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Temperature-Induced Denaturation of β-Glycosidase from the Archaeon *Sulfolobus solfataricus*[1]

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The β-glycosidase isolated from the extreme thermophilic archaeon *Sulfolobus solfataricus*, grown at 87°C, is a tetrameric protein with a molecular mass of 240 kDa. This enzyme is barely active at 30°C and has optimal activity, over 95°C, at pH 6.5. Its thermal stability was investigated at pH 10.1 and 10.6 by means of functional studies, circular dichroism and differential scanning calorimetry. There was no evidence of thermal activation of the enzyme and the temperature-induced denaturation was irreversible and not well represented by the two-state transition model. A more complex process occurred, involving the dissociation and unfolding of subunits, and subsequent nonspecific association and/or aggregation. Denaturation temperature was around 85°C, depending on protein concentration. The denaturation enthalpy change was between 7,500 and 9,800 kJ·mol^{-1}, depending on the pH. The collapse of the native structure around 85°C was confirmed by circular dichroism measurements and time-dependent activity studies. Finally, preliminary investigations were performed on the recombinant enzyme expressed in *Escherichia coli*.

Key words: β-glycosidase, *Sulfolobus solfataricus*, thermophilic enzyme, protein denaturation, scanning calorimetry.

In recent years many experimental investigations have been performed on extremophilic microorganisms (*1-5*). During their evolution, these microorganisms have developed the ability to survive over wide ranges of temperature, pressure, pH, and water activity (*6*). Much interest has been focused on the characterization of those living at high temperature and in particular on their enzymes. It is of interest not only to know how metabolic processes are sustained at such high temperatures, but also to understand the molecular bases of the exceptional stability of these enzymes. In fact, thermophilic globular proteins show much higher thermal stability than their counterparts from mesophilic sources, as well as greater resistance to other common protein denaturants, such as organic solvents, detergents, proteolytic enzymes, extremes of pH, *etc.* (*7-9*). The upper limit of thermal adaptation seems to be around 110-120°C due to the susceptibility of the covalent structure of the polypeptide chain to hydrolysis (*10*). The cloning and expression of thermophilic genes in mesophilic hosts, resulting in the production of native thermostable proteins, clearly demonstrate the intrinsic character of thermal stability, which is probably due to the cumulative effect of small modifications strategically located within the protein molecule. Finally, it should be stressed that, as for mesophilic proteins, the conformational stability of thermophilic proteins too becomes marginal at high physiological temperatures in order to ensure the necessary flexibility (*2, 11*). Despite the great importance attached to both fundamental research and biotechnological applications, the general features of thermophilic adaptation are still unknown, and there are no reliable strategies of biomolecular stabilization.

In our laboratories a thermophilic and thermostable β-galactosidase from the archaeon *Sulfolobus solfataricus* was purified and partially characterized (*12*). More recently, the β-galactosidase activity has been characterized as a glycosyl hydrolase and in consequence of the wide substrate specificity and exo-glucosidase activity, the enzyme has been classed as a β-glycosidase (Sβgly) (*5*). The gene of this enzyme has been cloned and expressed in *Escherichia coli* (*13*) and the recombinant enzyme has been purified to homogeneity and characterized (*14*). The native and recombinant enzymes have a molecular mass of 240 kDa, and are composed of four similar subunits of about 60 kDa. The two enzymes seem to be functionally similar: kinetic experiments have shown that they have the same wide substrate specificity and the same temperature-dependence of activity (*5*). Moreover, the N-terminal amino acid sequence and the content of secondary structure are comparable in the two enzymes (*9*).

Since denaturation and stability are closely related, in this paper we investigated the temperature-induced de-

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Abbreviations: Sβgly, β-glycosidase from *Sulfolobus solfataricus*; DSC, differential scanning calorimetry; CD, circular dichroism; ASA, accessible surface area.

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naturation of the wild-type *Sβ*gly. Differential scanning calorimetry (DSC) is a powerful tool for directly measuring protein denaturation parameters (*15, 16*). By means of DSC, the conformational stability of *Sβ*gly was examined at pH above 10.0. Furthermore, circular dichroism (CD) spectra and time-course measurements of activity were performed under the same pH conditions to further examine the thermostability. Finally, preliminary measurements were made on the recombinant *E. coli Sβ*gly to seek possible differences in the thermal stability of the two enzymes.

MATERIALS AND METHODS

Chemicals and Solutions—All chemicals were of reagent grade, purchased from Sigma. Three buffer systems were used: 50 mM sodium phosphate for pH 6.5; 10 mM Capso (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid) for pH 10.1 and 50 mM sodium glycine for pH 10.6. Protein solutions for DSC and CD measurements were exhaustively dialyzed by using Spectra Por MW 15,000-17,000 membranes against buffer solutions at 4°C. Doubly deionized water was used throughout. pH was measured with a Radiometer pHmeter PM 91 at 25°C.

