Guanidine-Induced Denaturation of β-Glycosidase from *Sulfolobus solfataricus* Expressed in *Escherichia coli*†

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Received March 3, 1998; Revised Manuscript Received June 16, 1998

ABSTRACT: Guanidine-induced denaturation of *Sulfolobus solfataricus* β-glycosidase expressed in *Escherichia coli*, Sβgly, was investigated at pH 6.5 and 25 °C by means of circular dichroism and fluorescence measurements. The process proved reversible when the protein concentration was lower than 0.01 mg mL^{-1}. Moreover, the transition curves determined by fluorescence did not coincide with those determined by circular dichroism, and the GuHCl concentration corresponding at half-completion of the transition increased on raising the protein concentration in the range 0.001−0.1 mg mL^{-1}. Gel filtration chromatography experiments showed that, in the range 2−4 M GuHCl, there was an equilibrium among tetrameric, dimeric, and monomeric species. These findings, unequivocally, indicated that the guanidine-induced denaturation of Sβgly was not a two-state transition with concomitant unfolding and dissociation of the four subunits. A mechanism involving a dimeric intermediate species was proposed and was able to fit the experimental fluorescence intensity transition profiles, allowing the estimation of the total denaturation Gibbs energy change at 25 °C and pH 6.5. This figure, when normalized for the number of residues, showed that, at room temperature, Sβgly has a stability similar to that of mesophilic proteins.

Studies devoted to determining in a quantitative manner the thermodynamic stability of globular proteins have reached great interest (*1*, *2*). Biotechnological applications of enzymes require high resistance to denaturation in order to lower the replacement costs in bioreactors (*3*). In this respect, enzymes isolated from hyperthermophilic microorganisms have considerable interest because they are stable at temperatures near or above 100 °C and operate optimally at such temperatures (*4*, *5*). Despite the increasing effort in this research area, the structural bases of thermostability have not been discovered yet.

Recently, in our laboratory, a β-glycosidase has been isolated from *Sulfolobus solfataricus*, Sβgly[1] (*6*), a thermoacidophilic archaebacterium that lives at 87 °C and acidic pH in volcanic hot springs (*7*). The protein-encoding gene has been cloned and expressed in *Escherichia coli* (*8*, *9*). The recombinant enzyme has proven to be identical to the natural form, the only difference being the absence of monomethylation in 15% of total lysine residues identified as *N*-ε-methyl-lysine (*9*). Sβgly is composed of four identical subunits, each composed of 489 residues with a molecular weight of 56 690 Da. It does not possess disulfide bridges, and the active site has been partially characterized (*10*). Recently, its structure has been solved at 2.6 Å (*11*). X-ray data showed that each subunit has the classic $(\beta\alpha)_8$ barrel fold, with substantial elaborations between the β-strands and α-helices in each repeat, lies at the corner of a slightly puckered square, and only contacts two other monomers in the tetramer. Interestingly, the crystallographic data showed that the enzyme contains more than double the proportion of ionic groups involved in ion pairs than is generally described in mesophilic proteins and that they are involved in clusters and networks that serve to cross-link noncontiguous parts at the protein surface (*11*). The knowledge of Sβgly structure opens the possibility to correlate the thermodynamic stability of Sβgly to its structural features.

In the present work we report on the guanidine-induced denaturation of Sβgly investigated by means of circular dichroism and fluorescence measurements. The overall denaturation process proved to be reversible in fluorescence, but not in circular dichroisn measurements. In addition, gel filtration chromatography measurements showed that the process did not conform to a simple two-state transition, and a mechanism involving a dimeric intermediate species was proposed. Such mechanism was able to fit the experimental fluorescence transition profiles and allowed the estimation of the Gibbs energy change associated with the reversible denaturation of Sβgly at pH 6.5 and 25 °C.

† Work was supported by grants from the Ministry for University and Scientific and Technological Research (MURST, Rome), the Italian National Research Council (CNR, Rome), and EU Contract "Extremophiles".

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[1] Abbreviations: Sβgly, *Sulfolobus solfataricus* β-glycosidase expressed in *Escherichia coli*; CD, circular dichroism; DSC, differential scanning calorimetry; FPLC, fast protein size-exclusion liquid chromatography; SDS−PAGE, sodium dodecyl sulfate polyacrylammide gel electrophoresis; ANS, 1-anilino-8-naphthalenesulfonic acid.

