildebrandt (1978) reported that at the relatively high protein concentration used (0.4 mg/mL; 0.1 M NH_4Ac and 0.15 M KCl, 20 °C), extensive aggregation of L7/L12 occurs at pH 3. We have performed similar sedimentation equilibrium experiments with L7/L12 at pH 2 (0.35 mg/mL; 20 mM

Table I: Secondary Structural Analysis[a] of Circular Dichroism Spectra Obtained at 25 and 90 °C as a Function of pH in MK_{360}

exptl conditions	% secondary structure		
	α helix	β structure	unordered structure
25 °C			
pH 5.88	71	11	18
pH 3.70	66	6	28
pH 3.30	45	18	37
pH 1.61	33	2	65
90 °C			
pH 5.88	44	16	40
pH 3.70	38	12	50
pH 3.30	34	18	48
pH 1.61	35	8	57

[a] Computer analysis according to the method of Chen et al. (1974).

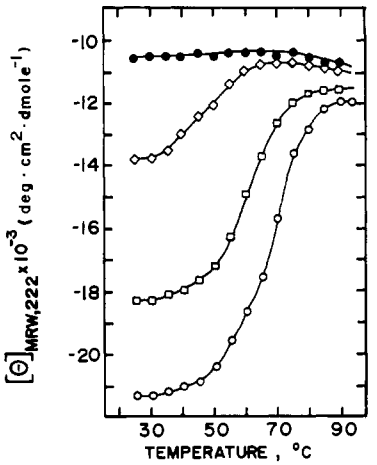

FIGURE 2: Thermal-denaturation profiles for protein L7/L12 at decreasing initial pH values in MK_{360} medium. pH 5.88 (○); pH 3.70 (□); pH 3.30 (◇); pH 1.61 (●). The profiles were obtained at protein concentrations ranging from 0.010 to 0.020 mg/mL.

$MgCl_2$ and 360 mM KCl, 20 °C) and confirmed his results. The ln C vs. r^2 plots are curved upward with point-average molecular weights from ~25 000 to ~80 000. Ultraviolet absorption spectra of acid-denatured L7/L12 at this high protein concentration showed significant linear increase of absorbance from 340 to 300 nm due to the light scattering of aggregated L7/L12. However, at the much lower concentration used in our spectroscopic studies (0.01–0.02 mg/mL), no linear increase in the absorbance due to light scattering was observed. Although such observation is used occasionally to indicate the absence of aggregation, an approximate calculation showed that the type of aggregation in question (~20 000 to ~80 000 molecular weight) may not be able to be detected by this technique.

Thermal Denaturation. Increasing the temperature of the L7/L12 dimer at nondenaturing pH results in a thermally denatured state which also retains considerable secondary structure as judged by $[\Theta]_{MRW,222}$. Analysis of the entire CD spectrum obtained at 90 °C reveals that ~44% α helix is present for protein L7/L12 as shown in Table I.

Increasing the temperature of samples adjusted to decreasing pH values along the acid-denaturation profiles results in thermal transition curves with decreasing T_m values (Figure 2). These curves are completely reversible. The CD spectra for the samples prior to thermal denaturation display an overall flattening in the CD signal and a progressive loss of the characteristic double trough with decreasing pH. Secondary structure analyses of the same spectra indicate that consid-

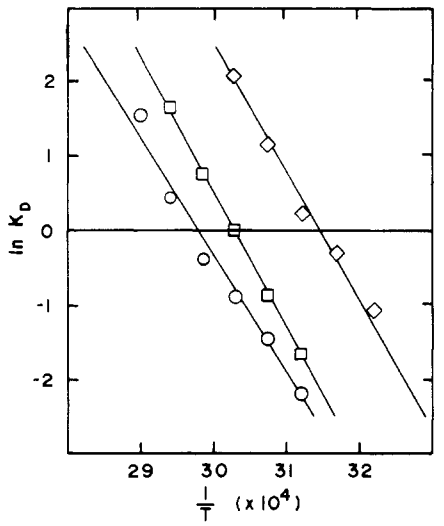

FIGURE 3: van't Hoff plots of the reversible thermal-denaturation data for protein L7/L12 at decreasing initial pH values. pH 5.88 (○); pH 3.70 (□); pH 3.30 (◇).