Enzyme Purification—*β*-Glycosidase [EC 3.2.1] was isolated from the archaeon *S. solfataricus*, strain MT4, and purified as described elsewhere (*5*). The purity of the homogeneous preparation was tested by SDS-polyacrylamide gel (*17*) and reversed-phase HPLC. Protein samples were dialyzed against appropriate buffers and concentrated by using an Amicon ultrafiltration apparatus for the following analyses.

Enzyme Assay—Enzyme 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, 0.25 μg 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 min^{-1} at 75°C, assuming an extinction coefficient of 3.1 $mM^{-1}\cdot cm^{-1}$ at 405 nm for *o*-nitrophenol (*18*).

Protein Concentration—Protein concentration was determined by the method of Bradford (*19*) or as described by Lowry *et al.* (*20*). Alternatively, spectrophotometric determination of protein concentration was performed by using $A_{280}=660{,}667\ M^{-1}\cdot cm^{-1}$ (unpublished results).

Optimal Temperature—The dependence of *Sβ*gly activity on temperature was determined by assaying aliquots of homogeneous enzyme (0.25-0.50 μg) in 10 mM Capso buffer, pH 10.1, and in 50 mM sodium glycine buffer, pH 10.6 at temperatures ranging from 30 to 95°C, as described for the enzyme assay.

Thermostability—The thermostability of *Sβ*gly was investigated by incubating 0.7 $mg\cdot ml^{-1}$ of enzyme solution in 10 mM Capso buffer, pH 10.1, and in 50 mM sodium glycine buffer, pH 10.6, in sealed Eppendorf tubes at 65, 75, 80, and 85°C. At each desired time, aliquots of the preincubated enzyme were withdrawn from the incubation mixtures and assayed at 75°C under the conditions described for enzyme assay.

Circular Dichroism Spectroscopy—Circular dichroism measurements were performed on homogeneous samples at protein concentrations ranging between 0.2 and 0.5 $mg\cdot ml^{-1}$ at pH 10.1 and 10.6. A spectropolarimeter, model J-710 (Jasco, Tokyo), equipped with a Neslab RTE-110 temperature controller (Neslab Instruments, Portsmouth, NH, USA) and calibrated with a standard solution of (+)-10-camphorsulphonic acid, was used. The following cuvettes (Hellma, Jamaica, NY, USA) were used: 190-240 nm region, 0.1 cm path length; near-UV region, 1.0 cm path length. A spectral acquisition spacing of 0.2 nm (1.0 nm bandwidth) was used in the 190-240 nm region, and 0.1 nm spacing (1.0 nm bandwidth) in the near-UV region. Photomultiplier absorbance did not exceed 600 V in the spectral regions measured. Each spectrum was signal-averaged at least five times, smoothed with Spectropolarimeter System Software Ver. 1.00 (Jasco) and baseline-corrected by subtracting the buffer spectrum. All measurements were performed at the indicated temperatures under a nitrogen flow. The results are expressed in terms of ellipticity (mdeg).

Secondary structure estimations were computed with a Jasco program that compares the actual protein spectrum from 190 to 240 nm with reference spectra for four conformations (*α*-helix, *β*-sheet, *β*-turn, and random or unordered). These spectra are based on the CD spectra of 15 reference proteins with known secondary and tertiary structure (*21*). In this program the calculations are constrained such that the sum of the percent of each type of structure equals 100%.

Scanning Calorimetry—Calorimetric measurements were carried out on a second-generation Setaram Micro-DSC apparatus, suitable for work on dilute solutions of biological macromolecules in the temperature range of 0-100°C. The instrument was interfaced with a data translation A/D board for automatic data acquisition. The calibration was performed exploiting the Joule effect by means of a specific Setaram apparatus. An electrical signal was applied for a time t, and the instrument output was recorded. The integration of the obtained curve represents a quantity A ($\mu V\cdot s$). This value is then divided by the product of the power P used and the time t, obtaining a coefficient $K=A/P\cdot t$, in $\mu V/mW$. The calibration was performed at different temperatures for a fixed scan rate. The experimental values were interpolated, generating a calibration curve in the range of 0-99°C.

A scan rate of 0.5 $K\cdot min^{-1}$ was chosen for the present study. All data analyses were accomplished with software developed in our laboratories (*22*). The raw data were converted to an apparent molar heat capacity by correcting for the instrument calibration curve and the buffer-buffer scanning curve and by dividing each data point by the scan rate and the number of moles of protein in the sample cell. Finally, the excess molar heat capacity functions, $\langle \Delta C_p \rangle$, were obtained after baseline subtraction, according to the procedure of Freire and Biltonen (*23*), *i.e.*, the baseline is given by the linear temperature dependence of the native state heat capacity. The total calorimetric enthalpy of denaturation $\Delta_d H$ was determined by direct integration of the area under the curve. It has been shown (*24*) that the van't Hoff enthalpy can be easily calculated from DSC curves with the formula:

$$\Delta H^{v.H.} = 4R(T_{max})^2 \langle \Delta C_p \rangle_{max} / \Delta_d H \qquad (1)$$

where $\langle \Delta C_p \rangle_{max}$ is the maximum height of the excess molar

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heat capacity, T_{max} is the temperature at which $\langle \Delta C_p \rangle_{max}$ occurs and is assumed to coincide with the so-called denaturation temperature, and R is the gas constant. The factor 4 concerning the denaturation of a single chain macromolecule, should be replaced by a factor 10 in the case of concomitant denaturation and dissociation of a tetrameric structure (*25, 26*). The unitary value of the $\Delta_d H/\Delta H^{v.H.}$ ratio is a necessary condition for the conclusion that a two-state transition takes place.