S0006-2960(98)00490-5 CCC: $15.00
Published on Web 09/19/1998

EXPERIMENTAL PROCEDURES

Protein Purification and Enzymatic Assay. Sβgly was overexpressed from the vector pT7 μ_B3 in *E. coli* and purified as previously described (*9*). The purity of the homogeneous preparation was tested by SDS−PAGE and reversed-phase HPLC. Protein samples were dialyzed against appropriate buffers and concentrated by using an Amicon ultrafiltration apparatus for the following analyses. Protein concentration was determined spectrophotometrically using $\epsilon_{280} = 660\,667$ M^{-1} cm^{-1}.

Enzymatic assay was carried out at 75 °C using a thermostated spectrophotometer (Varian DMS 200). The reaction mixture contained 50 mM sodium phosphate buffer, pH 6.5, 2.8 mM *o*-nitrophenyl β-D-galactopyranoside, 0.001 mg of enzyme, and distilled water to a final volume of 1.0 mL. One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1.0 μmol of substrate per minute at 75 °C, assuming an extinction coefficient of 3.1 mM^{-1} cm^{-1} at 405 nm for *o*-nitrophenol. Samples for activity measurements in GuHCl-containing buffers were incubated for 24 h at 4 °C, and a minimum of three assays were performed at each GuHCl concentration.

Chemicals and Solutions. All chemicals were of the highest purity grade, purchased from Sigma. Three buffer systems were used: 50 mM acetate at pH 5.0, 50 mM phosphate at pH 6.5, and 50 mM HEPPSO [2-(*N*-morpholino)ethanesulfonic acid] at pH 8.5. Protein solutions for fluorescence and CD measurements were exhaustively dialyzed by using Spectra Por MW15000-17000 membranes against buffer solutions at 4 °C. Doubly deionized water was used throughout. pH was measured, at 25 °C, with a Radiometer pHmeter, model PHM93.

GuHCl-Induced Denaturation Curves. Ultrapure guanidine hydrochloride was purchased from Aldrich, and stock solutions, 8 M, were prepared as previously described (*12*). Stock protein solutions were prepared in the appropriate buffer to be 10 times the desired final protein concentration. Buffer, GuHCl from the 8 M stock solution, and 200 μL of stock protein solution to give a final volume of 2-mL were added to 2 mL siliconized Eppendorf tubes. This yielded final GuHCl concentrations of 0−7 M and the desired protein concentrations. Since high GuHCl concentrations lower the pH, the pH value was kept constant by adding NaOH. Each sample was mixed by vortexing and was incubated for 24 h at 4 °C. Incubation times of 24 and 48 h produced identical fluorescence and CD signals, demonstrating that equilibrium was already reached after 24 h.

Fluorescence emission spectra were recorded with a Jasco FP777 spectrofluorimeter at protein concentrations in the range 0.001−0.1 mg mL^{-1} in the appropriate buffer and the specified amounts of GuHCl. The excitation wavelength was set at 295 nm in order to exclude the tyrosine contribution to the overall fluorescence emission. The experiments were performed at 25 °C by using a sealed quartz cuvette with 1-cm path length and a 5-nm slit width and were corrected for background signal. Both the decrease in fluorescence intensity and the shift in fluorescence maximum wavelength were recorded to monitor the unfolding transition.

Circular dichroism measurements were performed with a JASCO J-710 spectropolarimeter, calibrated with an aqueous solution of *d*-10(+)-camphorsulfonic acid at 290 nm (*13*). Molar ellipticity at 222 nm is reported as mean residue molar ellipticity, $[\theta]_{222}$ in deg cm^2 $dmol^{-1}$, calculated from the equation: $[\theta] = [\theta]_{obs}$·mrw/10 l·C, where $[\theta]_{obs}$ is the ellipticity measured in degrees, mrw is the mean residue molecular weight, 110 Da, C is the protein concentration in g mL^{-1}, and l is the optical path length of the cell in centimeters. Far-UV CD spectra were measured at a protein concentration of 0.1 mg mL^{-1} by using a cuvette with a 0.1-cm path length, a time constant of 4 s, and a 2-nm bandwidth. Spectra were signal-averaged over five scans and baseline-corrected by subtracting a buffer spectrum. Both instruments were equipped with thermostated cell holders, and temperature was held constant at 25 °C using a circulating water bath.