Table II: Thermodynamic Parameters of Thermal Unfolding of Protein L7/L12 in MK_{360}

pH	ΔH°_{vH} (kcal/mol)	T_m (°C)	ΔS°_u (cal/(K mol))	ΔG°_u, 25 °C (kcal/mol)
5.88	32	63	94	4
3.70	36	57	110	3
3.30	34	45	108	2

erable structural alterations have occurred as a result of the change in pH (Table I). Consequently, the thermally denatured states are achieved at correspondingly lower temperatures.

The thermally denatured states possess progressively lower $[\Theta]_{MRW,222}$ as a function of decreasing pH. This influence of pH on the thermally denatured state is even more pronounced in the CD spectra obtained at 90 °C for each pH value studied. The shapes of the CD spectra vary considerably, especially in the lower wavelength regions, and yield secondary structure analyses with correspondingly decreased amounts of α-helical structure. The acid-denatured state appears to be very similar structurally to the thermally denatured states achieved with samples whose initial pH values were below the midpoint of the pH profile. This final state is characterized as containing 33–35% α helix or approximately half of that existing in the native protein dimer. Thermodynamic information about the thermal-denaturation processes can be gained from van't Hoff plots, which are shown for the thermal denaturation of protein L7/L12 at decreasing pH in Figure 3. The thermal-denaturation data yield reasonably straight lines which are essentially parallel at all pH values in MK_{360}. The experimentally determined thermodynamic parameters obtained for thermal unfolding in MK_{360} are summarized in Table II.

Effect of Guanidinium Chloride. Since the acid-denatured state of protein L7/L12 still possesses considerable secondary structure, the possibility exists that further unfolding may occur upon exposure to additional denaturant action. Increasing the concentrations of GdmCl results in a broad reversible transition between 1 and 4 M, as monitored by $[\Theta]_{MRW,222}$ (Figure 4). The fact that the CD signal at 222 nm is still significant for the GdmCl-denatured state indicates that some residual secondary structure exists even under these conditions. Computer analyses of the spectra support this

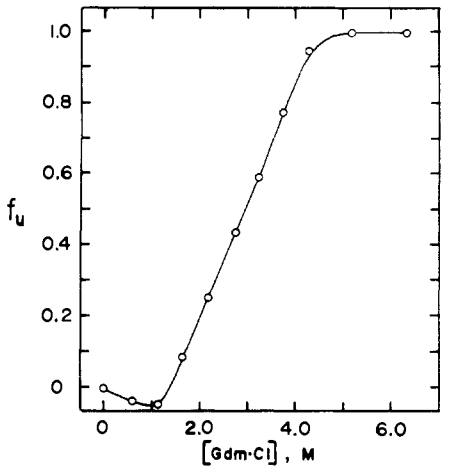

FIGURE 4: Guanidinium chloride denaturation profile of acid-denatured protein L7/L12. The results are reported as fractions of the total change in mean residue weight ellipticity at 222 nm, f_u, as a function of GdmCl concentration. Initial conditions: MK_{360} medium; pH 1.6; 25 °C; protein concentration, 0.032 mg/mL.

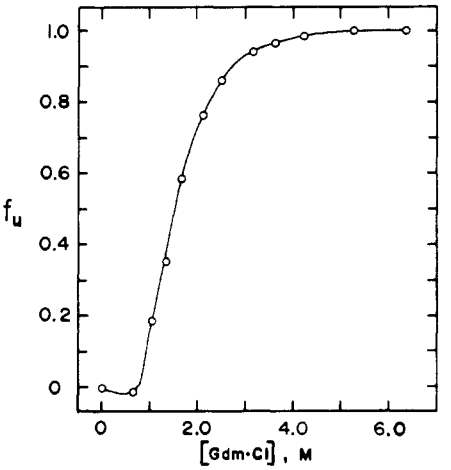

FIGURE 5: Guanidinium chloride denaturation profile of native protein L7/L12. The results are reported as fractions of the total change in mean residue weight ellipticity at 222 nm, f_u, as a function of GdmCl concentration. Initial conditions: MK_{360} medium; pH 6.5; 25 °C; protein concentration, 0.030 mg/mL.