RESULTS

Under conditions of maximum stability and activity (*i.e.*, pH 6.5, 50 mM sodium phosphate buffer), the wild-type *Sβ*gly shows an increasing enzymatic activity over 95°C (*12*). However, under these conditions thermal denaturation could not be investigated because aggregation phenomena occurred, well below 100°C, at the concentration values required by the sensitivity of the DSC instrument. Aggregation is frequently coupled with the thermal denaturation of oligomeric enzymes and often makes the quantitative analysis of DSC data infeasible (*27*). Similar results have been obtained for another thermophilic enzyme, α-glucosidase from *P. furiosus*, in 50 mM phosphate buffer, pH 6.0 containing 2.5 M GuHCl (*28*).

To perform reliable DSC measurements it was necessary to raise the pH above 10.0 in order to reduce the incidence of aggregation and to lower the denaturation temperature. All pH values reported refer to 25°C. It is worth noting that the buffers used for high pH experiments have large protonation enthalpies (about 40 kJ·mol^{-1}) and, as a consequence, the pH is a strong function of temperature (it decreases by about 0.025 pH units per degree) (*29*). This undesired effect cannot be circumvented. Under the conditions employed, thermal denaturation was irreversible as demonstrated by the absence of any calorimetric peak in the second scanning of the same sample. Side reactions occurring at high temperatures and aggregation phenomena probably prevent the correct refolding of the polypeptide chains (*30, 31*). All the DSC measurements were carried out at 0.5 K/min. The effect of the scan rate was not investigated due to the limited amount of wild-type *Sβ*gly obtainable by the fermentation process.

The denaturation parameters for the wild-type *Sβ*gly at pH 10.1 and 10.6 are reported in Table I. Figure 1 shows two DSC curves at different protein concentrations. At pH 10.1 the denaturation temperature was concentration-dependent: T_{max} increased from 86.5°C at protein concentration [C] = 2.4 μM to 88.9°C at [C] = 4.7 μM. The narrow concentration range investigated was imposed by instrument sensitivity for the lower limit, 0.60 mg·ml^{-1}, and by aggregation phenomena for the upper limit, 1.13 mg·ml^{-1}. In fact, problems of aggregation also occurred at high pH values. Figure 2 shows a characteristic calorimetric profile due to exothermic aggregation superimposed on the endothermic denaturation peak, obtained at pH 10.1 and [C] = 15 μM (corresponding to 3.6 mg·ml^{-1}) for the wild-type *Sβ*gly.

The increase of T_{max} can be ascribed to the dissociation of the four protein subunits during denaturation. The DSC peaks were asymmetric, showing a tail in the low-temperature side also indicative of dissociation. The simple application of equilibrium thermodynamics accounts well for the increase of T_{max} with increasing protein concentration and for the skewness of DSC peaks when the denaturation process is coupled with dissociation (*25, 32*).

The denaturation enthalpy change $\Delta_d H$ was about 9,500 kJ·mol^{-1} at pH 10.1 and 7,600 kJ·mol^{-1} at pH 10.6. These values greatly exceed that determined, by means of DSC for the glutamate dehydrogenase from *P. furiosus*, a hexamer of 270 kDa, $\Delta_d H = 1{,}730$ kJ·mol^{-1} at $T_{max} =$ 113°C, 10 mM imidazole buffer, pH 7.3, and 3.0 mM dithiothreitol (*33*). This value is much lower than those found for the wild-type *Sβ*gly, although they both refer to thermophilic enzymes. On the other hand, for the irreversible thermal denaturation of *E. coli* glucosamine-6-phosphate deaminase, a hexamer of 178.2 kDa, it was found that $\Delta_d H = 5270 \pm 500$ kJ·mol^{-1} at $T_{max} = 65.0$°C and pH 10.1, 10 mM phosphate buffer (*34*). Additionally, for tarantula hemocyanin, a 24-meric thermostable protein, $T_{max} =$ 90.0°C at pH 7.8, and the enthalphy change amounts to about 30,000 kJ·mol^{-1} (*35*).

At pH 10.6 the wild-type *Sβ*gly was less stable than at pH 10.1. In fact, both T_{max} and $\Delta_d H$ decrease. Figure 3

TABLE I. **Thermodynamic parameters from DSC measurements of the thermal denaturation of the wild-type *Sβ*gly.** (a) pH 10.1, 10 mM Capso buffer; (b) pH 10.6, 50 mM sodium glycine buffer. r is the ratio of the calorimetric enthalpy to the corresponding van't Hoff enthalpy.[a]

[C] (μM)	T_{max} (°C)	$\Delta_d H$ (kJ·mol^{-1})	$\Delta_d C_p$ (kJ·K^{-1}·mol^{-1})	r
(a)				
2.4	86.5	9,800	200	8
3.2	87.6	9,500	220	7
3.8	88.2	9,700	200	8
4.7	88.9	9,500	190	6
(b)				
1.9	85.8	7,700	180	8
4.6	84.2	7,600	190	7

[a]Each figure represents the value averaged over two or three measurements. The error in T_{max} does not exceed 0.4°C. The estimated (relative) uncertainties in $\Delta_d H$ and $\Delta_d C_p$ amount to 10% and 15–20%, respectively, of the reported values.