Gel Filtration Chromatography. Gel filtration chromatography was performed by FPLC (Pharmacia, Uppsala, Sweden) using a Superose-12 column at a flow speed of 0.1 mL min^{-1} (*14*). The column was equilibrated at the desired GuHCl concentrations before each run, and the temperature was kept constant at 25 °C. To calibrate the column in the presence of different GuHCl concentrations, the following standards were used: lysozyme (14 300 Da), carbonic anhydrase (28 800 Da), bovine serum albumin (66 300 Da), transferrin (81 000 Da), lactate dehydrogenase (141 000 Da), and tyroglobulin (660 000 Da). The protein samples, 50 μL (0.13 mg mL^{-1}), incubated for 24 h at 4 °C in different GuHCl concentrations, were isocratically eluted with 50 mM phosphate buffer, pH 6.5, and various GuHCl concentrations.

RESULTS

The GuHCl-induced denaturation of Sβgly was followed by spectroscopic techniques at three different pHs and 25 °C. The steady-state fluorescence emission spectrum with a maximum at 338 nm (the excitation was set at 295 nm) is dominated by the contribution of the 68 tryptophanyl residues. Upon complete denaturation in 6 M GuHCl, the exposure to water of tryptophanyl residues leads to a red shift of the maximum to 354 nm and to a drastic decrease in the fluorescence intensity at 338 nm (i.e., in 7 M GuHCl the fluorescence intensity amounts to 20% of its value for the native enzyme). The conformational transition, induced by increasing the GuHCl concentration and monitored by both the decrease in the steady-state fluorescence intensity at 338 nm and the shift in the fluorescence maximum wavelength, had a sigmoidal shape (see Figures 1 and 2). The values of GuHCl concentration corresponding at half-completion of the transition, indicated as $C_{1/2}$, and determined from both the fluorescence observables were 2.3 M at pH 5.0 and pH 8.5 and 2.9 M GuHCl at pH 6.5, with the concentration of Sβgly fixed at 0.01 mg mL^{-1}. Therefore, the protein tertiary structure is slightly more stable at pH 6.5, as already evidenced by the condition of maximal enzymatic activity (*15*). The denaturation was completely reversible, as renaturation of Sβgly, by suitable dilution of fully unfolded samples, keeping the concentration of Sβgly fixed at 0.01 mg mL^{-1}, showed a complete recovery of all the native spectroscopic features. The extent of renaturation did not depend on the incubation time, and in all probability, the low protein concentration used avoided aggregation of unfolded chains.

The coincidence of the $C_{1/2}$ values obtained for Sβgly with the two different fluorescence observables seems to contrast

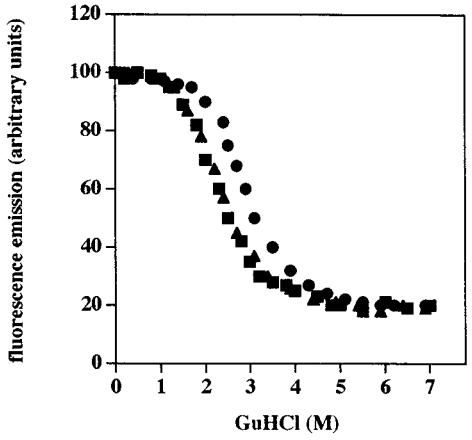

FIGURE 1: Dependence of the steady-state fluorescence intensity at 338 nm (excitation at 295 nm) on GuHCl concentration at 25 °C for Sβgly (c = 0.01 mg mL^{-1}): ■, pH 5.0; ●, pH 6.5; ▲, pH 8.5.

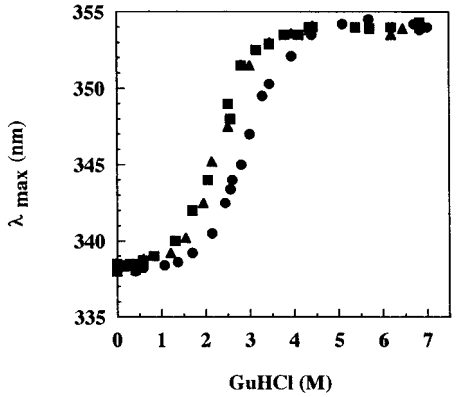

FIGURE 2: Dependence of the fluorescence emission maximum wavelength on GuHCl concentration at 25 °C for Sβgly (c = 0.01 mg mL^{-1}): ■ 5.0; ●, pH 6.5; ▲, pH 8.5.

with the general analysis performed by Eftink (*16*). In all probability, this experimental finding simply reflects the fact that Sβgly possesses a very large number of tryptophanyl residues (i.e., each monomer contains 17 tryptophanyl residues), and only the average properties of such a family of fluorophores are recorded. It is worth noting that these values of $C_{1/2}$ are close to those found at 20 °C by Jaenicke and co-workers for two tetrameric proteins from the hyperthermophilic bacterium *Thermotoga maritima*: 2.1 M GuHCl for D-glyceraldehyde-3-phosphate dehydrogenase (*17*) and 2.6 M GuHCl for L-lactate dehydrogenase (*18*).