Table III: Secondary Structural Analysis[a] of Circular Dichroism Spectra Obtained from Acid-Denatured L7/L12 Exposed to Increasing Concentrations of Guanidinium Chloride

	% secondary structure		
GdmCl (M)	α helix	β structure	unordered structure
0.61	43	18	39
1.14	45	20	35
1.65	40	19	41
2.18	37	12	51
2.74	32	14	54
3.22	32	8	60
3.72	24	12	64
4.24	20	7	73
5.17	22	8	70
6.31	21	13	66

[a] Computer analysis according to the method of Chen et al. (1974).

observation and suggest that ~20% α helix still remains in the GdmCl-denatured state (Table III). This corresponds to ~30% of the α helix found in the native state.

Denaturation of protein L7/L12 by GdmCl also drastically affects the native conformation as shown in Figure 5. Com-

Table IV: Secondary Structural Analysis[a] of Circular Dichroism Spectra Obtained from Native L7/L12 Exposed to Increasing Concentrations of Guanidinium Chloride

GdmCl (M)	% secondary structure		
	α helix	β structure	unordered structure
0.65	73	11	16
1.06	66	12	22
1.33	54	12	34
1.68	37	11	52
2.11	27	12	61
2.51	22	9	69
3.16	19	6	75
3.62	18	9	73
4.22	18	7	75
5.27	19	11	70
6.35	20	9	71

[a] Computer analysis according to the method of Chen et al. (1974).

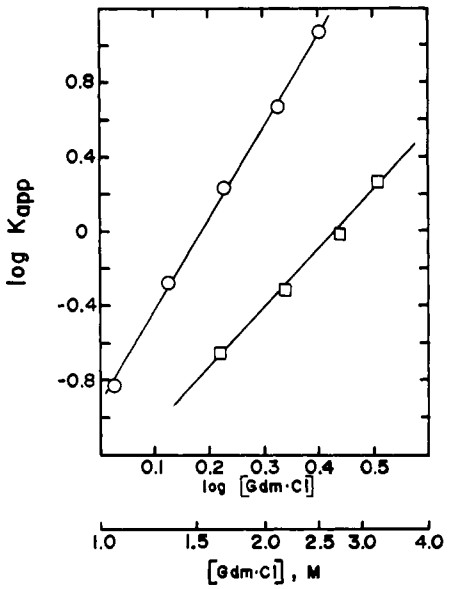

FIGURE 6: Plot of the logarithm of the apparent equilibrium constant, K_{app}, as a function of the logarithm of the guanidinium chloride concentration for the denaturation from native (○) and acid-denatured (□) protein L7/L12.

puter analysis of the CD data not only reflects this decrease in secondary structure (Table IV) but also indicates that ~18% α helix remains following denaturation of the native protein. The conformational transition is characterized by an initial sharp decrease in $[\Theta]_{MRW,222}$ followed by a more gradual change at higher denaturant concentrations.

The conformational transitions from the acid-denatured state and the native state for protein L7/L12 as a function of GdmCl can be plotted to demonstrate the dependence of the apparent equilibrium constant on the GdmCl concentration for the respective transitions. This is shown in Figure 6. Over a limited range of denaturant concentration near the midpoints of transition, the dependence is fairly linear and results in slopes of 3.2 and 5.0 for unfolding of the acid-denatured and the native states, respectively.

A comparison of the changes in $[\Theta]_{MRW,222}$ observed for the denaturation of the acid-denatured state and the native state as a function of GdmCl is shown in Figure 7. The plot suggests that the initial sharp change observed for the native state at low GdmCl concentrations does not occur with the acid-denatured state. Only the gradual change at higher GdmCl concentrations is common to both transitions. As