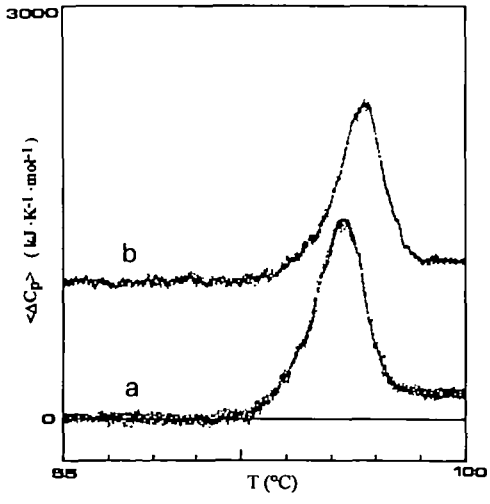

Fig. 1. **DSC profiles of wild-type *Sβ*gly at pH 10.1, 10 mM Capso buffer, at different protein concentrations: curve a [C] = 2.4 μM; curve b [C] = 4.7 μM.** The curves are shifted in the vertical axis for presentational purposes.

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shows the DSC profile obtained at pH 10.6 for [C] = 4.6 μM. Moreover, T_{max} was slightly dependent on concentration, but in contrast to what was observed at pH 10.1, at increasing concentrations, T_{max} was lowered. This may be due to predominance of nonspecific association phenomena of polypeptide chains, concomitant with or following denaturation, over the dissociation of subunits. It is well known that, for a denaturation process coupled with the nonspecific association of the unfolded polypeptide chains, T_{max} decreases with increasing protein concentration (*36*). The ratio between $\Delta_d H$ and $\Delta H^{v.H}$, calculated by inserting the factor 10 in Eq. 1, was in the range of 6–8 both at pH 10.1 and 10.6. This means that thermal denaturation cannot be described by a two-state transition model.

To confirm that the enzyme retains its overall native structure above pH 10.0 and that the process monitored by DSC is actually the breaking of native conformation, *i.e.*, denaturation, kinetic thermostability measurements were performed. Figure 4 reports the activity determined after incubating the wild-type *Sβgly* at pH 10.6 for different periods of time at various temperatures. The enzyme incubated at pH 10.6 and $T < 65$°C, when assayed at pH 6.5, showed normal kinetic activity. This demonstrates that *Sβgly* retains its tertiary and quaternary structures at pH 10.6. Furthermore, the enzyme incubated at $T = 85$°C for 20 min entirely lost its activity, in agreement with DSC determinations.

To validate further the DSC results, thermal denaturation of the wild-type *Sβgly* was investigated by means of CD measurements. Figure 5 reports the CD spectra in the far- and near-UV regions at pH 10.6 and at different temperatures. The spectra of the near-UV region clearly show that the tertiary structure of the enzyme was lost above 85°C, in agreement with DSC and enzyme activity results. The spectra in the far-UV region reveal that the content of secondary structure was still significant at 95°C. Table II gives an analysis of the spectra at 25 and 95°C, according to the procedure devised by Yang *et al.* (*21*). At high temperatures, a large fraction of secondary structure persisted. This finding shows that the thermally denatured state of *Sβgly* is not completely disordered and cannot be considered a random coil. In recent years, an increasing number of experimental investigations have emphasized the existence of compact denatured states, possessing a large amount of secondary structure (*37–39*). However, the protein data set used by Yang contains only spectra of mesophilic proteins at room temperature. Therefore, caution should be used when interpreting the quantitative analysis, although the statistical significance of the fit implies that the computed fractions of secondary structure may be reasonably indicative of the overall structure organization. We did not perform a van't Hoff analysis of the CD data because the DSC measurements clearly showed that the denaturation of *Sβgly* is irreversible.

Finally, preliminary calorimetric investigations were performed on *Sβgly* expressed in *E. coli* under the same conditions as used for the wild-type enzyme. The denaturation parameters of the recombinant protein are given in Table III. Figure 6 shows two DSC curves of *Sβgly* from *E.*

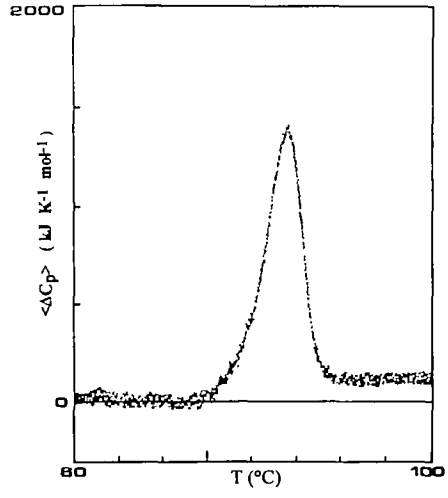

Fig. 3. **DSC curve of wild-type *Sβgly* at pH 10.6, 50 mM sodium glycine buffer, at [C] = 4.6 μM.**