Far-UV circular dichroism spectra were recorded at 25 °C and at a protein concentration of 0.1 mg mL^{-1}, to ascertain to which extent the secondary structure is affected by GuHCl. The molar ellipticity at 222 nm is reported in Figure 3 as a function of denaturant concentration for the three pHs investigated. In agreement with fluorescence measurements, CD data had a well-defined sigmoidal shape, indicative of a cooperative transition. However, the value of $C_{1/2}$ was close to 3.6 M GuHCl for all the pHs studied. In addition, the values of molar ellipticity at 222 nm for Sβgly were close to zero in 6−7 M GuHCl, indicating the loss of all secondary structure. These data demonstrate that the secondary structure stability is independent of pH in the range investigated and is greater than that of tertiary structure. This finding is in agreement with far-UV CD measurements performed as a function of temperature, which unequivocally

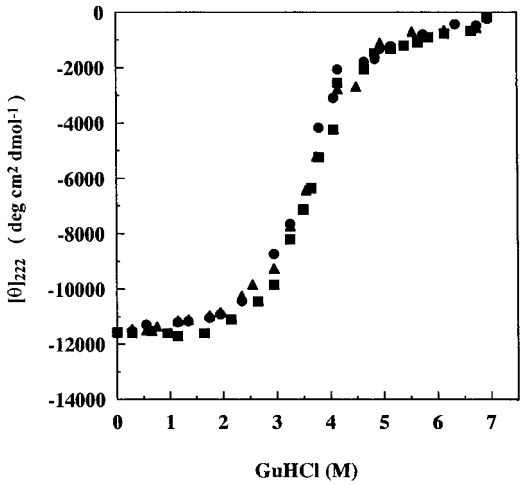

FIGURE 3: Dependence of the molar ellipticity on GuHCl concentration at 25 °C for Sβgly (c = 0.1 mg mL^{-1}): ■, pH 5.0; ●, pH 6.5; ▲, pH 8.5.

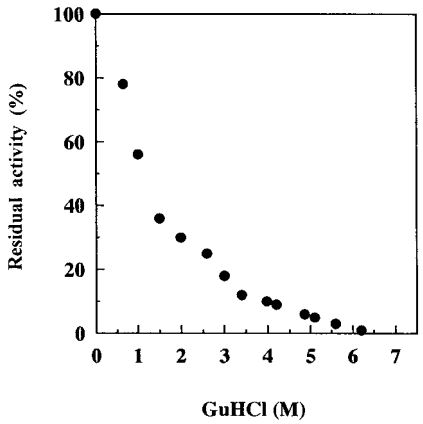

FIGURE 4: Percentage residual enzymatic activity of Sβgly as a function of GuHCl concentration at 75 °C and pH 6.5 (see Experimental Procedures for more details).

showed the existence of substantial secondary structure in heat-denatured samples of Sβgly (*19*, *20*). On the other hand, in these experimental conditions the process proved to be irreversible, probably because the high protein concentration used favors aggregation of unfolded chains, as already found in DSC investigations on the temperature-induced denaturation of Sβgly (*19*, *20*).

The noncoincidence between the transition curves obtained by fluorescence and CD spectroscopy is striking, even though different protein concentrations were used in the fluorescence and CD experiments. The data indicate that the tertiary structure is lost before the disruption of the secondary structure. We checked if there was a relationship between the breakage of tertiary structure and the loss of enzymatic activity. Measurements of enzymatic activity were performed on Sβgly samples incubated for 24 h with different GuHCl concentrations at 4 °C and pH 6.5 (the protein concentration was 0.001 mg mL^{-1}). The data are reported in Figure 4 and show that the biological function is reduced to less than 20% of its initial value at 3 M GuHCl, a concentration beyond the $C_{1/2}$ of 2.9 M GuHCl determined by fluorescence. The loss of enzymatic activity is correlated to the breakage of tertiary structure: a very small fraction of protein molecules are still active at 3.5 M GuHCl. It is important to remark that, by performing suitable dilution in

order to keep the protein concentration fixed, there is a complete recovery of enzymatic activity. This finding demonstrates that the GuHCl-induced denaturation of Sβgly is a reversible process when the protein concentration is sufficiently low to avoid aggregation of unfolded chains.