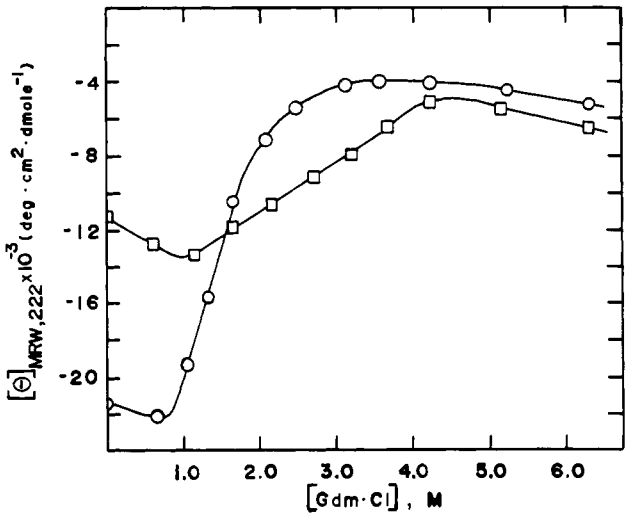

FIGURE 7: Comparison of guanidinium chloride denaturation of acid-denatured L7/L12 and native L7/L12 based on the absolute change of $[\Theta]_{MRW,222}$. Native protein (○); acid-denatured protein (□). Initial conditions: MK_{360} medium; 25 °C; protein concentrations, 0.030 and 0.032 mg/mL for the native and acid-denatured samples, respectively.

evidenced from this plot and the data in Tables III and IV, the final denatured states resulting from either initial state are extremely similar in residual secondary structural content. It is important to note that under these final denaturing conditions, protein L7/L12 is no longer a dimer. Sedimentation equilibrium results reveal a homogeneous species with a molecular weight of ~13 000. Removal of GdmCl by dialysis results in complete recovery of the native secondary structure, as judged by CD, and in a restoration of the dimer nature of the protein.

Discussion

The pH temperature studies indicate that considerable secondary structure is retained in both the acid- and thermal-denatured states. The protonation of the numerous glutamyl and aspartyl carboxyl groups at low pH and the disruption of hydrogen bonds and hydrophobic interaction at high temperature affect only half of the helical content of the L7/L12 molecule, leaving a substantial amount of secondary structure intact. This large residual conformation suggests the possibility of structural domains in the molecule possessing different conformational stabilities. The disruption of molecular forces by decreasing the pH or by increasing the temperature succeeds in affecting only certain portions of the molecule.

Decreasing the pH prior to thermal denaturation effectively produces partially acid-denatured states which were subjected to increasing temperature. With some or all of the electrostatic interactions disrupted, the thermal transitions were completed at correspondingly lower temperatures and, therefore, were characterized by decreasing T_m values. The residual structure remaining at 90 °C is shown to possess progressively smaller amounts of ordered structure at decreasing initial pH values. This trend is particularly significant in terms of the α-helical content (Table I).

The possible aggregation of L7/L12 at low pH even at the low concentration used in the present studies has not been ruled out. Its existence could contribute to the observed conformational transitions if the aggregation in question were accompanied by conformational changes of the protein as detected by CD. However, this aggregation should not affect the interpretations of the results on the conformational tran-

sitions, since Hildebrandt has shown (1978) that the pH-dependent aggregation results in no conformational changes of the protein as detected by far-UV CD studies. Thus, even though aggregation–disaggregation transition occurs, it should not contribute to the observed conformational transitions studied here; the presence of additional conformational states cannot be the result of aggregation.

The enthalpies for the thermal unfolding of protein L7/L12 at varying pH values, as determined from the van't Hoff plots, ΔH_{vH}, range from 32 to 36 kcal/mol. These ΔH_{vH} values are, in all cases, considerably lower than the calorimetric enthalpy of unfolding, ΔH_{cal}, for protein L7/L12 reported by Gudkov et al. (1978a,b). The fact that the ΔH_{vH} is less than the ΔH_{cal} indicates that the two-state approximation is not valid and that the thermal unfolding of protein L7/L12 is more complicated than an all-or-none process.

The T_m reported for the thermal unfolding in the calorimetric study is higher than that observed in the present study. Possible sources of this discrepancy could arise from the different ionic and pH conditions employed. Another difference lies in the time allowed for the sample to reach equilibrium during the temperature increase. In the calorimetric study, the temperature was increased at a constant rate of 1 °C/min while the present study allowed 15-min incubations for each temperature increase of from 3 to 5 °C. If the rate specified in the former study were too rapid for complete equilibrium to be attained, the T_m would appear to occur at a slightly higher temperature than it should.

Another obvious difference between the two studies concerns the protein concentration used. In the calorimetric study, concentrations of L7/L12 ranged from 1.7 to 2.5 mg/mL. Aggregation at high temperatures was reported to be a problem which was decreased, but apparently not totally eliminated, by increasing the pH to 9.2 (Gudkov et al., 1978b). Because of the sensitivity of CD in the far-UV region, the present study employed protein concentrations of between 0.01 and 0.02 mg/mL, resulting in no apparent aggregation over the entire temperature range studied. In addition, no conformational characterization of the thermal-denatured state is provided from the former study so that comparison of the final states from the two studies is not possible.