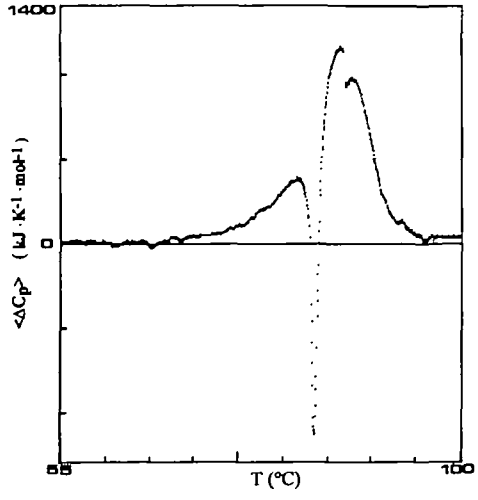

Fig. 2. **DSC curve of wild-type *Sβgly* at pH 10.1, 10 mM Capso buffer, at [C] = 15 μM.** An exothermic phenomenon of aggregation is superimposed on the denaturation peak.

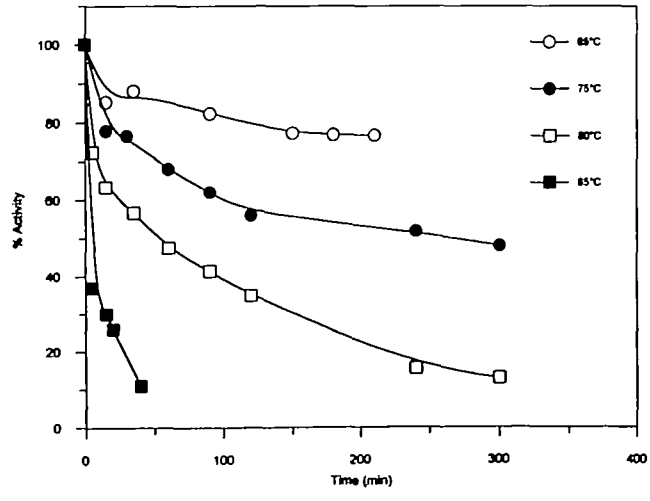

Fig. 4. **Thermal stability of *Sβgly* at various temperatures.** Enzyme solutions, 0.7 mg·ml^{-1} in 50 mM sodium glycine, pH 10.6, were incubated at the temperatures indicated.

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coli at pH 10.1 at different protein concentrations. As can be seen from the values of Table IIIa, the thermal stability of *Sβ*gly from *E. coli* was very similar to that of the wild-type form. The values of T_{max} and $\Delta_d H$ are in agreement with those determined for the wild-type *Sβ*gly. Even the ratio of $\Delta_d H$ to $\Delta H^{v.H.}$ was in the range of 6-8. Instead, a drastic change occurred at pH 10.6. The calorimetric profiles show two well separated peaks centred around 74 and 84°C. An example of this behavior is shown in Fig. 7 for [C] = 4.8 μM. Furthermore, $\Delta_d H$ was slightly higher than the corresponding value of the wild-type enzyme (8,400 kJ·mol^{-1} against 7,700 kJ·mol^{-1}). It has been found that about 20% of the lysine residues of the wild-type *Sβ*gly are mono-methylated, while such modification is not present in the recombinant enzyme. The methylation of lysine residues seems to be a post-translational modification that, besides its localized effect, may induce a global change in the protein (*40*). This may cause the marked difference between wild-type and *E. coli Sβ*gly, when passing from pH 10.1 to 10.6. Work is in progress in our laboratories to investigate the recombinant enzyme over a wide range of experimental conditions by means of different techniques in order to elucidate the differences from, and the similarities with, the wild-type *Sβ*gly.

TABLE II. **Percentage fractions of secondary structure of the wild-type *Sβ*gly at different pHs and temperatures.** The analyses were accomplished according to Yang's method.

pH	*T* (°C)	α-Helix	β-Sheet	β-Turn	Random
7.0	25	50.3	40.3	9.4	0.0
	95	49.9	40.0	9.9	0.2
10.1	25	50.0	39.8	10.2	0.0
	95	9.5	32.4	18.4	39.7
10.6	25	49.6	39.6	10.8	0.0
	95	12.1	30.4	20.9	36.6

DISCUSSION

*Sβ*gly is a tetrameric enzyme that possesses catalytic activity over 95°C at pH 6.5 (*5, 12*). Due to its exceptional resistance to high temperatures, it is a suitable model for investigating the molecular mechanisms involved in thermal stabilization. DSC and CD measurements were performed to quantify the thermal stability of *Sβ*gly. To obtain reliable DSC profiles and to prevent aggregation phenomena, we selected experimental conditions far from those of maximum activity and stability. The wild-type *Sβ*gly retained its quaternary structure at room temperature over a wide pH range (4.5-8.5), as demonstrated by ultracentrifugal determination of the relative molecular weight (data not shown). Furthermore, time-dependent thermostability measurements showed that, when assayed at pH 6.5, the enzyme incubated at pH 10.1 or 10.6 and T = 65°C for 2 h, retains 80% of its activity. These results allow us to conclude that, at pH 10.1 and 10.6, *Sβ*gly possesses its