All the experimental data suggest that Sβgly shows a rather complex behavior at increasing the GuHCl concentration, not compatible with a simple two-state denaturation mechanism. Therefore, we tried to gain information on the quaternary structure loss upon the GuHCl-induced denaturation. For a denaturation process associated with subunit dissociation, the protein concentration is a significant variable (*21*−*23*). To test whether monomerization occurred, measurements of the decrease in the steady-state fluorescence intensity were performed at 25 °C and pH 6.5 for different protein concentrations (in the range 0.001−0.1 mg mL^{-1}) as a function of GuHCl concentration. A significant effect of Sβgly concentration was detected as $C_{1/2}$ passed from 2.7 to 3.3 M GuHCl. It is worthwhile to note that, even for a protein concentration of 0.1 mg mL^{-1}, equal to that used in far-UV CD measurements, $C_{1/2}$ = 3.3 M GuHCl, a value lower than that determined from CD data (i.e., $C_{1/2}$ = 3.6 M). Furthermore, the reversibility of the process was lost when the protein concentration was equal to 0.1 mg mL^{-1}. This finding seems to confirm that the irreversibility of the denaturation process is not an intrinsic feature of the conformational transition, but a consequence of the strong tendency to aggregate of unfolded chains when their concentration is sufficiently high (*19*).

Gel filtration chromatography experiments with Sβgly in the presence of increasing amounts of GuHCl were also performed at 25 °C and pH 6.5. Figure 5 indicates that at 1 M GuHCl the native tetrameric protein is stable, while at 2.5 and 3.5 M GuHCl there is an equilibrium among tetrameric, dimeric, and monomeric species. At 5 M GuHCl the protein is completely monomeric. These data unequivocally demonstrate that the denaturation process is not a simple two-state transition with concomitant unfolding and dissociation of the four subunits.

Therefore, the GuHCl-induced denaturation of Sβgly can be described by the following two-step (i.e., three-state) mechanism:

$$N_4 \overset{K_1}{\longleftrightarrow} 2I_2 \overset{K_2}{\longleftrightarrow} 4D \quad (1)$$

where N_4 represents the native tetrameric structure, I_2 represents a dimeric intermediate species, and D represents the monomeric denatured state. Clearly, the dimeric intermediate species is not well-defined in structural terms. According to fluorescence, circular dichroism, and enzymatic activity data, it seems that in the I_2 species the subunits have partially lost the tertiary structure while the secondary structure is largely conserved. This recalls the molten globule intermediate state assumed by globular proteins during refolding (*24*). The latter is frequently identified by its ANS-binding capacity (*24*), and it would be of interest to test whether the partially dissociated Sβgly has an affinity toward this dye. However, the fluorescence spectrum of ANS in the presence of Sβgly, at 25 °C and pH 6.5, is not affected by increasing concentrations of GuHCl, being similar to that in buffer solution. Furthermore, the fluorescence intensity measured at 475 nm does not show any peak on increasing the GuHCl concentrations (data not shown). These findings suggest that the intermediate species I_2, populated in the GuHCl-induced denaturation of Sβgly, does not resemble a molten globule.

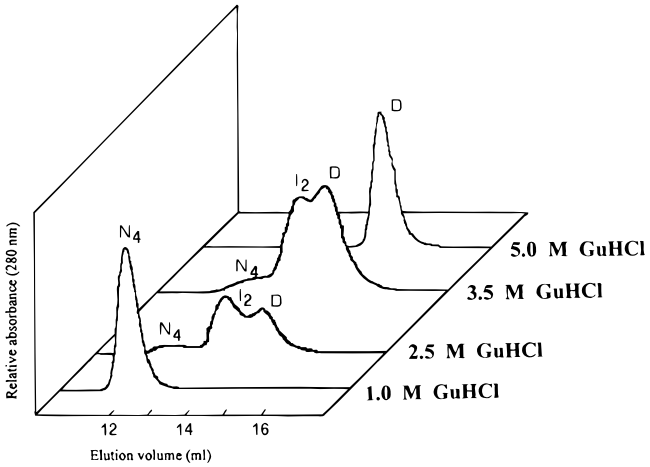

FIGURE 5: Elution profiles obtained by gel filtration chromatography for Sβgly at the GuHCl concentrations indicated, 25 °C and pH 6.5.