As characterized by CD, the acid- and thermal-denatured states have been demonstrated to retain considerable secondary structure. In an effort to probe the nature of the forces which stabilize this residual structure, the acid-denatured state was exposed to increasing concentrations of GdmCl under isothermal conditions. The further disruption of residual structure in acid- and heat-denatured proteins has been investigated with ribonuclease, lysozyme, and chymotrypsinogen (Aune et al., 1967). Addition of GdmCl to these three denatured proteins succeeded in demonstrating that further structural changes could be observed. The transition observed upon addition of GdmCl to acid-denatured protein L7/L12 resembles those reported by Aune et al. (1967) in that the transition is very broad. Assuming a two-state process for the further unfolding of the acid-denatured state, apparent equilibrium constants were determined. The slope obtained from the linear dependence of the logarithm of the equilibrium constant on the logarithm of the GdmCl concentration yields a relatively low degree of cooperativity observed for this further unfolding. A low degree of cooperativity is also observed for the unfolding of native L7/L12 with GdmCl, suggesting that the disruption of secondary structure represented by these transitions, as with the acid and thermal transitions, is also not a simple two-state process.

An interesting comparison can be made between the GdmCl denaturation of acid-denatured L7/L12 and that of native L7/L12. The decrease in native conformation as a function of increasing concentrations of GdmCl appears to possess at least two distinct transitions. The first transition results in large decreases in secondary structure over a narrow range of low GdmCl concentration. The second transition occurs at GdmCl concentrations in excess of approximately 1.7 M and is characterized by more gradual decreases in secondary structure over a broader range of denaturant concentrations.

Comparison of the GdmCl transitions of native and acid-denatured L7/L12 indicates that the second transition observed for the native protein may correspond to the conformational transition of the acid-denatured protein. Both transitions are very broad and are found to occur over approximately the same range of GdmCl concentration. If they represent the same transition, then the first transition seen in the native protein may correspond to conformational alterations which produce the acid-denatured state.

The results indicate that the molecular forces maintaining the unique conformation of the L7/L12 dimer are quite complex. The disruption of many of the electrostatic forces, hydrogen bonds, and hydrophobic interactions by acid and thermal denaturation only succeeds in destroying part of the native secondary structure. Further disruption of molecular forces using GdmCl, which generates monomeric L7/L12, still does not produce a random-coiled protein. Evidence for α-helical structure in protein L7/L12 at 5.5 M GdmCl has also been reported by Boublik et al. (1973). The existence of a stable conformational domain which is resistant to further disruption by extreme acid and heat and the retention of 18–20% α helix in 6 M GdmCl are quite different from many of the typical globular proteins.

The observations reported here are consistent with the molecular model of the L7/L12 dimer proposed recently (Luer & Wong, 1979). According to this model, which is based upon experimental data and semiempirical predictions, the dimer consists of two side by side monomeric L7/L12 proteins arranged in a parallel fashion. The amino-terminal half of each monomer is almost entirely α helical and possesses a prominent hydrophobic surface along one side of this helical segment. Interchain interaction is suggested to involve this surface with the possibility that dimerization might be stabilized through structural complementarity along the extensively hydrophobic surface. The carboxy-terminal half of the model is not proposed to participate significantly in the dimer formation and is not thought to be especially stable. There are very few hydrophobic interactions between monomer chains, and electrostatic interaction could easily occur with certain residues in this C-terminal portion of the molecule. In addition, the presence of segments of unordered structure provides the possibility of a certain degree of flexibility. Denaturation by extreme pH and heat and by GdmCl concentrations between 1.0 and 1.7 M could possibly reflect structural changes in this portion of the protein. Further unfolding of the acid-denatured state and the second transition observed with GdmCl concentration higher than 1.7 M could be explained in terms of the stable, highly helical amino-terminal domain proposed in the model. It is this amino-terminal domain which may remain intact following acid and thermal denaturation but which dissociates into monomers in the presence of high concentrations of GdmCl as evidenced by sedimentation equilibrium studies.

Acknowledgments

We are grateful to Drs. Clare K. Woodward and James

Hildebrandt of the University of Minnesota for helpful discussions and for providing a manuscript of their work prior to publication.

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