TABLE III. **Thermodynamic parameters from DSC measurements of the thermal denaturation of *Sβ*gly expressed in *E. coli*.** (a) pH 10.1, 10 mM Capso buffer; (b) pH 10.6, 50 mM sodium glycine buffer. r is the ratio of the calorimetric enthalpy to the corresponding van't Hoff enthalpy.[a]

[C] (μM)	T_{max} (°C)	$\Delta_d H$ (kJ·mol^{-1})	$\Delta_d C_p$ (kJ·K^{-1}·mol^{-1})	r
(a)				
2.4	85.8	9,400	180	8
3.3	86.4	9,000	170	8
4.8	87.9	9,200	200	7
(b)				
3.0	86.0	8,200	200	6
4.8	84.3	8,600	190	7

[a]Each figure represents the value averaged over two or three measurements. The error in T_{max} does not exceed 0.4°C. The estimated (relative) uncertainties in $\Delta_d H$ and $\Delta_d C_p$ amount to 10% and 15-20%, respectively, of the reported values.

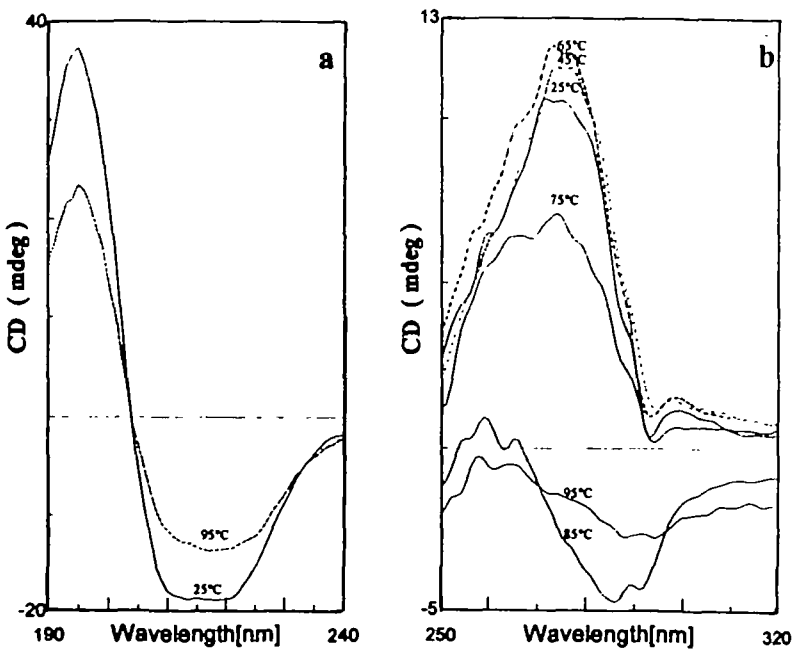

Fig. 5. **(a) Far-UV CD spectra of *Sβ*gly, pH 10.6, T = 25°C and 95°C. (b) Near-UV CD spectra of *Sβ*gly, pH 10.6, temperature range 25-95°C.**

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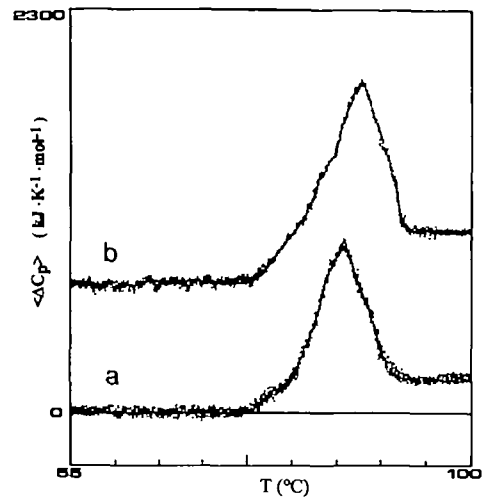

Fig. 6. **DSC profiles of *Sβgly* expressed in *E. coli* at pH 10.1, 10 mM Capso buffer, at different protein concentrations: curve a [C] = 2.4 μM; curve b [C] = 4.8 μM.** The curves are shifted in the vertical axis for presentational purposes.