The equilibrium constants of the proposed mechanism, K_1 and K_2, are related to the respective standard Gibbs energy changes, $\Delta_d G_1$ and $\Delta_d G_2$, by the fundamental thermodynamic equation: $\Delta_d G_i = -RT \ln K_i$. We assume valid the linear extrapolation model, LEM (*12*), in which $\Delta_d G_i$ is supposed to vary linearly with the denaturant concentration, C_{GuHCl}:

$$\Delta_d G_i = \Delta_d G_{H_2O,i} - m_i C_{GuHCl} \quad (2)$$

The total concentration of protein, P_{tot}, expressed in monomer units, is equal to

$$P_{tot} = 4[N_4] + 2[I_2] + [D] \quad (3)$$

that, by using the expressions of both equilibrium constants, can be transformed in

$$[D]^4 + [D]^2(K_1K_2^{1/2}/2) + ([D] - P_{tot})(K_1K_2/4) = 0 \quad (4)$$

Solution of this fourth degree algebraic equation allows us to evaluate [D], the concentration of denatured monomers, in the whole range of GuHCl concentrations investigated. Therefore, it is possible to determine the fractional population of molecules in the three states:

$$f_N = 4[N_4]/P_{tot} = 4[D]^4/P_{tot}K_1K_2 \quad (5)$$

$$f_I = 2[I_2]/P_{tot} = 2[D]^2/P_{tot}K_2^{1/2} \quad (6)$$

$$f_D = [D]/P_{tot} \quad (7)$$

Since the model is based on equilibrium thermodynamics, it was applied only to fluorescence data because the process proved to be reversible in those experimental conditions. Moreover, it is necessary to select a fluorescence signal that is proportional to the fractional population of thermodynamic states, as well-emphasized by Eftink (*16*). The fluorescence signal that fulfills this requirement is the steady-state fluorescence intensity measured at some pair of excitation

Table 1: Thermodynamic Parameters Obtained from the Nonlinear Regression of Fluorescence Intensity Measurements for GuHCl-Induced Denaturation of SβGly at 25 °C and Different pH Values[a]

pH	protein (mg mL^{-1})	m_1 (kJ mol^{-1} M^{-1})	$\Delta_dG_{H_2O,1}$ (kJ mol^{-1})	m_2 (kJ mol^{-1} M^{-1})	$\Delta_dG_{H_2O,2}$ (kJ mol^{-1})	Y_I	χ^2
5.0	0.010	9.5	60.1	12.5	116.0	65	0.09
6.5	0.001	9.7	67.2	13.0	125.1	64	0.11
6.5	0.005	10.0	67.7	12.9	125.9	65	0.09
6.5	0.010	10.1	68.4	13.2	127.8	64	0.07
6.5	0.050	10.3	68.9	13.4	128.5	63	0.08
6.5	0.100	10.5	68.2	13.0	128.3	64	0.12
8.5	0.010	9.8	61.0	12.6	117.2	65	0.09

[a] The data are analyzed as described in the text. For each protein concentration and for each pH, we performed three independent fluorescence intensity measurements. Each figure is the average of the values calculated by the nonlinear regression with respect to eq 8 over the three fluorescence measurements. The uncertainties in the estimates for m_i and $\Delta_dG_{H_2O,i}$ amount to 10% and 15%, respectively, of reported values, whereas the uncertainty on Y_I does not exceed 5% of reported values. The values of χ^2 are the highest obtained for each experimental condition. The nonlinear regression was also performed for the measurements at pH 6.5 and 0.1 mg mL^{-1}, which were not reversible, because the irreversibility is not an intrinsic feature of the conformational transition.

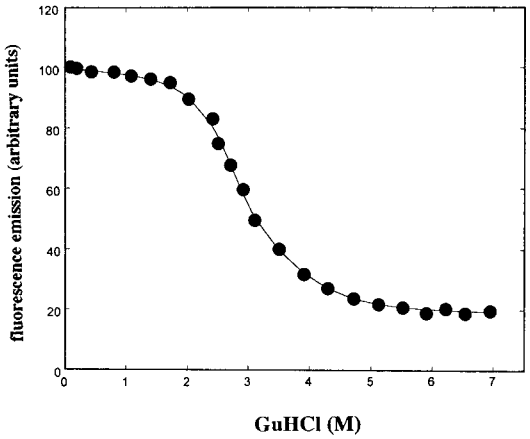

FIGURE 6: Dependence of the steady-state fluorescence intensity at 338 nm on GuHCl concentration at 25 °C and pH 6.5 for Sβgly (c = 0.01 mg mL^{-1}). Solid line represents the best fit obtained using the two-step model described in the text, eq 8.

and emission wavelengths. Therefore, the decrease in fluorescence intensity measured at 338 nm, Y, was calculated as a linear combination of each individual contribution (*25*), namely:

$$Y = f_N Y_N + f_I Y_I + f_D Y_D \quad (8)$$

where Y_N, Y_I, and Y_D are the intrinsic relative fluorescence intensities for the three thermodynamic states considered in the model. In addition, Y_N and Y_D are assumed to vary linearly with C_{GuHCl}, whereas Y_I is not known. A nonlinear least-squares regression was performed to estimate the fundamental parameters associated with the conformational transition (i.e., $\Delta_dG_{H_2O,1}$, m_1, $\Delta_dG_{H_2O,2}$, m_2, and Y_I) according to the minimum chi-square value. The nonlinear regression used the Levenberg−Marquardt algorithm (*26*), as implemented in the Optimization Toolbox of MATLAB.

The results are reported in Table 1, while the fitting of the data at pH 6.5 and Sβgly concentration equal to 0.01 mg mL^{-1} is shown in Figure 6. By summing the values of $\Delta_dG_{H_2O,1}$ and $\Delta_dG_{H_2O,2}$, the total denaturation Gibbs energy change of Sβgly at 25 °C is obtained, which is a measure of its thermodynamic stability in the specified conditions. This quantity amounts to 196 kJ/mol of monomer at pH 6.5, 178 kJ/mol of monomer at pH 8.5, and 176 kJ/mol of monomer at pH 5.0, when the concentration of Sβgly was fixed at 0.01 mg mL^{-1}. These values are per mole of monomer because the mass balance equation refers to the total concentration of monomeric subunits. By changing the protein concentration at pH 6.5 in the range 0.001−0.1 mg mL^{-1}, the values of $\Delta_dG_{H_2O,1}$ and $\Delta_dG_{H_2O,2}$ are little affected, as it should be according to equilibrium thermodynamics: their sum falls in the range 192−198 kJ/mol of monomer. The data emphasize that the native structure of Sβgly is more stable at pH 6.5 than at pH 5.0 or 8.5. Finally, the relatively high m values, about 10 kJ mol^{-1} M^{-1} for the first step and about 13 kJ mol^{-1}M^{-1} for the second step, point out that both the transitions are cooperative.

We would also like to remark an important point not often appreciated. For a denaturation process in which the number of particles increases (i.e., when unfolding is accompanied by dissociation of subunits), the ratio $\Delta_dG_{H_2O}/m$ does not correspond to $C_{1/2}$ but to the denaturant concentration where the equilibrium constant is equal to 1. Clearly, as dictated by thermodynamics, this ratio should be independent of protein concentration because, when temperature is constant, the value of the equilibrium constant is fixed and cannot depend on concentration. This requirement is fulfilled by the values reported in Table 1, within the limits of experimental uncertainty, confirming that the GuHCl-induced denaturation of Sβgly is an equilibrium process.

According to the parameter estimates collected in Table 1, the Gibbs energy change involved in the transition from the native tetramer to the dimeric intermediate corresponds to about 35% of the total stabilization Gibbs energy. Thus, the dimeric intermediate is not close in stability to the native state, probably because the dissociation to dimers is accompanied by substantial destruction of the tertiary structure. On the other hand, the Gibbs energy change involved in the second part of the denaturation process corresponds to about 65% of the total stabilization Gibbs energy. This finding suggests that the dissociation to monomers and the complete breakage of secondary and tertiary structures require a large amount of energy.

DISCUSSION

Even though a large quantity of information has been learned on the folding process and thermodynamic stability of globular proteins, by performing experiments on small globular proteins, it is clear that a general understanding of such problems cannot be reached by studying exclusively small model proteins. Most proteins are multimeric, with the subunits assembled together by noncovalent interactions to give rise to the active quaternary structure (*27*, *28*).