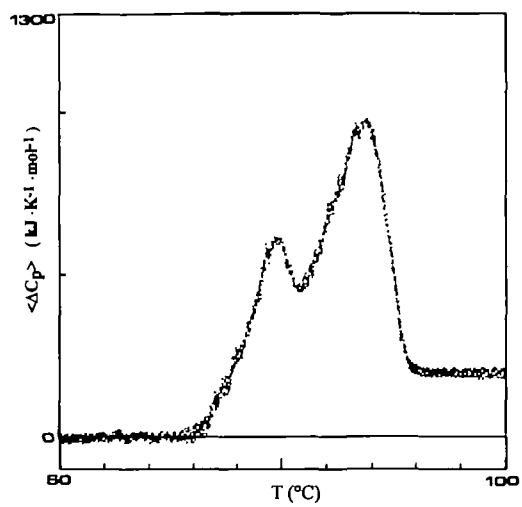

Fig. 7. **DSC profiles of *Sβgly* expressed in *E. coli* at pH 10.6, 50 mM sodium glycine buffer, at [C] = 4.8 μM.** Two well-separated peaks are present.

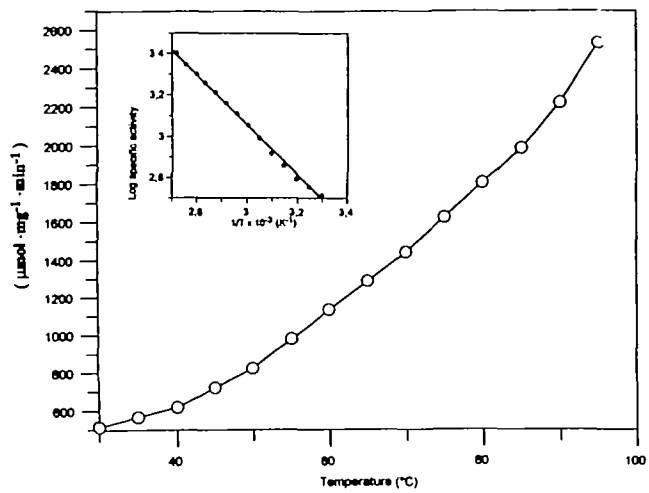

Fig. 8. **Temperature-dependence of *Sβgly* activity at pH 10.6.** The enzyme was assayed under the conditions described in "MATERIALS AND METHODS" at the temperatures indicated. In the inset the corresponding Arrhenius plot is shown.

native tetrameric structure. For these reasons, it appeared interesting to investigate the thermal denaturation of *Sβgly* at these pHs. DSC measurements showed an expected high value of the denaturation temperature, as well as a trend of T_{max} values as a function of protein concentration, both at pH 10.1 and 10.6. This trend implies competition between the dissociation of subunits coupled with denaturation and their subsequent nonspecific association or aggregation. Depending on the solution conditions, pH, ionic strength and protein concentration, the dissociation may prevail over association or *vice versa*. The nonspecific association and/or aggregation of denatured subunits also competes with the refolding of the enzyme, and this may be one of the factors rendering irreversible the thermal denaturation (*31*). Because of the irreversible nature of the denaturation we did not calculate entropy and Gibbs energy difference between the native and denatured states. Indeed entropy calculations from experimental heat capacity data are based upon Clausius equality, which does not hold for an irreversible process (*41*). On the other hand, it should be noted that, according to the first law of thermodynamics, the total heat absorbed equals the denaturation enthalpy change, even though the denaturation process is irreversible.

An important result of our DSC measurements is the relatively high value of the enthalpy change involved in the overall process. The value of 9,800 kJ·mol^{-1} (equivalent to 40.8 J·g^{-1}) measured at pH 10.1, [C] = 4.7 μM and centred around 88.9°C, is comparable with the value of 5,270 kJ·mol^{-1} (equivalent to 29.5 J·g^{-1}) found for the irreversible thermal denaturation of *E. coli* glucosamine-6-phosphate deaminase (a hexamer of 178.2 kDa) at pH 10.1, considering the lower value of T_{max} = 65°C and that the denaturation heat capacity change amounts, on average, to 0.4-0.5 J·K^{-1}·g^{-1} (*15*). In both cases the specific enthalpy values are comparable with those of monomeric globular proteins from mesophilic organisms that are in the range 25-30 J·g^{-1} at 60°C (*15*). Therefore it seems that the quaternary structure of *Sβgly* is as compact as those of monomeric globular proteins for which close-packing interactions are considered to play a pivotal role in the stability (*42*). This finding is consistent with the preliminary analysis of the crystals of the wild-type *Sβgly* (*43*). These authors found that *Sβgly* crystals show strong diffraction, unusual physical stability and substantial insensitivity to radiation damage. However, in order to elucidate the molecular origin of the exceptional stability, the *Sβgly* tertiary structure should be examined at high resolution.

The ratio of calorimetric to van't Hoff enthalpy gives a rough indication of the number of cooperative domains in the macromolecule. Our results indicate the presence of about 8 cooperative domains in the *Sβgly* structure. Further thermodynamic and structural investigations, however, are necessary to clarify the denaturation mechanism and the overall folding pattern of *Sβgly*. The finding that the thermal denaturation of *Sβgly* is not a two-state transition is not unexpected in view of the high molecular

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weight of each subunit, 60 kDa, and the assumed maximal size of the cooperative folding unit of proteins, which is of the order of 200 amino acids (*44-46*). Due to the irreversibility of the overall process and the uncertainty in the mechanism of the unfolding transition, we did not attempt to perform a deconvolution analysis of DSC curves (*23, 32*).