Relatively few studies have been devoted to characterize the thermodynamic stability of multimeric proteins, especially from thermophiles. This prompted us to investigate the stability of Sβgly as a function of GuHCl concentration. In addition, when the denaturation of large enzymes was studied in detail, the presence of intermediates in the unfolding pathway was pointed out (*25*, *29*, *30*). Sβgly is a very large molecule, in view of the fact that each subunit largely exceeds the maximum dimension of a single domain (i.e., maximum number of residues around 200). Therefore, a complex denaturation process could be expected and was indeed found. By combining the data from steady-state fluorescence and CD measurements with the results of gel filtration chromatography, it was evident that the GuHCl-induced denaturation of Sβgly cannot be described by a two-state mechanism. In contrast, we proposed a two-step mechanism involving an equilibrium among tetrameric, dimeric, and monomeric species, which was able to account for experimental data in a satisfactory manner.

It has to be noted that, in the crystal structure, the asymmetric unit is a dimer, related to the other dimer in each molecule by a crystallographic 2-fold axis (*11*). In addition, each monomer contacts only two other monomers in the molecule: (a) the interface between the two subunits related by noncrystallographic symmetry involves residues in the loops from 38 to 47, 161 to 166, and 216 to 230; (b) the interface between the two subunits related by the crystallographic 2-fold axis involves residues in the loop from 394 to 400 and the C-terminal strand from 464 to 489 (*11*). These structural data seem to support the existence of dimeric species in the course of GuHCl-induced denaturation of Sβgly, even though a more detailed examination of the two interfaces would be necessary to bring out a significant difference in the interactions involved.

It may be surprising that the fluorescence measurements do not show two, more or less, separated transitions, but this finding can be explained. Each subunit of Sβgly possesses 17 tryptophanyl residues, which, according to the X-ray data, are not homogeneously dispersed in the three-dimensional structure (*11*). In fact, eight tryptophans are located in the hydrophobic core of each subunit and are very close among each other, five lie near the external protein surface, and the last four are localized near the catalytic site. The emission properties of these indolic residues are different, as already demonstrated by frequency-domain fluorescence investigations of Sβgly (*31*, *32*). Therefore, in view of the high number of tryptophans involved and their different location in each subunit, it would be very unlikely to observe a plateau in the fluorescence measurements as a function of GuHCl concentration.

In the crystal structure of Sβgly an unusually large number of ion pairs was observed (*11*), and it is believed that these may contribute favorably to the conformational stability of the protein. To test this supposition, we performed some measurements with a neutral denaturant-like urea. Fluorescence intensity measurements at 25 °C and pH 6.5 with a protein concentration of 0.01 mg mL^{-1} proved that Sβgly is stable in the presence of urea up to a concentration of about 5 M. At higher urea concentrations the fluorescence intensity decreases, but it is not possible to obtain a complete sigmoidal transition, since the post-transition baseline is practically lacking (data not shown). For this reason we did not further investigate the urea-induced denaturation of Sβgly. In addition, the enzymatic activity of Sβgly slightly increases in the presence of urea (*33*). These findings seem to confirm the fundamental role played by ion pairs in determining the conformational stability of Sβgly.

It is important to perform a comparison between the total stabilization Gibbs energy of Sβgly with that of mesophilic proteins. For a large number of small monomeric globular proteins (i.e., in the range of 50−200 residues) isolated from mesophilic sources, the values of the stabilization Gibbs energy have been calculated at 25 °C from both temperature-induced (*2*) and denaturant-induced (*34*) unfolding processes. By dividing such values by the number of amino acid residues in a given protein, the stabilization Gibbs energy per residue always falls in the range 200−550 J molres^{-1}. These figures are largely below the random thermal energy at room temperature (i.e., $RT \approx 2500$ J mol^{-1} at 25 °C), and so, they imply that the stability of native structure is marginal and must involve cooperativity (*1*, *4*). Actually, similar values of the stabilization Gibbs energy per residue have been determined for oligomeric mesophilic proteins (*21*, *22*, *35*) and, interestingly, for three small globular proteins from thermophilic sources (*36*−*38*), whose denaturation process was carefully investigated by DSC measurements. The total stabilization Gibbs energy per residue of Sβgly at pH 6.5 and 25 °C, in the whole concentration range investigated, amounts to about 400 J molres^{-1}. Such a figure falls in the middle of the range determined for mesophilic globular proteins. Therefore, the analysis of Sβgly data seems to confirm that the high thermal stability of proteins from thermophiles is not correlated to an extra stability at room temperature.

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BI980490W