The heat capacity change between the denatured and native states, $\Delta_d C_p$, amounts to about 200 $kJ \cdot K^{-1} \cdot mol^{-1}$ at both pH 10.1 and 10.6. Various authors have shown that $\Delta_d C_p$ can be *a priori* calculated with accuracy from the accessible surface area, ASA, of native and denatured (*i.e.*, assumed random coil) structures and the specific contributions of polar and nonpolar groups to heat capacity (*47-49*). Some of us (*50*) have developed a simple model that couples the specific contributions of a peptide group, CONH, and a nonpolar hydrogen atom, CH, determined from the transfer process of liquid alkyl-amides, with general and average structural features of globular proteins (*i.e.*, molecular weight, number of residues, expected number of hydrogen bonds and fraction of buried nonpolar ASA). By applying this approach to Sβgly with 2,164 residues and a molecular weight of 240 kDa, $\Delta_d C_p$ comes out at 186 $kJ \cdot K^{-1} \cdot mol^{-1}$, a figure in good agreement with experimental determinations. The closeness of the two values implies the probable occurrence of compensating errors (*i.e.*, the denatured state of Sβgly does not seem to correspond to a random coil). It seems unlikely that a thermophilic globular protein can be described by the same average structural features obtained from a large set of mesophilic proteins.

Several researchers have described a thermal activation process for thermophilic proteins: a conformational change that switches protein toward a structural organization which is temperature resistant and more active (*33, 51-53*). Sβgly shows a linear Arrhenius plot without discontinuity for the enzymatic activity, at pH 6.5 (*5*), pH 10.1 and 10.6. The inset of Fig. 8 shows the Arrhenius plot corresponding to pH 10.6. There is no indication of a temperature-driven conformational change into a more active structure. DSC measurements confirm the kinetic data, because no humps in the DSC profiles are visible in the temperature range of 25-65°C. These findings demonstrate that the existence of a structural change that switches the enzyme to a more active form is not a general rule for thermophilic proteins (*28*).

The CD data deserve further comment. An increasing number of experimental investigations report the existence of compact denatured states, using both GuHCl and temperature as denaturing agents (*37-39, 54, 55*). These compact denatured states have a substantial content of secondary structure, but do not have a unique folding pattern (*i.e.*, the tertiary structure is destroyed). The term "molten globule" is used in the literature to identify these compact denatured states, even though the scientific community does not agree on the thermodynamic characterization and behavior of these conformations (*56-58*). In our case, the far-UV CD measurements indicate that the thermally denatured Sβgly retains a high content of secondary structure, although the quaternary and tertiary structures are destroyed. However, we do not believe it is correct to consider the thermally denatured state of Sβgly as a "molten globule," because the dimensions and the degree of compactness of this state have not been determined.

As recognized by some authors (*59, 60*), the interaction with water molecules of polar groups, previously buried in the protein core, is strongly exothermic. The existence of a high content of secondary structure in the thermally denatured state of Sβgly would prevent the complete "hydration" of the polypeptide chains. Therefore, the $\Delta_d H$ values of Sβgly would not contain the exothermic contribution of the interaction with water molecules of various polar groups.

It is noteworthy that Sβgly possesses only one free cysteine residue per subunit, according to the gene sequence (*13*) and the Ellman reaction (*12*). Therefore, this protein does not possess disulfide bridges. It is usually believed that, by reducing the conformational entropy gain associated with denaturation, disulfide bridges increase protein thermostability. The insertion of three disulfide bridges in the T4 lysozyme, by means of site-directed mutagenesis, resulted in a marked increase of denaturation temperature (*61*). However, a comparison of the primary sequences of proteins from different sources shows that thermophilic enzymes have a low content of cysteine residues (*62, 63*). Moreover, the *P. furiosus* α-glucosidase has been shown to exhibit considerable stability and activity in the presence of high levels of dithiothreitol, indicating that disulfide linkages are not important contributors to the stability of this protein (*28*). This finding is presumably related to the susceptibility of disulfide bridges to oxidation at temperatures close to or above 100°C.

The gene sequence of Sβgly also implies the presence of a large number of charged residues (*13*). They may be involved in the formation of several salt bridges, which are considered of fundamental importance to increase the thermostability of globular proteins, as suggested two decades ago by Perutz (*64*). In fact, X-ray crystallographic studies have shown that thermophilic enzymes possess a larger number of salt bridges than their mesophilic counterparts (*46, 65*). The putative presence of several salt bridges in the structure of Sβgly may account, at least in part, for its high thermal stability.

In conclusion, we attempted to characterize the thermal stability of Sβgly. The temperature-induced denaturation of this tetrameric enzyme is a complex and irreversible process, in which subunit dissociation is coupled with unfolding, while unfolded polypeptide chains tend to associate in a nonspecific manner. The results can be interpreted on the basis of preliminary structural information on Sβgly and well-established thermodynamic properties of globular proteins. A comparison with a mesophilic counterpart would be useful, the ultimate goal being the correlation of the intrinsic thermal stability of Sβgly with its tertiary structure. Such a study is in progress.

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